**AGRICULTURAL UNIVERSITY OF ATHENS**

**DEPARTMENT OF FOOD SCIENCE AND HUMAN NUTRITION**

**LABORATORY OF FOOD PROCESS ENGINEERING, PROCESSING AND PRESERVATION OF AGRICULTURAL PRODUCTS**

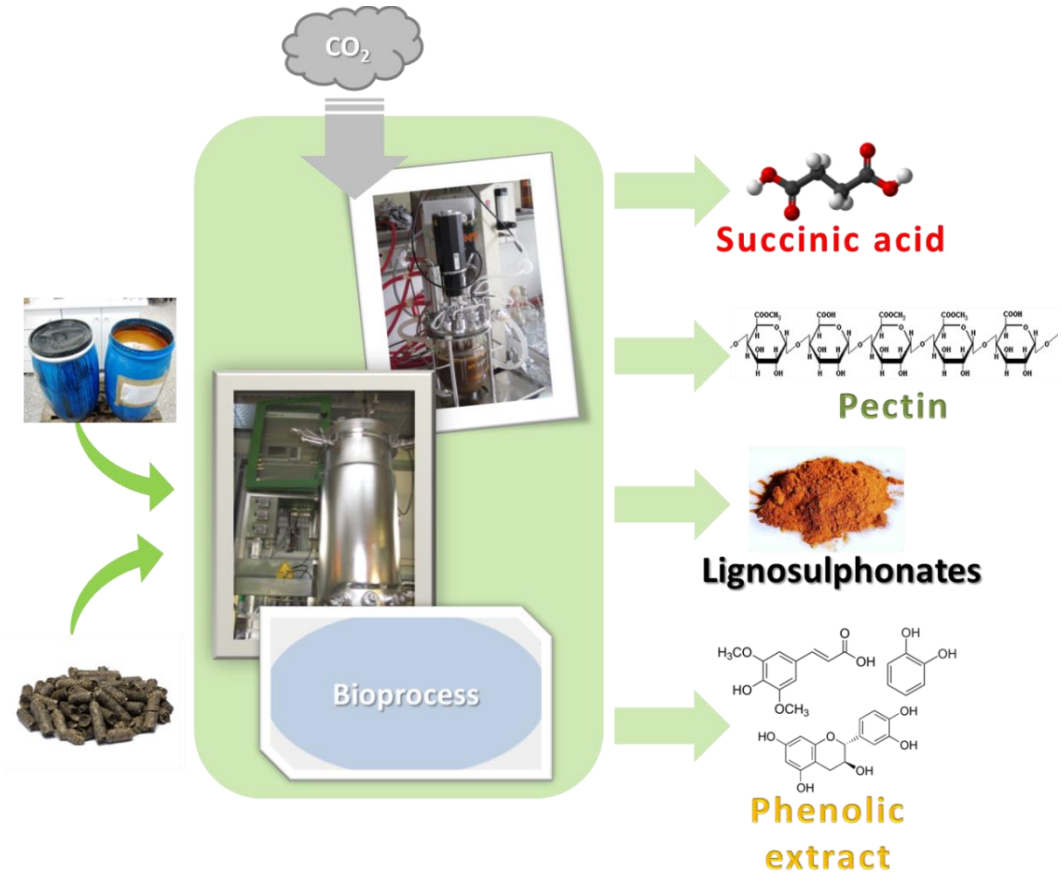
# "Valorisation of industrial waste and by-product streams for the extraction of phenolic compounds and the development of bioprocesses for the production and purification of succinic acid"

A thesis submitted for the degree of

**DOCTOR OF PHILOSOPHY**

by

**MARIA D. ALEXANDRI**




**ATHENS**

**JUNE 2017**

**SUPERVISOR: Dr KOUTINAS APOSTOLOS**

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**ΤΜΗΜΑ ΕΠΙΣΤΗΜΗΣ ΤΡΟΦΙΜΩΝ ΚΑΙ ΔΙΑΤΡΟΦΗΣ ΤΟΥ ΑΝΘΡΩΠΟΥ**

**ΕΡΓΑΣΤΗΡΙΟ ΜΗΧΑΝΙΚΗΣ, ΕΠΕΞΕΡΓΑΣΙΑΣ ΚΑΙ ΣΥΝΤΗΡΗΣΗ ΓΕΩΡΓΙΚΩΝ ΠΡΟΙΟΝΤΩΝ**

# "Αξιοποίηση βιομηχανικών αποβλήτων και παραπροϊόντων για την ανάκτηση εκχυλισμάτων πλούσιων σε φαινολικά συστατικά και την ανάπτυξη βιοδιεργασιών για την παραγωγή ηλεκτρικού οξέος υψηλής καθαρότητας"

## ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

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## ΠΕΡΙΛΗΨΗ

Ο σκοπός της διατριβής έγκειται στην αξιολόγηση δύο διαφορετικών βιομηχανικών παραπροϊόντων ως θρεπτικά μέσα ζύμωσης για την παραγωγή ηλεκτρικού οξέος και για την εκχύλιση φαινολικών καθώς και άλλων προϊόντων προστιθέμενης αξίας. Η αποτελεσματική κλασμάτωση αυτών των παραπροϊόντων θα μπορούσε να οδηγήσει στη δημιουργία καινοτόμων βιοδιυλιστηρίων.

Το απόβλητο της βιομηχανίας χαρτοπολτού (spent sulphite liquor-SSL), προκύπτει από την όξινη επεξεργασία του ξύλου (εν προκειμένω του δέντρου *Eucalyptus globulus*) με χρήση θειωδών. Το SSL περιέχει σάκχαρα τα οποία προκύπτουν από την υδρόλυση της ημικυτταρίνης (με κυριότερο σάκχαρο την ξυλόζη), λιγνοσουλφονικά, φαινολικά, οξικό οξύ και άλλες ουσίες οι οποίες μπορούν να δράσουν σαν παρεμποδιστές κατά τη ζύμωση του αποβλήτου από μικροοργανισμούς. Αρχικά μελετήθηκε η εκχύλιση των φαινολικών συστατικών του SSL χρησιμοποιώντας τη μέθοδο εκχύλισης υγρού-υγρού με τη χρήση οξικού αιθυλεστέρα ως διαλύτη εκχύλισης. Ακολούθησε η βελτιστοποίηση των κυριότερων παραμέτρων που επηρρεάζουν την εκχύλιση των φαινολικών όπως η αρχική τιμή pH του αραιωμένου SSL καθώς επίσης και η αναλογία διαλύτη-SSL χρησιμοποιώντας παραγοντικό σχεδιασμό. Στις βέλτιστες συνθήκες (σε τιμή pH της υδατικής φάσης ίσης με 2,22 και αναλογίας διαλύτη-SSL ίσης με 3,67: 1) επιτεύχθηκε η πιο αποτελεσματική εκχύλιση φαινολικών συστατικών με συγκέντρωση ολικών φαινολικών ίση με 7,5 g σε ισοδύναμα γαλλικού οξέος ανά λίτρο SSL. Το ελλαγικό και το γαλλικό οξύ ήταν τα κυριότερα φαινολικά που ταυτοποιήθηκαν στο εκχύλισμα. Η απομάκρυνση των φαινολικών από το SSL οδήγησε στην επίτευξη υψηλότερης παραγωγής ηλεκτρικού οξέος από τα στελέχη *Actinobacillus succinogenes* και *Basfia succiniciproducens* σε σχέση με τις ζυμώσεις που πραγματοποιήθηκαν με SSL χωρίς κάποια προεπεξεργασία. Τα πειραματικά αποτελέσματα αποδεικνύουν ότι η απομάκρυνση των φαινολικών συστατικών του SSL οδηγεί όχι μόνο στην παραγωγή ενός προϊόντος προστιθέμενης αξίας όπως τα φαινολικά αλλά ταυτόχρονα συμβάλει στη δημιουργία ενός λιγότερο τοξικού υποστρώματος, κατάλληλου για την βιοτεχνολογική παραγωγή ηλεκτρικού οξέος.

Στη συνέχεια μελετήθηκε η δημιουργία βιοδιυλιστηρίου μέσω της κλασμάτωσης του SSL σε λιγνοσουλφονικά, φαινολικά συστατικά και ενός ρεύματος πλούσιου σε σάκχαρα κατάλληλου για την παραγωγή ηλεκτρικού οξέος. Σε πρώτο επίπεδο μελετήθηκε η απομάκρυνση των λιγνοσουλφνικών με τη μέθοδο της εκχύλισης υγρού-υγρού

χρησιμοποιώντας ακετόνη και ισοπροπανόλη ως διαλύτες. Η ισοπροπανόλη αποδείχθηκε πιο αποτελεσματικός διαλύτης καθώς η χρήση της οδήγησε στην απομάκρυνση περίπου 80% των ολικών λιγνοσουλφονικών του δείγματος. Το υπόστρωμα που προέκυψε μετά την απομάκρυνση των λιγνοσουλφνικών χρησιμοποιήθηκε σε ζυμώσεις ημισυνεχούς λειτουργίας με τα στελέχη *A. succinogenes* και *B. succiniciproducens* οδηγώντας στην παραγωγή 19 g/L ηλεκτρικού οξέος και στις δύο περιπτώσεις. Από την άλλη πλευρά, μελετήθηκε η χρήση υποστρώματος που προέκυψε μετά την απομάκρυνση από το SSL των λιγνοσουλφονικών με τη μέθοδο της νανοδιήθησης σε συνδυασμό με την εκχύλιση των φαινολικών συστατικών η οποία οδήγησε στην παραγωγή 39 g/L ηλεκτρικού οξέος από το στέλεχος *B. succiniciproducens*. Το ισοζύγιο μάζας για τη συγκεκριμένη μέθοδο έδειξε ότι είναι δυνατή όχι μόνο η επίτευξη υψηλών συγκεντρώσεων ηλεκτρικού οξέος αλλά και ταυτόχρονη παραγωγή 28,1 g λιγνοσουλφονικών καθώς επίσης και 1,15 g εκχυλίσματος πλούσιου σε φαινολικά συστατικά από κάθε 100 g SSL. Μια σημαντική ποσότητα βαρέων μετάλλων απομακρύνθηκε από το SSL είτε με τη χρήση νανοδιήθησης είτε με τη χρήση εκχύλισης με ισοπροπανόλη. Αυτό το καινοτόμο βιοδιυλιστήριο θα μπορούσε να ενσωματωθεί στις σημερινές συμβατικές βιομηχανίες παρασκευής χαρτιού που βασίζονται στη μέθοδο της όξινης επεξεργασίας του ξύλου με θειώδη.

Προκειμένου να αυξηθεί η τελική συγκέντρωση ηλεκτρικού οξέος μελετήθηκε η χρήση ακινητοποιημένων βιοκαταλυτών σε υποστρώματα ακινητοποίησης όπως απολιγνινοποιημένα κυτταρινούχα υλικά (ΑΚΥ) και σφαιρίδια αλγινικών. Η χρήση ακινητοποιημένων κυττάρων του *A. succinogenes* σε αλγνικά οδήγησε στην επίτευξη υψηλότερης απόδοσης μετατροπής σακχάρων προς ηλεκτρικό οξύ (0,81 g/g) σε σχέση με τη χρήση ακινητοποιημένου βιοκαταλύτη σε ΑΚΥ (0,65 g/g). Η τελική συγκέντρωση ηλεκτρικού οξέος καθώς και η απόδοση που επιτεύχθηκε από τις ακινητοποιημένες καλλιέργειες του στελέχους *B. succiniciproducens* σε αλγνικά (45 g/L και 0,66 g/g) ήταν υψηλότερες σε σχέση με τις αντίστοιχες τιμές που προέκυψαν από το στέλεχος *A. succinogenes* σε αλγνικά (35,4 g/L και 0,61 g/g) χρησιμοποιώντας επεξεργασμένο με νανοδιήθηση SSL ως θρεπτικό μέσο ζύμωσης. Ακινητοποιημένες καλλιέργειες του στελέχους *B. succiniciproducens* σε αλγνικά επαναχρησιμοποιήθηκαν σε τέσσερεις συνεχόμενες ζυμώσεις ημι-συνεχούς λειτουργίας με SSL επεξεργασμένο με νανοδιήθηση, οδηγώντας στη συνολική παραγωγή 64,7 g ηλεκτρικού οξέος με την απόδοση του προϊόντος να κυμαίνεται μεταξύ 0,42-0,67 g/g και παραγωγικότητα μεταξύ 0,29-0,65 g/L/h. Οι ακινητοποιημένες καλλιέργειες βελτίωσαν την αποδοτικότητα της παραγωγής ηλεκτρικού οξέος σε σχέση με τις καλλιέργειες ελεύθερων κυττάρων.

Εκτός από το SSL, μελετήθηκε επίσης η αξιοποίηση ενός ακόμα σημαντικού παραπροϊόντος που προκύπτει από τη βιομηχανία ζάχαρης, της πούλπας από σακχαρότευτλα (ή ζαχαρόπιτα). Η πούλπα από σακχαρότευτλα περιέχει κυτταρίνη, ημικυτταρίνη, πηκτίνες και φαινολικά. Η κλασμάτωση του συγκεκριμένου αποβλήτου ξεκίνησε με τον διαχωρισμό των φαινολικών συστατικών χρησιμοποιώντας τη μέθοδο εκχύλισης με υδατικό διάλυμα αιθανόλης. Ακολούθησε κατακρήμνιση των πηκτινών και τα υπολειπόμενα στερεά υδρολύθηκαν αρχικά με τη χρήση διαλύματος $H_2SO_4$ και έπειτα με ένζυμα (με τη χρήση του εμπορικού μείγματος ενζύμων Accellerase 1500), με στόχο την παραγωγή ενός υποστρώματος πλούσιου σε σάκχαρα, κατάλληλου για ζυμώσεις ηλεκτρικού οξέος. Η συγκέντρωση των στερεών της υδρόλυσης, η διάρκεια της όξινης υδρόλυσης καθώς και η κατάλληλη συγκέντρωση ενζύμων αποτέλεσαν τις κύριες παραμέτρους που μελετήθηκαν και βελτιστοποιήθηκαν. Υπό τις βέλτιστες συνθήκες (10% συγκέντρωση στερεών, 0,5% $H_2SO_4$, 30 λεπτά υδρόλυσης στους 121 $^oC$ και 0,5 mL Accellerase ανά g κυτταρίνης) το υδρόλυμα που προέκυψε περιείχε 40 g/L σάκχαρα, κυρίως γλυκόζη και αραβινόζη. Εν συνεχεία αξιολογήθηκε η ικανότητα παραγωγής ηλεκτρικού οξέος από τα στελέχη *A. succinogenes* και *B. succiniciproducens* χρησιμοποιώντας τα σάκχαρα του υδρολύματος ως πηγή άνθρακα. Το στέλεχος *A. succinogenes* έδωσε καλύτερα αποτελέσματα όσον αφορά την τελική συγκέντρωση ηλεκτρικού οξέος σε σχέση με το στέλεχος *B. succiniciproducens* σε καλλιέργειες διαλείποντος έργου με υδρόλυμα της πούλπας από σακχαρότευτλα ως θρεπτικό μέσο ζύμωσης. Ζυμώσεις ημι-συνεχούς λειτουργίας με το στέλεχος *A. succinogenes* που πραγματοποιήθηκαν σε εργαστηριακή κλίμακα, οδήγησαν στην παραγωγή 30 g/L ηλεκτρικού οξέος με απόδοση 0,94 g/g και παραγωγικότητα ίση με 0,83 g/L/h. Η διεργασία που αναπτύχθηκε εργαστηριακά μεταφέρθηκε επιτυχώς σε πιλοτική κλίμακα με την πραγματοποίηση ζυμώσεων ημισυνεχούς λειτουργίας σε βιοαντιδραστήρα χωρητικότητας 50 L οι οποίες έδωσαν συγκρίσιμα αποτελέσματα με τις ζυμώσεις σε εργαστηριακή κλίμακα.

Ο διαχωρισμός και ο καθαρισμός του βιοτεχνολογικά παραγώμενου ηλεκτρικού οξέος μελετήθηκε με την αξιολόγηση πέντε διαφορετικών μεθόδων: με κατακρήμνιση με τη χρήση ασβεστίου, με εξαλάτωση, με απευθείας κρυστάλλωση είτε με οξίνιση του μέσου είτε με τη χρήση κατιονανταλλακτικών ρητινών, με εκχύλιση χρησιμοποιώντας αμίνες σε οργανικούς διαλύτες και με τη μέθοδο της ηλεκτροδιάλυσης. Από όλες τις μεθόδους που χρησιμοποιήθηκαν η μέθοδος της απευθείας κρυστάλλωσης με τη μέθοδο των κατιονανταλλακτικών ρητινών έδωσε τα καλύτερα αποτελέσματα, καθώς η απόδοση ανάκτησης του ηλεκτρικού οξέος ήταν της τάξεως του 79% με καθαρότητα 96%, ακολουθούμενη από τη μέθοδο εκχύλισης με τη χρήση αμινών σε οργανικό διαλύτη με την

οποία ανακτήθηκε το 73% του ηλεκτρικού οξέος από το θρεπτικό μέσο με καθαρότητα 97,2%.

**Επιστημονική περιοχή:** Αξιοποίηση βιομηχανικών αποβλήτων μέσω χημικών και βιοχημικών διεργασιών

**Λέξεις κλειδιά**: απόβλητο βιομηχανίας χαρτοπολτού (SSL), ηλεκτρικό οξύ, αντιοξειδωτικά, πούλπα από σακχαρότευτλα, *Actinobacillus succinogenes*, *Basfia succiniciproducens*, βιοδιυλιστήριο

## ABSTRACT

The scope of this thesis is the evaluation of by-product streams derived from two different industrial sectors as fermentation substrates for succinic acid production as well as the extraction of phenolic compounds and other value-added products. The effective fractionation of these by-product streams could result in the development of advanced biorefinery concepts.

Spent sulphite liquor (SSL), is the by-product stream of the acidic sulphite pulping process of *Eucalyptus globulus* wood in the pulp and paper industry. SSL contains fermentable sugars derived from hemicellulose degradation (with xylose being the predominant one), lignosulphonates, phenolic compounds, acetic acid and other compounds with potential inhibitory effect towards fermenting microorganisms. At the beginning of this study, the effective removal of the phenolic compounds present in SSL was evaluated by employing solvent extraction with ethyl acetate as extracting solvent. The main parameters affecting the extraction of phenolic compounds such as the initial pH of the diluted SSL and the solvent-to-liquid ratio were optimized by applying experimental design. At the optimum conditions (pH value of the aqueous phase equal to 2.22 and a solvent-to-liquid ratio of 3.67:1) the highest total phenolic content in the extract was obtained (7.5 g gallic acid equivalents per L SSL). The extract showed strong antioxidant activity (Antioxidant activity index of 3.64). The main phenolic compounds identified were ellagic and gallic acids. The removal of phenolic compounds from SSL led to higher succinic acid production efficiency by both *Actinobacillus succinogenes* and *Basfia succiniciproducens* than in the case that untreated SSL was employed as fermentation substrate. The results indicated that the effective removal of the phenolic compounds could result in a value-added product and at the same time this process could lead to a detoxified fermentation medium, suitable for the biotechnological production of succinic acid.

SSL has been effectively fractionated into lignosulphonates, antioxidants and a sugar-rich substrate for bio-based succinic acid production. Initially, solvent extraction using acetone and isopropanol was tested in order to remove the lignosulphonates from SSL. Isopropanol was proved to be a more suitable solvent as it led to the separation of approximately 80% of the total lignosulphonates content. The fed-batch fermentations carried out using the pretreated SSL with isopropanol resulted in a final succinic acid concentration of 19 g/L using both *A. succinogenes* and *B. succiniciproducens*. Fractionation of SSL via nanofiltration for lignosulphonate separation and solvent extraction with ethyl acetate for the

removal of phenolic compounds produced a detoxified sugar-rich stream that led to the production of 39 g/L of succinic acid by the strain *B. succiniciproducens*. This fractionation scheme resulted also in the production of 28.1 g lignosulphonates and 1.15 g phenolic-rich extract per 100 g of SSL. Both pretreatment schemes separated significant quantities of heavy metals. This novel biorefinery concept could be integrated into conventional acidic sulphite pulping mills.

In order to achieve higher final succinic acid concentrations, cell immobilisation was also tested, using two immobilisation supports, namely: delignified cellulosic material (DCM) and alginate beads. Fed-batch immobilized cultures with *A. succinogenes* in alginates resulted in higher sugar to succinic acid conversion yield (0.81 g/g) than the respective yield achieved (0.65 g/g) when DCM immobilized cultures were used. The final succinic acid concentration and yield achieved in fed-batch immobilized cultures of *B. succiniciproducens* in alginates (45 g/L and 0.66 g/g) were higher than *A. succinogenes* immobilized cultures (35.4 g/L and 0.61 g/g) using nanofiltrated SSL as fermentation medium. Immobilized cultures of *B. succiniciproducens* in alginate beads were reused in four sequential fed-batch fermentations of nanofiltrated SSL leading to the production of 64.7 g of succinic acid with a yield range of 0.42-0.67 g/g and productivity range of 0.29-0.65 g/L/h. The immobilized cultures improved the efficiency of succinic acid production as compared to free cell cultures.

Besides SSL, sugar beet pulp (SBP), a by-product stream derived from the sugar industry, was also evaluated for succinic acid production and extraction of value-added products. SBP contains cellulose, hemicellulose, pectins, and phenolic compounds. Fractionation started with the removal of phenolic compounds using solvent extraction with an aqueous ethanol solution. Subsequently, pectins were separated from SBP by precipitation. The remaining solids were subjected to acid pretreatment with $H_2SO_4$ and enzymatic hydrolysis (with the commercial enzyme preparation Accellerase 1500) in order to obtain a sugar-rich hydrolysate that could be used as fermentation substrate. Solid loading, duration of acid hydrolysis and enzyme loading were evaluated. At the optimum conditions (10% solids, 0.5% $H_2SO_4$, 30 min at 121 $^oC$ and 0.5 mL Accellerase per g cellulose) the SBP hydrolysate contained 40 g/L of reducing sugars, mainly glucose and arabinose. The performance of *A. succinogenes* and *B. succiniciproducens* regarding succinic acid production using the sugars present in the hydrolysate was evaluated. *A. succinogenes* was a more efficient strain than *B. succiniciproducens* regarding succinic acid production when SBP-derived hydrolysates were used. Fed-batch fermentations with *A. succinogenes* carried out in lab-scale bioreactors resulted in 30 g/L of succinic acid concentration with a yield of 0.94 g/g and productivity of

0.83 g/L/h. The process was successfully scaled-up to 50 L bioreactor volume with comparable results to the lab-scale fermentations.

The downstream separation and purification of bio-based succinate from the fermentation broths has been evaluated using five different downstream processes, namely calcium precipitation, salting-out, direct crystallisation with acidification or cation exchange resins, reactive extraction, and bipolar electrodialysis. Among the evaluated downstream separation schemes, the most promising one was direct crystallisation with cation-exchange resins, which led to a succinic acid recovery yield of 79% and a purity of succinic acid crystals of 96%, while reactive extraction led to a succinic acid recovery yield of 73% and a purity of 97.2%.

**Scientific area**: Valorisation of industrial wastes via chemical and biochemical processes

**Keywords**: spent sulphite liquor, succinic acid, antioxidants, sugar beet pulp, *Actinobacillus succinogenes*, *Basfia succiniciproducens*, biorefinery

**“Αξιοποίηση βιομηχανικών αποβλήτων και παραπροϊόντων για την ανάκτηση εκχυλισμάτων πλούσιων σε φαινολικά συστατικά και την ανάπτυξη βιοδιεργασιών για την παραγωγή ηλεκτρικού οξέος υψηλής καθαρότητας”**

## Dissemination activities and research visits

### Publications related to the thesis

**Alexandri M.**, Papapostolou H., Stragier L., Verstraete W., Papanikolaou S., Koutinas AA. (2017). Succinic acid production by immobilized cultures using spent sulphite liquor as fermentation medium. Bioresour Technol, 238: 214-222

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### Other publications

Dal Prá, V., FLunelli, F.C., Vendruscolo, R.G., Martins, R., Wagner, R., Lazzaretti Jr., A., Freire, D., **Alexandri, M.,** Koutinas, A., Mazutti, M.,da Rosa, M. 2017. Ultrasound-assisted extraction of bioactive compounds from palm pressed fiber with high antioxidant and photoprotective activities, Ultrasonics Sonochemistry, 36, 362-366

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## Conferences

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**Alexandri M.**, Schneider R., Venus J., Koutinas A. 2016. Valorization of Sugar Beet Pulp

(SBP) for bio-based succinic acid production. EUBis, Wageningen, Netherlands (*Oral presentation*)

**Alexandri M.**, Papapostolou H., Gardeli C., Komaitis M., Papanikolaou S., Koutinas A. 2016. Immobilization of *Actinobacillus succinogenes* for enhanced succinic acid production in Spent Sulphite liquor. WasteEng, Albi, France (*Poster presentation*)

**Alexandri M.,** Vlysidis A., Papapostolou H., Kookos I., Koutinas A. 2016. Evaluation of different methods for the recovery of biotechnologically produced succinic acid. RRB, Ghent, Belgium (*Poster presentation*)

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Koutinas A., Ladakis D., Vlysidis A., **Alexandri M.**, Pateraki C., Kookos I., Papanikolaou S. Techno-economic evaluation of an integrated biorefinery scheme producing succinic acid from wood pulp industry wastes. 11$^{th}$ International Conference on Renewable Resources and Biorefineries, York-UK, 3-5 June 2015 (*Poster presentation*)

Georgaka N.M., **Alexandri M.**, Papapostolou H., Papanikolaou S., Koutinas A. Development of an advanced biorefinery concept based on valorization of winery wastes. 11$^{th}$ International Conference on Renewable Resources and Biorefineries, York-UK, 3-5 June 2015 (*Poster presentation*)

**Alexandri M.**, Papapostolou H., Gardeli C., Llano T., Komaitis M., Papanikolaou S., Coz A., Koutinas A. Detoxification of spent sulphite liquor for succinic acid production. 10$^{th}$ European Symposium on Biochemical Engineering Sciences and 6$^{th}$ International Forum on Industrial Bioprocesses, September 7-10, 2014, Lille, France (*Oral presentation*)

Llano T., **Alexandri M.**, Koutinas A., Gardeli C., Papapostolou H., Komaitis M., Coz A. Solvent extraction of phenolics from lignocellulosic waste. 5$^{th}$ International Conference on

Engineering for Waste and Biomass Valorisation, August 24-28, 2014, Rio de Janeiro, Brazil.

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Pateraki C., **Alexandri M.**, Ladakis D., Papapostolou H., Vlysidis A., Papanikolaou S., Koutinas A. Pretreatment of pulp and paper industry by-product stream for the production of lignosulphonates and succinic acid. 247th American Chemical Society National Meeting & Exposition, Chemistry and Materials for Energy, March 16-20, 2014, Dallas, USA. (*Oral presentation*)

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**Alexandri M.**, Pateraki C., Vlysidis A., Papapostolou H., Koutinas A. An integrated biorefinery concept for the production of paper and biochemicals. 2nd Iberoamerican Congress

On Biorefineries, 10-12 April, 2013, Jaen, Spain. (*poster presentation*)

**Research visits**

In 2016 I spent 9 months at the Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB) in Potsdam (Germany) within the framework of Erasmus+ placement and DAAD scholarship (Research Grants-Short-Term Grants, 2016) at the Department of Bioengineering and under the supervision of Dr. Joachim Venus.

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Μαίρη Αλεξανδρή

Αθήνα, 2017

# TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

# CHAPTER 1
# INTRODUCTION

According to the International Energy Outlook (IEO, 2016), the total energy consumption worldwide is expected to increase from 549 quadrillion Btu in 2012 to 815 quadrillion Btu in 2040, an increment of 48%. This tremendous growth of energy demand is directly related to the parallel economic growth, as the global gross domestic product (-GDP, expressed in purchasing power parity items) increases every year by 3.3%, from 2012 to 2040 (IEO, 2016). Renewable resources are considered as a very fast-growing source of energy for the period 2012-2040, but still fossil fuels are projected to continue dominating as energy sources until 2040 by 78% (IEO, 2016). As a consequence,, $CO_2$ emissions are also envisaged to increase from 32.2 billion metric tonnes in 2012 to 35.6 billion metric tonnes in 2020 (IEO, 2016). Many countries have started to develop strategies in order to reduce the $CO_2$ emissions in accordance to the 21$^{st}$ Conference of Parties in Paris (2015).

According to the IEO, 2016 Reference case, 54% of energy consumption can be attributed to the industrial sector in 2012, and its energy demand rises by 1.2% per year. Food and pulp and paper industries are categorized as energy-intensive manufacturing industries, having a gross output increment by 3.5% per year (IEO, 2016). The worldwide energy consumption based on renewables was 17.4 quadrillion Btu in 2012, but it is expected to grow to 25.1 quadrillion Btu in 2040.

This rising demand for energy, chemicals and polymers together with the eventual depletion of fossil resources and increasing environmental concerns has turned society's attention towards the development of sustainable alternatives based on the utilization of renewable feedstocks (Du et al., 2008). In this context, the conventional petroleum refineries could be replaced by *biorefineries*. Biorefineries, using the tools of white (formerly industrial) biotechnology will implement the adequate bio-processes for the sustainable production of fuels, chemicals and biopolymers, opening the way to the bio-economy era. The cost-competiveness of the modern biorefineries over the petroleum based refineries lies on the efficient biomass fractionation in order to introduce several marketable products at both an efficient and profitable manner (Koutinas et al., 2014). For example, the production of chemicals and polymer resins utilizing sugars and biomass leads to 2-7 times more profit together with 6-8 times more job positions, whilst the demand for raw materials is much lower in comparison to biofuel production (Carus et al., 2011). Hence, renewable resources could be exploited for the production of fuels and chemicals, in a way that both preserve the

environment and at the same time creates profit for the society. Nevertheless, there are still many impediments blocking the large-scale production of commodity chemicals from biomass, such as feedstock availability, logistics and the development of the adequate unit operations for the conversion of the renewable feedstock to marketable products (Koutinas et al., 2014). Sustainable production of chemicals could be achieved through the combination of biofuel production with the extraction of value-added products, like antioxidants, that could be applied in high-value applications (*e.g.*, cosmetics), making the overall process more cost-effective (Koutinas et al., 2014). Waste and by-product streams from various industrial sectors such as pulp and paper industry, food industry, biodiesel and bioethanol production, could be further valorized through their fractionation to value-added compounds and their transformation to nutrient-rich fermentation feedstocks.

Logistics is the major problem of the bio-economy era, as transportation of biomass is highly costly. In order to overcome this problem, the biorefinery plants should be constructed where the renewable resources are readily available. The integration of these facilities into existing industrial plants where waste and by-product streams are produced on site, could be a feasible approach. Moreover, this concept would facilitate the transition from the petrochemical to the bio-economy era. Food industries and the pulp and paper industry could be transformed into advanced biorefineries through the exploitation of their waste and by-product streams. Since, only 7% of petroleum is applied for chemical production, these waste streams could be valorized for the sustainable production of chemicals (Koutinas et al., 2014).

Pulp and paper industry constitutes an important industrial sector that produces high amounts of wastes. The worldwide production of chemical wood pulp in 2015 was 135,256,751 tonnes, from which 97,672,333 tonnes were sulphate, bleached pulp and 2,017,555 tonnes were sulphite, bleached pulp (FAOSTAT). In Europe, 31,427,085 tonnes of chemical wood pulp were produced in 2015, from which 21,251,380 tonnes were attributed to sulphate, bleached pulp and 1,456,055 tonnes were sulphite, bleached pulp (FAOSTAT). Spent sulphite liquor (SSL), the by-product stream from the acidic sulphite pulping process is generated at quantities of about 8-9 $m^3$ during the production of 1 tonne of pulp (Lawford and Rousseau, 1993). In the sulphate (Kraft) process, it is estimated that the production of 1 tonne of pulp generates 7 t of weak black liquor (Biermann, 1993). For both waste streams, the solid content is about 10-20% and it is concentrated to a solid content of 60-75% (Koutinas et al., 2014). The composition of the spent liquors varies depending on the cooking method and conditions as well as on the raw material (*e.g.* softwood or hardwood). The current practice involves burning these spent liquors in the pulp mills, for heat and energy production and for

the recovery of cooking chemicals (IPPC, 2001). Some mills recover the lignosulphonates, as they already have market value (Varanasi et al., 2013). However, these liquors also contain high amounts of reducing sugars- derived from hemicellulose hydrolysis -and could be valorized via microbial conversion. This concept is already employed in Sweden for ethanol production (IEA Bioenergy, 2001). Their utilization for the biotechnological production of chemicals and biopolymers is still under investigation.

Food industries also produce vast quantities of waste and by-product streams. For every kilogram of raw material treated in the dairy and wine sector the yield of by-product streams can account from 2-90% (Koutinas et al., 2014). For the sugar production from sugar beets (one of the most important crops worldwide), the by-products can reach 86%. Recently, there are many publications dealing with the development of novel biorefineries based on food waste streams, by employing different fractionation and valorization strategies (López et al., 2010; Pinto et al., 2009).

One possible direction towards waste valorization involves the production of key platform chemicals like succinic acid. The biotechnologically produced succinic acid is considered one of the most important chemicals of the bio-based economy. Its high value applications range from cosmetics and food additives to the production of resins, coatings and bio-polyesters. Succinic acid is characterized by the U.S. Department of Energy as one of the twelve most promising bio-based chemicals. With a market price of $2.4-3 per kg, succinic acid market potential is expected to constantly increase annually by 19%, especially if it is used by the bioplastic sector for the production of polybutylene succinate and polyurethanes (*e.g.* polyethylene succinate) (MarketsandMarkets, Succinic acid market by applications and geography. Global trends and forecasts (2011–2016), March 2012, http://www.marketresearch.com/Marketsand).

It is evident that the establishment of novel valorization strategies of existing waste streams would benefit our society. White biotechnology is an important tool towards environmentally benign and cost-effective production of fuels and chemicals. The proposed biorefineries should be designed in a manner that favors both productivity and conversion yields and always following the sustainability concept. The upstream and downstream methods followed should lead to major biomass exploitation and to minimum waste generation. Besides the operational and capital costs that are still the main barriers, it is also the society that should realize the waste disposal as huge problem, as a loss of profit and that its exploitation will create job opportunities. Consequently, the transition to the bio-economy

era will occur not only by surpassing the technological challenges, but also by introducing sustainable processes in the chemical industry.

# CHAPTER 2
# INDUSTRIAL WASTES AND BY-PRODUCT STREAMS

## 2.1. Spent sulphite liquor (SSL)

Spent sulphite liquor (SSL) constitutes the by-product stream derived during the acidic sulphite pulping process of mainly hardwoods in the pulp and paper industry. During the acidic sulphite pulping process, the production of cellulose pulp is achieved under high temperatures (135 $^{o}$C-145 $^{o}$C) and low pH values (1-2) and by employing aqueous solutions of $SO_2/MHSO_3/MSO_3$ (where M stands for Na, Ca, Mg or $NH_3$) (Marques et al., 2009). With this "cooking" process, lignin is fractionated via sulphonation and therefore removed as salts of lignosulphonic acid (LS). Hemicellulose is also hydrolysed, resulting is C5 and C6 sugar monomers, with xylose being the predominant one. The cellulose pulps produced by the acidic sulphite pulping process account almost 6% of total pulp production, as sulphate pulping (Kraft) is the most commonly used process (Pereira et al., 2013). The use of hardwoods such as *Eucalyptus globulus* started to be very common in the recent years (Pereira et al., 2013). The use of bases containing $Na^+$ of $Mg^+$ is preferable since they can be regenerated and reused (Sjöström, 1993; Sixta, 2006). After the cooking process, cellulose pulp is washed and then further process in order to obtain the final product. The water from the washing stage together with all the other residues of the process (lignosulphonates, sugars) is called "thin" SSL, which is subjected to multiple evaporators in order to reduce its volume and to recover the steam. The current practice involves the recovery of LS and the generation of energy from SSL (Figure 2.1). It is estimated that for every ton of cellulose pulp produced, almost 1 ton of solid waste are suspended in the SSL (Fernandes et al., 2012). It is obvious that SSL constitutes an important industrial by-product stream as its annual production is more than 90 billion tons (Lawford and Rousseau, 1993).

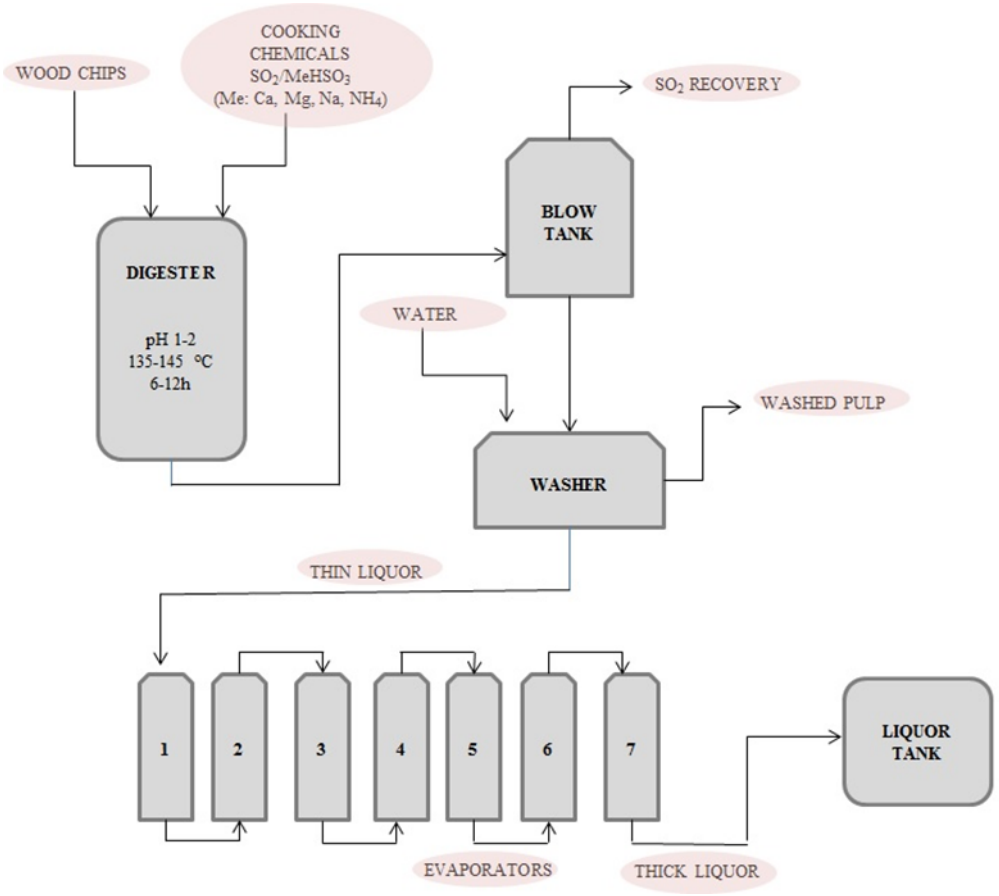



**Figure 2.1** Acidic sulphite pulping process for the production of cellulosic pulp and side production of SSL in the industry

### 2.1.1. Composition of spent sulphite liquors

The composition of SSL varies significantly depending on the wood that is used for the pulping process. The botanical classification of trees devides them in two different categories: the gymnosperms and the dicot angiosperm trees. The wood derived from angiosperms is called harwood (e.g. oak, eucalyptus, poplar), whereas the wood from gymnosperms is called softwood (e.g. pine, spruce, larch). The difference between these two types of woods lies in fibre morphology as well as in their chemical composition. Cellulose, hemicellulose and lignin are the main components in both softwoods and hardwoods but their proportions are significantly different.

Cellulose is composed of β-D-glucopyranose monomer units, connected by β-(1,4) glycosidic bonds, forming a linear homopolysaccharide. The degree of polymerisation (DP) is about 5000-6000 glucose units (Sixta, 2006; Rowell et al., 2005). The disaccharide of D-glucopyranose linked in β-(1,4) glycosidic bonds is called cellobiose, and it is the basic repeated unit of cellulose. In general, softwoods contain a bit less cellulose than hardwoods.

Cellulose together with hemicellulose is the main polyssacharide supporting the plant cell walls. Hemicellulose in hardwoods is partly acetylated 4-*O*-methyl-glucurono-β-D-xylan (glucuronoxylan), but also a minor part of non-acetylated glucomannan is also found. The main difference between hardwoods and softwoods lies in the composition of hemicellulose, as hardwood hemicellulose is mainly composed from pentosans (basically glucuronoxylan), whilst softwood hemicelluse is mainly hexosan (galactoglucomannans).

Lignin is an amorphous polymer that is responsible for the rigidity of plant cell walls, giving also antiseptic and hydrophobic properties to the plants. The structural component of lignin is phenyl propane units, connected by ether and/or carbon to carbon bonds (Sjöström, 1993, Rowell et al., 2005). The most well-known precursors of lignin are *p*-coumaryl alcohol, conoferyl alcohol and sinapyl alcohol which form the *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) sub-units (Pereira et al., 2013). The lignin derived from softwood and hardwood has different proportions of S, G and H units. Softwood lignin belongs to the "G-type" (guaiacyl lignin) as it is formed of polymerised coniferyl-alcohol units. On the other hand, hardwood lignin belongs to the "GS-type" (guaiacyl-syringyl lignin), as it is a co-polymer of coniferyl and sinapyl alcohols, in a ratio varying from 1:4 or 2:1. The main inter-unit bond is β-*O*-4, but lignin can also be connected to polysaccharides, resulting in a lignin-carbohydrate network. Hardwood lignins possess more ether linkages than softwoods, property that together with the fact that their total lignin content is almost 50% lower, results in easier delignification during pulping (Sixta, 2006; Rowell et al., 2005).

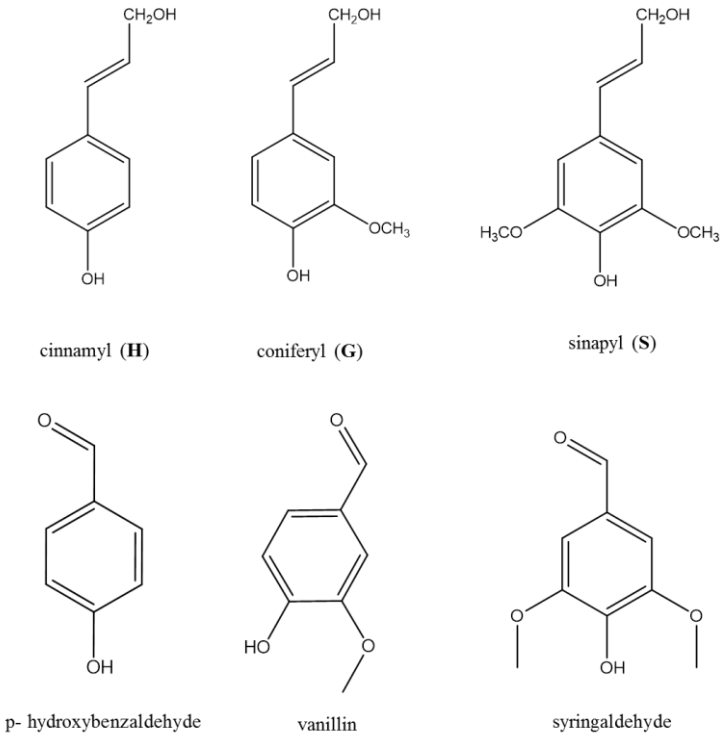



**Figure 2.2** Lignin precursors and their corresponding oxidation products

During the acidic sulphite pulping process, cellulose fibers are recovered, whereas lignin is sulphonated and removed and hemicellulose is hydrolysed to its sugar monomers. This liquor contains a high concentration of organic matter, making its disposal impossible. The composition of SSL depends mainly on the type of wood chips used for the pulping. When *Eucalyptus globulus* wood is used for the process, the main components are the LS, C5 and C6 sugars, phenolic compounds, acetic acid and other organic compounds that are mainly products of sugar degradation. The LS are the major component found in SSLs, having a concentration of about 400 g/L (Marques et al., 2009; Pateraki et al., 2013), followed by sugar monomers derived from hemicellulose hydrolysis having a concentration of approximately 150 g/L (Pateraki et al., 2013). The main sugar monomer derived from hardwoods is xylose (~ 100 g/L), followed by galactose (~ 26.3 g/L), glucose (~ 14.9 g/L), mannose (~ 6 g/L) and arabinose ( 2.3 g/L) (Marques et al., 2009; Pateraki et al., 2013). Acetic acid is also present in SSL in quantities of about 6 g/L and it is a product of sugar degradation or cellulose deacetylation (Palmqvist and Hahn-Hägerdal, 2000a). Among the volatile compounds, furfural and HMF are also present but in lower amounts (Marques et al., 2009; Pateraki et al., 2013; Pereira et al., 2013). More specifically, furfural is mainly a degradation product of xylose, whereas HMF is derived when hexoses are exposed at high temperatures (Palmqvist and Hahn-Hägerdal, 2000a). Formic acid (product of furfural and HMF degradation) and

levulinic acid (product of HMF degradation) could also be found in SSL but in traces (Palmqvist and Hahn-Hägerdal, 2000a). SSL also contains an important fraction of phenolic compounds, which are also products of lignin degradation during pulping. In the study of Marques et al., (2009), that employed GC-MS analysis of ethyl-acetate extracts of SSL, it was found that it was rich in aromatic acids, such as gallic, syringic and vanillic acids. The compositional analysis of SSL hinders its possible applications. Its high carbohydrate content makes it a good substrate for microbial bioconversion. Lignosulphonates are already a commercial product, but during the recovery of LS, sugars are destroyed. The development of a multifunctional process that would exploit all the main components of SSL, could transform the conventional process into a novel biorefinery.

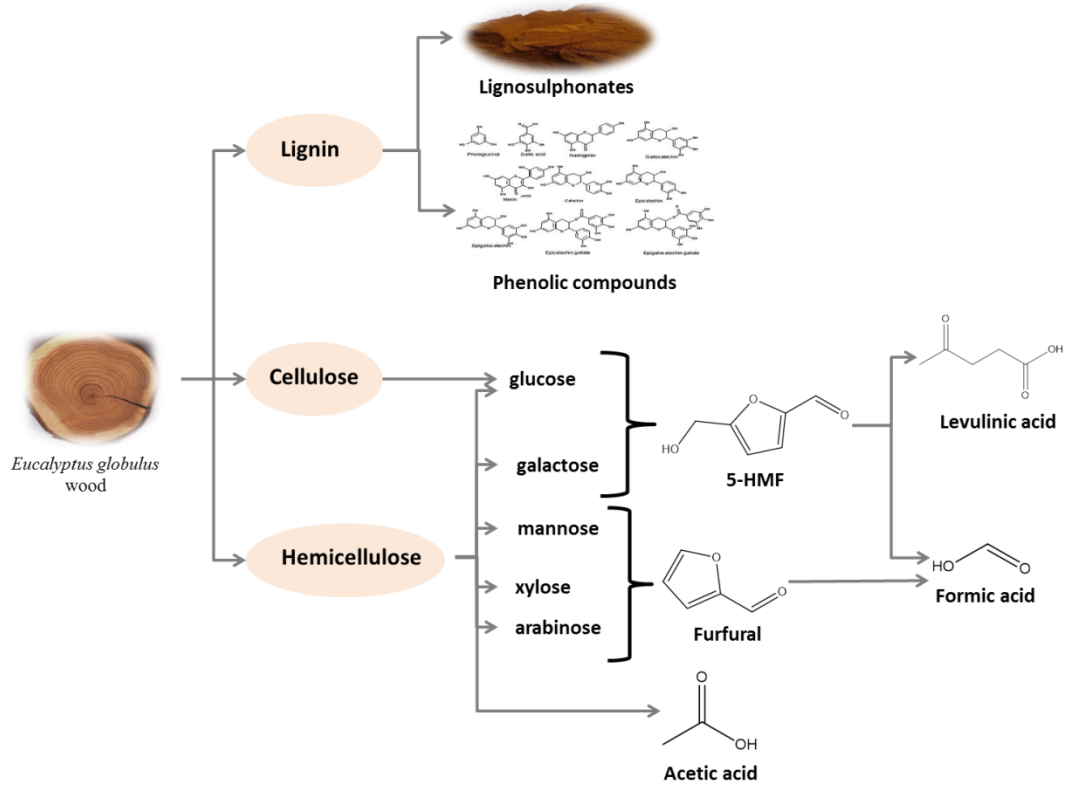



**Figure 2.3** Generation of inhibititors in SSL

*a) Antioxidants derived from SSL*

As previously mentioned, SSL contains an important phenolic fraction, which is derived from lignin degradation. The composition and the nature of the phenolic compounds that are present in SSL, strongly depends on lignin composition and the guaiacyl-syringyl ratio. Lignin is composed of monolignols, trans-coniferyl alcohol and trans-sinapyl alcohol that are methoxylated to different degrees, as well as trans-p-coumaryl alcohol that is a non-

methoxylated monolignol. The three building blocks forming the phenylpropanoid structure are generated from different combinations of the aforementioned monolignols. Guaiacyl monolignol, syringyl monolignol and p-hydroxyphenyl monolignol are lignin's building blocks (Balakshin et al., 2013). During pulping, the low molecular weight phenolics are released from the partial depolymerisation of these building units as sulphite units are introduced in the ether linkages. Syringaldehyde and vanillin are two examples of low molecular weight phenolic compounds derived from syringyl (S) and guaiacyl (G) units, respectively (Barakat et al., 2012).

There are not many studies in the literature about the phenolic compounds contained in the SSL. In the study of Marques et al., (2009), the main phenolic compounds present in the ethyl acetate extract of SSL were identified using GC-MS analysis. The main phenolic compound was gallic acid, followed by pyrogallol, ellagic acid, vanillic acid and syringic acid. One year later, Faustino et al., (2010b) studied the extraction of phenolic compounds as well as the antioxidant activity of the obtained extracts from Kraft and Spent sulphite liquors. The authors studied two different solvents (ethyl acetate and diethyl ether) as well as different pH values of the liquors. The ethyl acetate extracts from sulphite liquors exhibited higher total phenolic content (about 240 mg GAE/g) in comparison to the extracts from the kraft process (181.6 mg GAE/g). The antioxidant activity of the extracts derived from both processes was equally high. More specifically, when the extraction of the kraft liquors was carried out at pH value of 6 the antioxidant activity index (AAI) was 3.41, whilst in case of sulphite liquors the highest AAI value (3.29) was obtained at a pH of 2. AAI was proposed by Scherer and Godoy, (2009) according to which extracts having an index higher than 2 should be considered as strong antioxidants. The authors also used column chromatography in order to obtain different fractions, which were then analyzed with NMR. The fractions from the SSL extracts contained among others syringylacetone, syringic acid, acetosyringone and syringaldehyde. Llano et al., (2015) also evaluated various parameters affecting the extraction of phenolic compounds from SSL. The diethyl ether extract had a total phenolic content (TPC) equal to 6.31 GAE/L, with an AAI equal to 4.9. Ellagic and gallic acids were the main phenolics identified in the extract.

Phenolic compounds derived from lignin have various applications such as active ingredients in order to prohibit the oxidative degradation induced by high temperatures, light (both visible and ultraviolet) and oxygen in plastics, elastomers, bio-oils, plastics. Another use is as stabilizers of lubricants to prevent oxidation, or as antioxidants in adhesives, paints, dispersions (in biocides) even in packaging, as well as wood preservatives and in paper

making as an agent against yellowing (Balakshin et al., 2013; Willför et al., 2003). Phenolic resins are used as binders for thermal and acoustic insulators, glass fiber mats and printed circuit boards (Caillol, 2012,)

Besides their various applications, the removal of phenolic compounds from SSL, could increase its fermentability as they are well known for their inhibition towards microorganisms (Palmqvist and Hahn-Hägerdal, 2000a). The main inhibitory mechanism lies to their ability to pass into the biological membranes, resulting to integrity loss and subsequently affecting their ability to function as selective barriers (Heipieper et al., 1994).The inhibition of phenolic compounds towards fermenting microorganisms in discussed in a larger extent in CHAPTER 7.

*b) Lignosulphonates (LS)*

Lignosulphonates (LS) are salts of lignosulphonic acid resulting from lignin sulphonation, during the acidic sulphite pulping process. As a consequence, LS are lignin fragments with a specific amount of sulphonic groups, the number of which is highly depended on the concentrations of calcium and $SO_2$ used in the pulping process (Bjørsvik and Liguori, 2002). During sulphonation, lignin breaks down into its smaller fragments, which are also water soluble, liberating the cellulose fibers.

The chemical reactions taking place in the acidic sulphite pulping process are described in Figure 2.4,Figure **2.5** and Figure **2.6**. More specifically, the first reaction that takes place involves the protonation of the benzyl ether bond of lignin (Figure 2.4), followed by substitution in the same position with the nucleophile $HSO_3$-, leading to the formation of sulphonated lignin (LS) (Bjørsvik and Liguori, 2002). Due to the process conditions, more successive reactions could also occur leading to the formation of condensation products (Figure 2.5). These condensation products are formed when LS fragments lose their sulphite group, producing a carbocation that can be subjected to a Friedel-Craft alkylation reaction. Furthemore, α-hydroxysulphonic acid could also be produced via substitution of α-carbonyl groups and sulphonation of the aldehyde groups (Figure 2.6)

Chemical analysis of thin and thick sulphite liquors from *Eucalyptus globulus* wood carried out by Marques et al., (2009), revealed that LS were mainly composed of syringyl units, having a ratio of syringyl to guaiacyl equal to 81:19. Sulphonation was also high, with a substitution ratio ranging from 11-20% (w/w). Their molecular weight was low and varied

from 1000-1300 Da. Lignin condensation was mainly at C5 and C6 position in the aromatic rings.

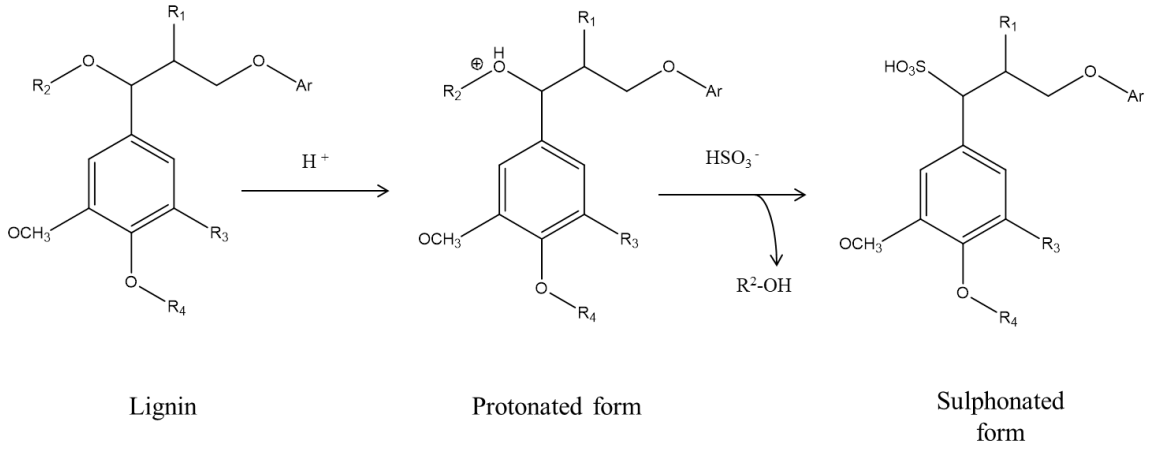



**Figure 2.4** Chemical reactions occurring in acidic sulphite pulping process during lignin sulphonation: protonation and then substitution of an $HSO_3$- group at the benzyl ether bond of lignin (Scheme adapted from Bjørsvik and Liguori, 2002).

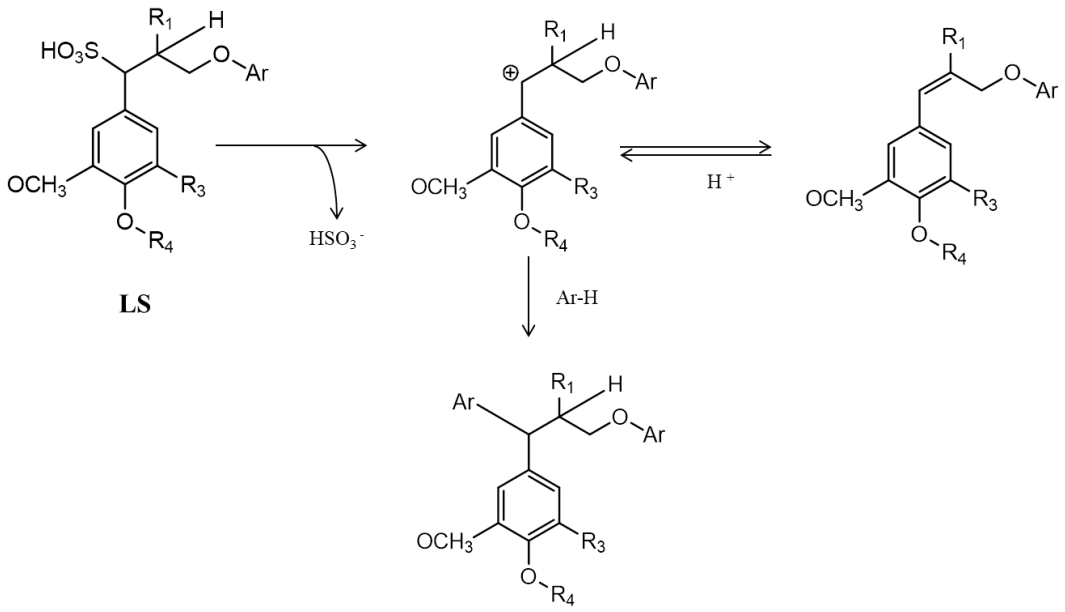



**Figure 2.5** Formation of condensation products from LS (Scheme adapted from Bjørsvik and Liguori, 2002).

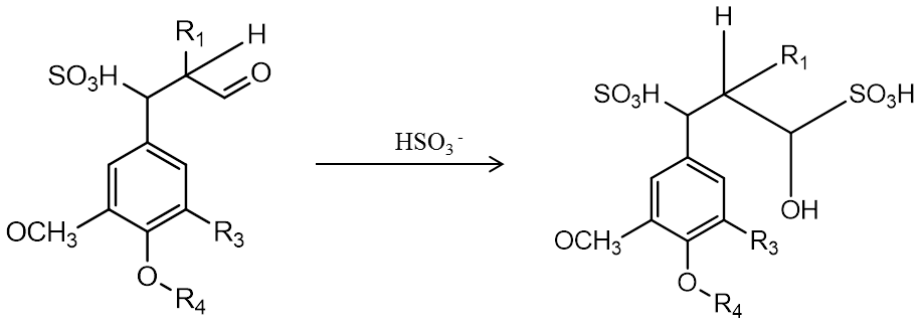



**Figure 2.6** Formation of α-hydroxysulphonic acid LS (Scheme adapted from Bjørsvik and Liguori, 2002).

Lignosulphonates are a product of major importance since it is already used in a variety of industrial applications, mainly as concrete plasticizers. LS also find application in the production of plasterboard and cement due to their water-bonding capacity. Another important application is in oil drilling mud, as LS can reduce the viscosity of mineral slurries, but also in dyes, pesticides and carbon black (Bjørsvik and Liguori, 2002). The most important fine chemical derived from oxidation and hydrolysis of LS is vanillin. Vanillin is used as flavoring agent in food and cosmetics, but also as a precursor for the synthesis of various second-generation fine chemicals, such as protocatechuic acid, veratric acid, veratralhehyde and protocatechualdehyde (Bjørsvik and Liguori, 2002).

The Howard process is applied to recover LS from SSL with a yield of 90 -95 %, via precipitation with sodium or calcium hydroxide. There are also studies proposing the use of other methods for LS recovery such as ultrafiltration and ion-exchange resins. Finally, LS are recovered in the form of calcium, magnesium, sodium or ammonium salts, depending on the base that was applied. Vanillin could also be produced via oxidation of in alkaline environment (Santos et al., 2011).

*c) Sugars present in SSL and their applications*

SSL contains an important amount of sugar monomers, derived mainly of the hydrolysis of hemicellulose during the pulping process. As a consequence, the main sugar monomer present in SSL is xylose, accounting the 74% of the total sugars. Galactose, glucose, mannose and arabinose are also present but in lower amounts. The high sugar content of the SSL renders it a potential substrate for microbial bioconversion. The fact that it is mainly composed of C5 sugars in combination to the presence of inhibitory compounds in the liquor, has hindered its applications so far. The numerous inhibitory compounds could also

have synergistic effects, leading to low, or even no microbial growth. Phenolic compounds are reported to present synergistic effects (Alexandri et al., 2016a), and furans in combination to organic acids, mainly acetic acid have been proven to be highly inhibitory towards fermenting microorganisms (Coz et al., 2017). In order to enhance the fermentability of SSL, various detoxification methods were proposed in order to minimize or eliminate the inhibitory compounds (Table 2.1).

SSL has been already used as fermentation substrate mainly for the production of ethanol. In order to minimize the inhibitory effect of the various components of SSL, different detoxification methods are proposed and are shortly reviewed in the Table 2.1. Besides ethanol, recent publications are proposing the use of SSL as substrate for the production of short-chain organic acids (Queir et al., 2017), as well as for succinic acid (Pateraki et al., 2016b).

**Table 2.1** Fermentations using Spent Sulphite Liquor (SSL) derived from hardwoods

| Pretreatment method | Strain | Product | Reference |
|---|---|---|---|
| $CaO,CO_2$ and ion exchange resins | *Pichia stipitis* | Ethanol | Takahashi et al., 2013 |
| pH neutralization with KOH, aeration with compressed air and centrifugation | Mixed microbial culture (MMC) | Short-chain organic acids (SCOAs) | Queir et al., 2017 |
| No pre-treatment/strain adaptation | *Scheffersomyces stipitis* | ethanol | Pereira et al., 2015 |
| No pre-treatment | Genetically modified *S. cerevisiae* | ethanol | Novy et al., 2013 |
| No pre-treatment | Genome shuffled *Pachysolen tannophilus* | ethanol | Harner et al., 2015 |
| Ion exchange resins | *S. stipites* NRRLY-7124 | ethanol | Xavier et al., 2010 |
| Biological treatment with *P. variotii* | *S. stipites* NRRLY-7124 | ethanol | Pereira et al., 2012 |
| Ultrafiltration/nanofiltration | *A. succinogens* *B. succiniciproducens* | Succinic acid | Pateraki et al., 2016b |

### 2.1.2. Pretreatment of SSL and other SSL-like lignocellulosic hydrolysates

Various detoxification methods are proposed in the literature based on physical processes (evaporation, membrane filtration), chemical processes (such as overliming, activated charcoal treatment, solvent extraction, ion exchange resins) as well as biological treatment (mainly enzymatic treatment using laccases) (Chandel et al., 2011). The detoxification methods aim to partially or completely remove specific compounds. Moreover, the selection of the most adequate detoxification method, is highly depended on the selected strain, as each microorganism exhibits different tolerance towards specific inhibitory compounds (Mussatto and Roberto, 2004; Palmqvist and Hahn-Hägerdal, 2000a, 2000b).

*a) Evaporation*

Evaporation is a physical method that could be employed in order to remove volatile components of the lignocellulosic hydrolysate such as acetic acid, furfural and HMF, and it is considered as a primary detoxification stage. In the study of Larsson et al., (1999) almost 90% of furfural was successfully removed from wood hemicellulosic hydrolysate when vacuum evaporation was employed. Llano et al., (2017) reported a removal of total volatile compounds equal to 8.61%. The removal of acetic acid and furfural was critical for the efficient production of xylitol from rice straw hydrolysate and other hemicellulosic hydrolysates (Converti et al., 1999; Silva and Roberto, 1999). Evaporation was also employed as a primarily detoxification step, before activated charcoal in sugarcane bagasse hemicellulosic hydrolysate in the study of Rodriguez et al. (2001). The authors observed a furfural removal of 98 %, whilst acetic acid was only partially removed. Butanol production by the strain *Clostridium beijerinckii* grown in wood pulping hydrolysate was significantly improved when ion exchange resins treatment was coupled with evaporation (Lu et al., 2013). Finally, Dalli et al., (2017) reported that combination of evaporation with solvent extraction led to significant removal of acetic acid (87.5%) and furfural (97.4%), producing a more detoxified substrate for xylitol production by an immobilized *Candida tropicalis* strains.

*b) Liming and Overliming*

Overliming is a very common detoxification technique, applied to various hydrolysates prior to fermentation. This method involves precipitation of toxic components via treatment of the hydrolysate with an alkali (mainly $Ca(OH)_2$ or NaOH), at high pH values (preferable around 9-10) and temperatures (Coz et al., 2016). Aldehydes and ketones are efficiently removed when this method is employed, resulting to a hydrolysate with enhanced

fermentation properties (Martinez et al., 2001; Mussatto and Roberto, 2004; Palmqvist and Hahn-Hagerdal, 2000). A significant disadvantage of this method is the fact that generates notable amounts of calcium sulphate precipitate. Another important drawback is that depending on the severity of the process, high co-precipitation of sugars is also observed. For this reason, overliming conditions should be adjusted to the material that would be treated (Meinita et al., 2012).

When overliming is employed to SSL, lignosulphonates could also be precipitated as they form calcium lignosulphonates that are insoluble in water (Llano et al., 2017). Besides LS, phenolic compounds, HMF and furfural are also precipitated. Llano et al., (2017) tested the effect of two different alkali, namely $Ca(OH)_2$ and $NH_4OH$ for the removal of LS, furfurals and phenolic compounds from SSL derived from *Eucalyptus globulus*. The use of $Ca(OH)_2$ resulted in higher LS removal (36.5%) in comparison to $NH_4OH$ (13.3%), but also to the higher sugar losses (11.5%). A removal of 36.5% of phenolic compounds and 77.6% of furfural was also observed.

*c) Adsorption using activated charcoal or ion exchange resins*

Adsorption is a detoxification technique that is implemented in order to separate specific compounds from hydrolysates. The main advantages of the adsorption techniques involve the relatively easy process operation and scale-up, as well as the possibility of regeneration and re-use, minimizing the overall cost (Coz et al., 2016). It is also considered a state-of-the art method for product separation and purification in biorefineries (Datta,1992).

The treatment of hemicellulosic or lignocellulosic hydrolysates using activated charcoal has been widely studied in the literature as it results in high inhibitor removal yields while sugar losses are much lower when compared to overliming (Canilha et al., 2008; Chandel et al., 2007; Mussatto and Roberto, 2001). The efficiency of this process is depended on various process parameters such as temperature, the pH of the hydrolysate, contact time as well as the liquid to activated charcoal ratio (Prakasham et al., 2009).

Detoxification of hydrolysates using ion exchange resins is quite effective in terms of removing inhibitory compounds generated from lignin degradation, such as acetic acid, furfural and phenolic compounds (Chandel et al., 2011). Actually, the detoxification effect is so high that the treated hydrolysate could be comparable to a pure sugar solution (Barbosa-Pereira et al., 2013). Treatment of hemicellulosic hydrolysates of *Eucalyptus* wood with ion exchange resins led to enhanced xylitol production by the strain *Candida guilliermondii*

(Villarreal et al., 2006). The highest disadvantage of ion exchange resins is the high cost that still hinders their industrial applications (Chandel et al., 2011). Combination of cation and anion exchange resins was tested for the removal of $Mg^{2+}$ and other cations as well as carboxylic acids and phenolic compounds (Fernandes et al., 2012; Xavier et al., 2010).

Llano et al., (2017) studied a cationic (Dowex 50WX2) and an anionic (Amberlite IRA-96) resin in order to assess the effect on inhibitor removal from spent sulphite liquor. The authors tested the effect of each resin separately and in series. The most promising results were given by the anionic resin, achieving removal yields of 96.1% of LS, 98.1% of total phenolic compounds, 61.3% of acetic acid, 65% metals and almost 100% removal of furfurals. Another advantage of this detoxification method is the possibility to regenerate and re-utilise the resins and recover the adsorbed compounds. As it is already stated, LS are commodity chemicals and phenolic compounds could also be valorised as end products since they exhibit a considerable antioxidant activity (Faustino et al., 2010; Llano et al., 2015).

*d) Solvent Extraction*

The extraction of inhibitory compounds with organic solvents is another proposed method in order to increase the fermentability of hemicellulosic hydrolysates. Ethyl-acetate is the most well-studied solvent, as it highly removes the phenolic compound fraction generated by lignin degradation (Zhuang et al., 2009). Cruz et al., (1999) studied the solvent extraction of *Eucalyptus globulus* acid hydrolysates with ethyl acetate and diethyl ether, resulting to a removal of phenolic compounds of about 84%. Solvent extraction with ethyl acetate has also been employed for the removal of phenolic compounds from pulp liquors of both kraft and acidic sulphite pulping process (Faustino et al., 2010; Llano et al., 2015). Extraction efficiency is highly related to the pH of the hydrolysate as well as the liquid-to-solvent ratio, whereas extraction time and temperature seem to have a lower effect (Cruz et al., 1999; Faustino et al., 2010; Llano et al., 2015). Faustino et al., (2010) studied the solvent extraction of kraft and acidic spent sulphite liquors in different pH values. In spent sulphite liquors, decreasing the pH yielded to the highest phenolic extraction, as well as increased antioxidant activity of the extracts. Llano et al., (2015) evaluated different solvents (diethyl ether, hexane, benzene, chloroform and trichloroethylene) and different extraction conditions (liquid-to-solvent ratios, extraction time, and settling time) for the removal of phenolic compounds, acetic acid, HMF and furfural from spent sulphite liquor. The authors reported a removal of 18.5% HMF, 12.5% furfural and 41.7% acetic acid when solvent extraction with diethyl ether was employed.

*e) Membrane separation*

Membrane-based separation technologies are becoming more and more attractive since they present high separation efficiency, low cost and easy scale up (Datta et al., 2014; Pinelo et al., 2009; Li et al., 2008). Membrane technologies find applications in food, chemical and pharmaceutical industries (Coz et al., 2016). Depending on the target compound that needs to be separated a different membrane material could be applied, having also the desirable pore size. The most common materials used for membrane manufacturing is silica, alumina, cellulose acetate, polyamides and polyethersulfone (Coz et al., 2016). Microfiltration (MF), with membrane pore size of 0.1-10 μm involves the separation of fine particles. Ultrafiltration (UF) is applied mainly for protein separation and macromolecules with a membrane pore size of 1-100 nm. Finally, in nanofiltration (NF) technologies, the membranes used have a pore size of about 0.5-1 nm. Separation is achieved via a combination of sieving and molecule's charge, due to ionisable groups on the surface of the membrane (Zaman et al., 2017).

Membrane separation technologies have been well-established for the treatment of wastewaters and other effluents (Afonso et al., 1992). Ultrafiltration and reverse osmosis have also been applied for separation and purification of LS or for concentration of diluted spent sulphite liquor (Afonso et al., 1992). In the study of Neytzell-De Wilde, (1987), UF membranes were tested for LS recovery from spent liquor derived from calcium bisulphite pulping process of eucalyptus wood. Even though a permeate stream with high sugar concentration was obtained and at the same time the retentate stream was rich in LS, SSL caused a series of problems throughout the process, like flux drop and membrane fouling. More than 80 % of LS present in spent sulphite liquor were separated in the retentate stream in the study of Bhattacharya et al., (2005), using membranes with a MWCO of 20 to 100 kAMU. In Norway and Finland, there are pulp and paper industries operating ultrafiltration plants that produce high molecular weight LS (Fernández-Rodríguez et al., 2015). Membrane separation of LS has some important benefits such as the easy handling of the waste, as no pH or temperature adjustment is required, as well as the possibility to produce different LS streams according to their molecular weight, based on the membrane MWCO applied (Jönsson et al., 2008).

## 2.2. Sugar beet pulp (SBP)

Sugar beet (*Beta vulgaris*) is a biennial plant, harvested in autumn (from September to October) and together with sugar cane, is one of the main sugar sources worldwide. It is mainly cultivated in Northern Europe and North America, as it yields better in low temperate climates. The total sugar beet production worldwide is quite stable. Sucrose is accumulated in the roots of sugar beets during winter, and its cultivation is often after cereals in order to increase both yields and quality. The process of sugar extraction includes various steps starting by diffusion, juice purification, evaporation, crystallization, pelletization of the remaining pulp and also sugar recovery from the molasses. More specifically, the process of sugar extraction firstly involves the washing and cutting of sugar beets into thin cossettes. The extraction of sugar is then carried out through the process of reverse osmosis, using hot water in diffusion towers. From this step, the raw juice is obtained (Figure 2.7). The spent beet pulp is dried, pelletized and sold as animal feed. Then, the calcium carbonate and carbon dioxide are used in order to separate the non-sugar substances from the raw juice. After filtration, a clarified juice contained 60% of sugar is obtained. This juice is then subjected to evaporation until its sugar contained reaches approximately 70% (thick juice). Further concentration of the thick juice leads to the formation of the sugar crystals. The crystals are separated from the syrup via centrifugation, leaving another by-product, the molasses. After centrifugation, sugar can be re-dissolved and let to crystallize again, in order to reach its refined from. The final product is dried and cooled (Figure 2.7).

This process generates two by-product streams: the sugar beet molasses (SBM) and the sugar beet pulp (SBP), which is the solid residue of the process. Leaves and foliage are also considered waste streams generated from the crop processing, that are mainly used for biogas production (Scoma et al., 2016).

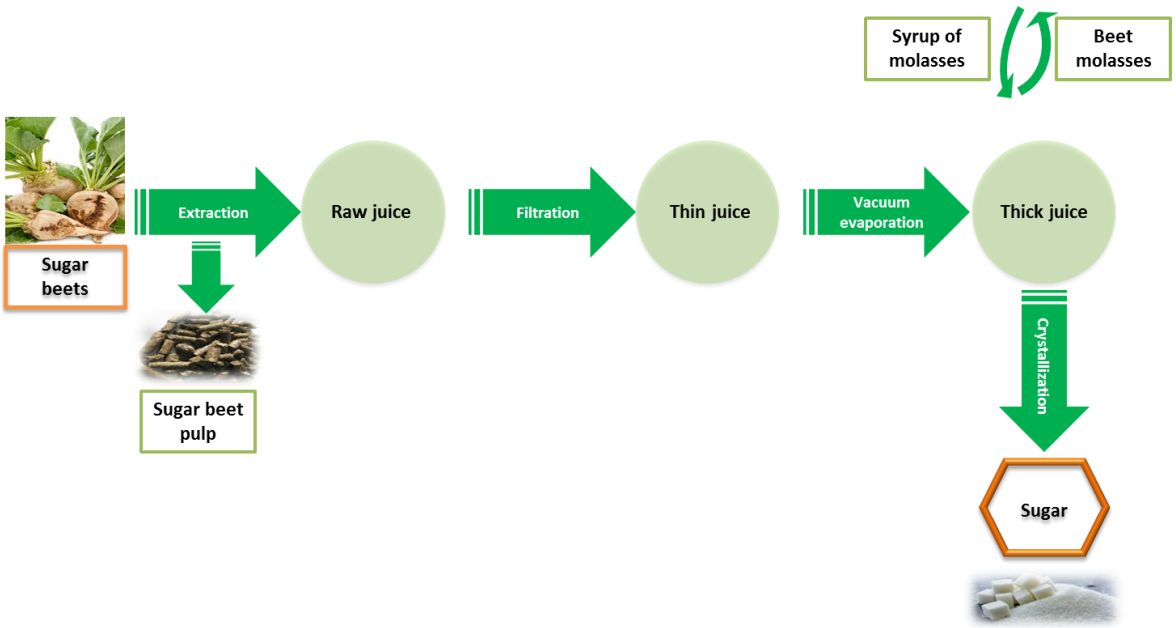



**Figure 2.7** Extraction of sugar from sugar beets and the generation of by-product streams

### 2.2.1. Composition of SBP

There are two main by-product streams from the sugar industry with sugar beet as raw material: Sugar beet molasses (SBM) and Sugar beet pulp (SBP).

Sugar beet molasses (SBM) are high concentration glucose syrup, having a density of approximately 1.4 g/cm. Besides sucrose, SBM could also contain nitrogen, accounting about 2% of the dry weight (Scoma et al., 2016).

Sugar beet pulp (SBP) is mainly consisted of cellulose (20-25%), hemicellulose (25-36%), pectins (20-25%), proteins (10-15%), while it is also characterised by the very low lignin content (1-2%) (Micard et al., 1996) (Table 2.2). The current practice for SBP processing involves drying and pelletizing in a cost-intensive process and then it is sold as low-value animal feed. Zheng et al., (2013) reported in some specific places, the treatment of SBP amounts about 30-40% of the overall energy costs in the sugar industry. For this reason, the valorisation of SBP through microbial conversion to bio-based materials would minimize the cost in the sugar industry, giving at the same time more value to the crop.

**Table 2.2** Chemical composition of SBP according to the literature

| Component (%) | Zheng et al., 2013 | Bellido et al., 2015 | Berlowska et al., 2016 | Olmos & Hansen, 2012 | Martínez et al., 2009 |
|---|---|---|---|---|---|
| Cellulose | 22.70 | 20.34 | 33.6 | 23 | 21.1 |
| Hemicellulose | 36.64 | 33.7 | 40.5 | 44.8 | 35.5 |
| Xylan | 5.14 | 1.73 | - | - | - |
| Galactan | 5.92 | 21.81 | - | - | - |
| Arabinan | 23.73 | 9.54 | - | - | - |
| Mannan | 1.85 | 0.62 | - | - | - |
| Pectin | 22.84 | 27.34 | - | 9.8 | - |
| Acid insoluble lignin | 1.04 | 1.93 | - | - | - |
| Acid soluble lignin | 0.12 | 0.03 | - | | |
| Lignin | | | 1.4 | 4.5 | 3.4 |
| Crude protein | 11.42 | 7.36 | 0.12 | - | - |
| Ash | 2.51 | 3.41 | - | - | - |
| Other | 2.73 | - | - | - | - |

*a) Pectins*

Pectins are considered an important structural polysaccharide of plant cell walls that is found in fruits like citrus and apples and in sugar beets. The structural unit of pectins is D-galacturonic acid, which is connected to neutral sugars in the side chains, mainly L-rhamnose, arabinose, galactose and xylose (Ma et al., 2013). The D-galacturonic acid units are linked with a-(1-4) bonds, while they can be also methyl-esterified at 6-position and also acetyl-esterified at *O*-2 and *O*-3 (Ma et al., 2013). Pectin is an important commodity, finding used in textile industry and in food industry where it serves as thickener, emulsifier and stabilizer (Liu

et al., 2006). Besides there various applications, pectins could also be converted to sugar monomers, that could be further valorized via bioconversions for the production of bioethanol and biogas (Doran et al., 2000; Hutnan et al., 2000). There are various enzymes (polymethylgalacturonase, (endo)-polyglalacturonase pectin depolymerase, pectinase, exopolygalacturonase, exopolygalacturanosidase) able to hydrolyze pectins by introducing a water molecule in the polygalacturonic acid chain (Jayani et al., 2005). Moreover, in sugar beet, the arabinan side chains contain ferulic acid residues. Actually, about of 45-50% of ferulic acid is bonded also with the galactan residues to the *O*-6 position (Voragen et al., 2009).

*b) Antioxidants from Sugar beet*

In plants, phenolic compounds are secondary metabolites synthesized via the shikimate pathway. As it is already mentioned, pectins in sugar beets are connected to ferulic acid. Ferulic acid is therefore the most abundant phenolic acid present in sugar beets and it is mainly concentrated in plant's cell-wall polysaccharides (Thibault et al., 1998). Sugar beet pulp is considered a potential source of ferulic acid that founds various applications not only as antioxidant, but also for the synthesis of vanillin (Aarabi et al., 2016) . According to the study of Aarabi et al., (2016), high amounts of ferulic acid can be extracted from SBP using alkaline hydrolysis, whereas extraction with organic solvents (such as methanol) leads to pure extraction of this phenolic acid.

Mohdaly, (2013) studied the total phenolic content and the antioxidant potential of sugar beet pulp ethanolic and methanolic extracts. According to their findings, the methanolic extract presented a TPC value of 1.79 mg GAE/g DW (from which 1.24 mg QE/g DW were total flavonoids), whilst the ethanolic extract contained 1.52 mg GAE/g DW (from which 0.91 mg QE/g DW were total flavonoids). The corresponding antioxidant activity of the extracts, estimated by the DPPH radical, was 62% and 42%, respectively, meaning that both extracts exhibited high antioxidants activities comparable to the ones of synthetic antioxidants (BHA and BHT). The antioxidant activity of plant extracts can be explained by their ability to act as hydrogen donors. Antioxidants are able to interrupt the free radical chain oxidation by donating hydrogen, resulting to the formation of a stable end-product, stopping the lipid oxidation (Madhava Naidu et al., 2008). HPLC results from the same work showed that the main phenolic compounds present in these extracts were chlorogenic, caffeic, 4-hydroxybenzoic, p-coumaric, vanillic, trans-*o*-hydroxycinnamic, cinnamic and sinapic acids. The same extracts were also tested in stabilization of sunflower oil.

These results indicate that SBP is a potential source of antioxidants that could be considered a value-added co-product. The extraction of phenolics could enhance even more the market potential of the initial crop.

*c) Cellulose and hemicellulose of SBP*

Cellulose is the main constituent of SBP and of the plant cell wall in general. It is already mentioned in previous chapter that cellulose is an homopolymer consisted of β-D-glucopyranose residues linked with β-(1,4) glycosidic bonds. The degree of polymerization of cellulose varies from 10,000 glucopyranose units to unit to 15,000 in cotton (Agbor et al., 2011). Cellobiose is a disaccharide formed by two glucose units and it is the repeating unit of cellulose. Cellulose chains are gathered together to form microfibrils, which are stacked together to produce the cellulose fibres. The general structure of cellulose is determined by covalent bonds, hydrogen bonds and Van der Waals. The interchain hydrogen bonds also determine the crystalline or the amorphous structure of the cellulose (Laureano-Perez et al., 2005).

Hemicellulose accounts the 20-50% of lignocellulosic biomass and the main difference to cellulose is its chemical heterogeneity. As it is also already stated in previous chapter, hemicelluloses are branched polymers consisted mainly from C5 sugars (xylose and arabinose), hexoses (glucose, galactose, mannose), which can be also acetylated (Agbor et al., 2011). Their molecular weight is in general lower than cellulose and they bear branches with side-chains, rendering hydrolysis easier (Saha, 2003). The composition of hemicellulose can differ depending on the type biomass. Hemicelluloses in softwoods contain mainly glucomannans, whilst in straw and grasses they are mainly formed by xylan. Xylans in plants are heteropolysaccharides, consisted mainly of xylose but also from arabinose, glucuronic acid, acetic, ferulic and p-coumaric acids (Agbor et al., 2011) In SBP, the main xylan is manly composed from arabinose, followed by galactose, xylose and galactose (Table 2.2). Xylan can be easily degraded under acidic or alkaline conditions (Agbor et al., 2011).

Generally, in comparison to cellulose and lignin, hemicellulose is the most easily hydrolysable biomass component, as it is also characterized by a high thermal sensitivity (Agbor et al., 2011). The removal of hemicellulose is necessary in order to improve cellulose digestibility. Pretreatment severity should be optimized in order to avoid or minimize the formation of some toxic compounds such as furfural, HMF and acetic acid that could have an inhibitory effect towards fermenting microorganisms (Palmqvist and Hahn-Hagerdal, 2000b).

For this reason, hydrolysis optimisation is a combination of high sugar recovery together with the lowest formation of inhibitory compounds.

Finally, lignin is the third most abundant polymer in biomass and it is responsible for the rigidness of plant cell walls. It also serves as a barrier to microbial attack and the oxidative stress. More details about lignin are discussed in previous chapter. SBP contains low amounts of lignin (Table 2.2), which is a very important advantage for its utilization as renewable feedstock. Lignin removal is mandatory in order to increase biomass digestibility. The severity of the process depends on the amount of lignin present in the biomass. Delignification is employed with different chemicals and results in biomass swelling and disruption of lignin structure (Agbor et al., 2011).

In order to valorize SBP via microbial fermentation to bio-based chemicals and fuels, pretreatment is required, in order to convert the structural polysaccharides into fermentable sugars. Its low lignin content is an important advantage but there are also many other factors affecting the degree of hydrolysis of the structural polymers. These factors involve the crystallinity and the degree of polymerization of cellulose, the fiber strength as well as the cellulose covering by hemicellulose (Mosier et al., 2005). Below, some of the most common pretreatment methods of lignocellulosic biomass are reviewed, emphasizing in dilute acid pretreatment and enzymatic hydrolysis as these are the well-employed methods in SBP. Table 2.3 presents the pretreatment methods that have been applied so far on SBP.

### 2.2.2. Pretreatment methods

Agbor et al., (2011) classified the biomass pretreatment methods into four different categories: physical, chemical, biological and combination of treatments. The chemical pretreatment could be further classified into acidic, alkaline and neutral according to the pH used for the process (Galbe and Zacchi, 2007). The use of more than one pretreatment method is most of the time necessary as combination is required in order to hydrolyse cellulose, hemicellulose and lignin.

The adequate pretreatment method is characterized by various parameters, starting by low capital and operational costs. It should lead to the highest sugar recovery as possible and at the same time with no or low amounts of lignin and sugar degradation products that could inhibit microbial growth or/and enzyme activity (Chandra et al., 2007). Other important parameters involve the cost and the possibility of regeneration of the catalyst, production of lignin-based value-added products and efficient hemicellulose hydrolysis in order to minimize

the need of additional enzymes (hemicellulases) (Galbe and Zacchi, 2007; Mosier et al., 2005).

Many different pretreatment methods have been studied and evaluated in the literature and many are still under development (Agbor et al., 2011). It is, in general, difficult to compare the different pretreatment processes, but in a biorefinery, the estimation of the mass balances could be applied in order to assess the efficacy of a given pretreatment (or combination of methods) method in a given feedstock (Agbor et al., 2011). According to Agbor et al., (2011), the evaluation of the efficiency of a pretreatment method could be carried by:

- Sugar analysis in the obtained hydrolysate, combined by the determination of the carbohydrate content of the remaining solids after the pretreatment
- The efficiency of the enzymatic hydrolysis
- The fermentability of the obtained hydrolysate
- The possibility of obtaining different streams that lead to more value added products

*a) Mechanical treatment*

Grinding, milling, chipping and shredding are most the commonly used methods for mechanical treatment of lignocellulosic biomass (Palmowski and Muller, 1999). According to Sun and Cheng, (2002), these methods are employed in order to increase the surface area of the biomass and decrease cellulose crystallinity and polymerization degree. After chipping, particle size is about 10-30 mm, whereas after grinding and milling particle size can reach 0.2-2 mm. Chipping increases heat and mass transfer, while grinding and milling are more efficient for the reduction of cellulose crystallinity (Agbor et al., 2011).

Important drawback of the mechanical pretreatment is the energy requirements. Of course the energy required depends on the specific biomass to be treated. For example, hardwood milling demands higher energy outputs than agricultural residues like SBP (Agbor et al., 2011). Even though milling is reported to positively affect the product of various fermentation products, the energy demands make its application on industrial scale non-feasible (Hendricks and Zeeman, 2009). Latest studies, have proposed that if milling is applied after chemical pretreatment, energy consumption could be lower (Zhu et al., 2010, 2009).

*b) Biological pretreatment*

Biological pretreatment is mainly carried out with white-rot fungi but also with soft- and brown-rot ones as they can produce enzymes that can degrade both lignin and hemicellulose. White-rot fungi seem to be the most efficient as they produce laccases; enzymes capable of hydrolyzing lignin (Agbor et al., 2011). Some examples of white-rot fungi already studied in the literature are *Phanerochaete chrysosporium*, *Phlebia radiate*, *Dichmitus squalens*, *Rigidosporus lignosus* (Hatakka, 1994). The main impediment of this method is the hydrolysis time that could reach days to achieve a significant result, even though delignification but these fungi can be selective and effective (Hatakka, 1994).

*c) Physicochemical pre-treatments*

- *Dilute acid pretreament (acidic hydrolysis)*

Acid pretreatment is one of the most common methods for hemicellulose hydrolysis of lignocellulosic biomass. The process involves the use of an inorganic acid (mainly sulphuric acid but HCl and phosphoric acid are also employed) in order to hydrolyze hemicellulose into its structural monosaccharides. This step is carried out in order to increase the accessibility of enzymes to cellulose. Depending on the substrate, acid hydrolysis will be carried out either using low acid concentration at high temperatures, either at high acid concentrations at lower temperatures. Operating at high acid concentrations and at lower temperatures is consider more economic, however this practice generates some problems such as equipment damaging (if high corrosive acids are employed), sugar degradation that in the end leads to the generation of inhibitory compounds (such as furans and acetic acid) as well as difficulty in acid recovery. On the other hand, at higher temperatures, sugar degradation products such as furfural, could be further be degraded to toxic compounds such as formic and levulinic acids (Larsson et al., 1999). Nevertheless, this method has been widely employed for the hydrolysis of hemicellulosic biomass, as high hydrolysis yields are achieved. In the study of Hsu et al., (2010), 1 % (w/w) $H_2SO_4$ was used for the pretreatment of rice straw. Hydrolysis was carried out for 1-5 min at 160 or 180 $^oC$. This method together with the enzymatic hydrolysis of cellulose resulted in 83 % yield of total sugars. Lu et al., (2009) used the same acid in order to hydrolyse rapeseed straw. Pretreatment was carried out for 10 min at 180 $^oC$, followed by enzymatic hydrolysis. The combination of both methods resulted in 75.12 % of hemicellulose and 63.17 % of cellulose hydrolysis to xylose and glucose respectively. Pappas et al., (2014), studied different concentrations of $H_2SO_4$ and at different temperatures (110 and 130 $^oC$) for different hydrolysis times (30 and 60 min) in order to pretreat *Phalaris aquatica* L. before

enzymatic hydrolysis. Hydrolysis using 2% (w/w) sulfuric acid for 60 min and at 130 $^{o}$C resulted in the highest glucose yield (85.05%).

Dilute acid pre-treatment has been tested not only at lab but also at pilot scale. In the USA, the National Renewable Research Laboratory (NREL) treats about 1 ton per day of corn stover in a pilot scale process, using dilute acid pretreatment (Jørgensen et al., 2006). The company SEKAB in Sweden uses the same method in a pilot plant for the hydrolysis of wood residues. The company DONG Energy in Denmark operates since 2005 a pilot plant with 100-1000 kg/h capacity, using various pre-treatment methods, including acid hydrolysis with $H_2SO_4$ (Jørgensen et al., 2006).

- *Steam pre-treatment or steam explosion (SP or SE)*

In this process, lignocellulosic biomass that is already mechanically treated is subjected to hydrolysis using high pressure saturated steam at high temperatures (160-240 $^{o}$C). Hemicellulose is mainly solubilized via this process, whilst lignin's structure is also altered due to the high temperatures employed. As a result, cellulose becomes more accessible to enzymatic attack.

The main advantages of this process include the limited use of chemicals, the low energy consumption and the low environmental impact. On the other hand, the disadvantages, depending on the treated biomass, are: insufficient hydrolysis yield, generation of inhibitory compounds at high temperatures, and loss of soluble sugars during hydrolysate washing (Agbor et al., 2011). Nevertheless, steam explosion has been already tested at pilot scale in NREL pilot plant in Golden and SEKAB in Sweden (Agbor et al., 2011).

- *Liquid hot water pre-treatment (LHW)*

LHW pre-treatment concept is similar to steam explosion, with the only difference that hot water (optimum temperatures ranging from 180- 190 $^{o}$C) is used instead of steam. This process is also known as solvolysis or aquasolv. Both hemicellulose degradation and delignification are achieved with this method, while cellulose is more accessible to enzymatic hydrolysis (Yang and Wyman, 2004). The formation of acetic acid due to the cleavage of *O*-acetic groups in hemicellulose assists the overall process of sugar release from the substrate (Mosier et al., 2005). At the same time, depending on the severity of the process, unwanted aldehydes could also be formed, but if the pH is maintained in the range of 4 and 7, the

concentration of toxic compounds is reported to be relatively low (Kohlmann et al., 1995). Mosier et al. (2005) scaled up this process in a 163 L reactor for the pretreatment of corn fibre slurry, showing that this process could be employed for large scale applications.

- *Ammonia fibre/freeze explosion (AFEX)*

The main characteristic of AFEX is the use of liquid ammonia at high pressures, but lower temperatures (> 90 $^{o}$C) in comparison to steam explosion and LHW. When this process is applied to the lignocellulosic biomass, delignification as well as alteration of cellulose crystallinity are carried out. It is reported that when this pre-treatment step is employed, the yield of the enzymatic hydrolysis of cellulose is almost close to the theoretical (Foster et al., 2001; Kim and Lee, 2002). AFEX presents many advantages: the ammonia used could be easily recovered and recycled; the mild conditions used led to minor or no formation of inhibitors; this method is highly selective towards lignin. However, this process is not effective at high lignin concentrations (18-30%) and the cost of ammonia is an important drawback on its large scale application (Agbor et al., 2011).

- *Lime pre-treatment (LP) and wet oxidative pre-treatment (WOP)*

During lime pre-treatment, hemicellulose and lignin are solubilised with aqueous $Ca(OH)_2$ at relatively low temperatures (25-130 $^{o}$C) and pressures. In wet oxidative pre-treatment, an oxidizing agent is used (oxygen, hydrogen peroxide or even water) in order to achieve delignification of the biomass and hemicellulose hydrolysis (Agbor et al., 2011). The main advantage of both methods is the low cost. Lime has low value and it is easy to recover. The low temperatures applied minimize the energy inputs. Moreover, delignification yields are quite high (about 80%) even in lignocellulosic materials rich in lignin content (like hardwoods) (Wyman et al., 2004). However, both methods lack selectivity leading to hemicellulose and cellulose losses. Conditioning (washing and neutralisation) is required rendering downstream process difficult. Finally, during delignification, aromatic compounds could be produced that are important inhibitors for the fermentation of the obtained hydrolysate (Hendriks and Zeeman, 2009).

- *Organosolv pre-treatment*

This process is carried out using solvents with low boiling point (such as methanol and ethanol), alcohols with high boiling point (such as glycerol and ethylene glycol) or other types of organic reagents (such as organic acids, ethers or ketones) at elevated temperatures ranging from 100-250 $^{o}$C. When alcohol is utilised, delignification is carried out to a large extend and almost complete hemicellulose hydrolysis is achieved (Angbor et al., 2011). This method is highly selective resulting in three different streams: a lignin fraction, a hydrolysate containing monosaccharides derived from hemicellulose degradation and a solid fraction containing cellulose, with increased digestibility (Angbor et al., 2011). The main disadvantage of this process is the highest cost of all the pre-treatment methods reviewed so far. Moreover, inhibitory compounds could be formed, making the hydrolysate an unsuitable substrate for microbial fermentation. Safety and environmental risks are also important disadvantages of this process.

- *Carbon dioxide ($CO_2$) explosion pre-treatment (CDE)*

In this process, supercritical $CO_2$ is introduced under high pressure (1000-4000 psi) to the lignocellulosic biomass. Depending on the biomass to be treated, high temperatures (up to 200 $^{o}$C) are applied. For the successful hemicellulose hydrolysis, it is important that the biomass will have some moisture (Kim and Hong, 2001). Significant benefits of the process are the low cost of $CO_2$ and the low formation of inhibitors. Nevertheless, the high cost of the equipment needed due to the high pressures is still an important impediment for the large scale application of the process (Agbor et al., 2011).

- *Ionic liquid pre-treatment (IL)*

Treatment with ionic liquids is a novel method and involves the use of solvents that are characterised by low melting points (below 100 $^{o}$C), high thermal stability, polarity and are mainly anions (Agbor et al., 2011). The most commonly used ionic liquids for biomass pre-treatment are imidazolium salts. A possible mechanism of action suggests that ionic liquids disrupt the biomass as they compete with the polymers for hydrogen bonding (Moulthrop et al., 2005). The pre-treatment with ILs is considered an environmental friendly method as the solvent can be easily recovered via reverse osmosis, pervaporation, salting out or ion exchange chromatography (Agbor et al., 2011). However, their efficiency on biomass fractionation is still under investigation.

*d) Enzymatic hydrolysis*

Pre-treatment of sugar beet pulp (or lignocellulosic biomasses in general) serves not only for sugar production from hemicellulose, but also assists the enzymatic degradation of cellulose. Hydrolysis of cellulose can be carried out both chemically and enzymatically, with the latter being more favourable as it can be carried out under mild pH and temperatures. As a result, enzymatic hydrolysis does not cause corrosion problems in the equipment, by-product generation is low and at the same time high sugar yields are achieved (Canilha et al., 2004). In order to maximize sugar recovery, optimization of the enzymatic hydrolysis parameters should be carried out; namely the adequate substrate concentration, enzyme loading, hydrolysis time, pH and temperature. An important impediment of this method lies also to the fact that its efficiency can be highly affected by end product inhibitions or from the general biomass structure (Agbor et al., 2011).

Three different classes of enzymes, called cellulases, are required for the enzymatic hydrolysis of cellulose: a) endo-$\beta$-1,4-glucanases (EG, E.C.3.2.1.4), that hydrolyse randomly internal cellobiose bonds, resulting in free chain ends; b) cellobiohydrolases or exoglucanases (CBH, E.C.3.2.191) that remove cellobiose from the free end- chains (either from the reducing or the non-reducing end) and c) $\beta$-glucosidases (E.C.3.2.1.21) which produce glucose through cellobiose degradation (Sun and Cheng, 2002). Synergistic effects of the main cellulose hydrolysing enzymes have been also observed (Ganner et al., 2012). Example of synergism is the formation of chain ends via EG action in order to enhance the hydrolyzing effect of CBH (Jalak et al., 2012). More specifically EG attacks the amorphous disordered cellulose regions, thus revealing the crystalline and ordered nanofibrils. As a result, these regions are easily hydrolysed by CBH, leading to the production of more amorphous regions (Bornscheuer et al., 2014). It is difficult to establish kinetic models of cellulose degradation by cellulases not only due to the complexity of the substrate but also the complexity of the enzyme itself (Bornscheuer et al., 2014). For example, cellulose has both amorphous and crystalline areas that have also different size, thus, different enzyme access. As a result, enzyme hydrolysis is carried out at different rates. Subsequently, during the enzymatic degradation cellulose structure is slowly altered in terms of: number of bonds, chain length, and crystallinity. The main factors affecting the yield of the enzymatic hydrolysis are the following: i) cellulose accessibility, ii) surface availability, iii) degree of crystallinity, iv) degree of polymerisation, v) remaining lignin and cellulose, vi) structural changes that occurred during hydrolysis, and vii) pre-treatment (Zhang and Lynd, 2004). During enzymatic hydrolysis, the first step involves the adsorption of the enzyme at the heterogeneous region,

followed by diffusion and then complexation. The next reaction is catalysis and de-complexation and finally desorption (Fox et al., 2013).

For the efficient application of enzymatic hydrolysis, pre-treatment steps should be optimised, end-product inhibition minimized and most importantly to reduce the enzyme production costs (Bornscheuer et al., 2014). Both Genencor and Novozymes are working since 2000 in the reduction of enzyme production costto less than 5.3 cents per liter from more than US$ 1.3 that is the current cost (Bornscheuer et al., 2014). Of course the goal is to achieve high final titration and yields of ethanol that is the main bio-based product from biomass (Table 2.3). Enzymatic hydrolysis can be carried out either before the fermentation and it is called SHF (separate hydrolysis and fermentation) or at the same time (the so called SSF: simultaneous saccharification and fermentation). The main advantage of SSF is that there is no end-product inhibition towards the enzymatic activity, as the produced sugars are consumed by the fermenting microorganism. However, the development of new enzyme complexes favors the SHF. It is reported that the use of CellicCtec 2 (Novozymes) leads to enhanced ethanol yields, when it is used as a single step process, before the fermentation (Cannella and Jørgensen, 2014).

A number of pilot and/or even commercial facilities exist around the world: POET (USA), Iogen Corporation (Canada), Renmatrix, Inbicon (Denmark), Abengoa (Spain) (Bornscheuer et al., 2014). Iogen Corporation in Ottawa (operating since 2004) produces bio-ethanol from barley, wheat and oat straw using a combination of pre-treatment techniques. Enzymatic hydrolysis is carried out using enzymes produced by the fungus *Trichoderma reesei*. A demonstration plant in Denmark (in Kalundborg) treats straw with enzymes from Novozymes, DSM and Danisco Genencor (Bornscheuer et al., 2014).

To conclude, enzymatic hydrolysis is a well-established method, which can also find industrial application. The production of new enzymatic complexes introduces new aspects in the already existing mechanisms. The use of various cellulases and oxygenases gives the opportunity to hydrolyse a broad variety of substrates (Bornscheuer et al., 2014). Research of new enzyme cocktails could lead even in minimizing the negative effect of residual lignin and hemicellulose. In this respect, the holistic approach of pre-treatment, biocatalyst design as well as reaction engineering will expand the production of bio-based fuels and chemicals (Bornscheuer et al., 2014).

## 2.3. Conclusions

SSL and SBP are two industrial by-products that could be further valorized via the biotechnological production of succinic acid. SSL contains an important fraction of reducing sugars, but at the same it contains many inhibitory compounds such as lignosulphonates, phenolic compounds and acetic acid. Their removal will probably enhance fermentation efficiency and at the same more value-added co-products can be produced (LS and antioxidants), increasing the cost-competiveness of the process. For the valorization of SBP, pretreatment is also required in order to convert its structural polysaccharides into readily fermentable sugars. Studies have already shown promising results for mainly ethanol production. Succinic acid production is not yet reported by any of these streams.

**Table 2.3** Overview on pretreatment methods on SBP and the target bio-based product produced

| Pretreatment method | Conditions | Sugar Yield | Target Product | Reference |
|---|---|---|---|---|
| Acid and enzymatic hydrolysis | 0.66% $H_2SO_4$, 120 $^oC$, 30 min 6 % solid loading/Celluclast 1.5L and Novozyme 188, 2% solids, 50 $^oC$, 72h | 62% | ethanol | Zheng et al., 2013 |
| Acid and enzymatic hydrolysis | 0.6% Sulphurous acid at 190 $^oC$, for 10 min, 1:6 solid to liquid ratio/CellicCTec2, 50 $^oC$, solid:liquid ration 1:30 | 33.6% | - | Kharina et al., 2016 |
| Autohydrolysis/ enzymatic hydrolysis | pH 4, 120 $^oC$, 5 min, 6% solid loading/ Celluclast 1.5L and Novozyme 188, 7.5 % solids, 50 $^oC$, 72h | 79.5% | Acetone-butanol-ethanol | Bellido et al., 2015 |
| Dilute acid hydrolysis | 0.3 M $H_2SO_4$, 6h in agitated isothermal pyrex reactor at 110 $^oC$, with 10:1 liquid to solid ratio | 45.3 g/L | ethanol | Günan Yücel and Aksu, 2015 |
| Conditioning/enzymatic hydrolysis | Citrate-phosphate buffer (0.1 M, pH 5), ration 50 mL/ 6g, 120 $^oC$, 20 min/cellulases, β-glucosidase, xylanase, exo-polygalacturonase | 71.8 g/L | Ethanol & lactic acid | Díaz et al., 2016 |
| Ammonia treatment/ enzymatic hydrolysis | Ammonia load of 0.5:1, SBP with 66% moisture content /Celluclast 1.5L, Novozym 431, Viscozyme L, 40 $^oC$, 5% (w/w) solid loading, 48 h | 608 mg/g biomass | Ethanol | Foster et al., 2001 |

| | | | | |
|---|---|---|---|---|
| Pressure thermal pretreatment/enzymatic hydrolysis | 2% (w/w) $H_2SO_4$, 121 $^oC$ for 30 min, 12% solid loading/ Viscozyme & Ultraflo Max | 86.4% | Ethanol | Berłowska et al., 2016 |
| Alkaline/ microwave-alkaline & thermal alkaline methods | pH 12 with 2 M NaOH/700 W, 170 $^oC$, 30 min/ pH 12, 30 min, 121 $^oC$, 1.5 atm, 30 min | 6739, 19129, 20884 soluble COD concentrations | Bio-Hydrogen | Ozkan et al., 2011 |
| Enzymatic hydrolysis | Commercial enzyme mixtures in horizontal rotating tubular bioreactor for 24 h | 30% total mass solubilization | - | Andlar et al., 2017 |
| Grinding/ thermal-pressure pretreatment/enzymatic hydrolysis | 2.5 mm particle size/ 10% solids (w/v), 120 $^oC$, 4 bars pressure, for 10, 15 and 20 min/ Celustar XL and Agropect pomace (ratio 3:1), 40 $^oC$ and 10% solid loading | 50-60 mg/mL | Biogas | Ziemiński and Kowalska-Wentel, 2016 |
| Thermo-chemical pretreatment & enzymatic hydrolysis | High pressure steam (10 bar, 184 $^oC$)/ 5% solid loading, cellulose 13L-CO13L (mixture of cellulose, β-glucosidase and β-glucanase) at 50 $^oC$ | Solubilizing of 97% arabinose and 10% glucose | Fractionation for the production of value-added products | Hamley-Bennett et al., 2016 |
| Dilute acid pretreatment | 55 g/L SBP (32-50 mesh), 1.1 g $H_2SO_4$ / g SBP and treatment for 90 min at 80 $^oC$ | 86.3% hemicellulose hydrolysis | - | Chamy et al., 1994 |
| Simultaneous saccharification and fermentation | Enzymatic pretreatment at pH 5 and 40 $^oC$ with Novo SP249 (mixture of pectinases and hemicellulases) and Rut-C30 NRRL (cellulose) | 45.9 g/L total sugars | Single cell protein | Nigam and Vogel, 1991 |

# CHAPTER 3
# SUCCINIC ACID PRODUCTION BY FERMENTATION

## 3.1. Succinic acid, an important platform chemical

Succinic acid, a C4-dicarboxylic acid, has been established as an important platform chemical as has attracted great interest for commercialization (Bozell and Petersen, 2010). Conventionally, succinic acid finds application in the food industry as pH modifier and as flavoring/ anti-microbial agent, in pharmaceuticals and for the production of coatings, pigments and resins (Pateraki et al., 2016b). The applications that will expand the demand for succinic acid in the bio-economy era are for the production of bio-plastics, plasticizers, polyurethanes and as a precursor for bulk chemicals.

In 2011, the global succinic acid market was approximately 40,000 MT. Unfortunately; more than 97% was produced petrochemically from hydrogenation of maleic anhydride, whereas only 3 % was produced from bio-processes. The use of succinic acid for the production of pigments, resins and coatings accounted about 19.3% of its global market, followed by the pharmaceutical industry (15.1%) and the food industry (12.6%). Its utilization for the production of PBS and PBST and for polyester polyols was estimated to be the 9% and the 6.2%, respectively (www.bioconsept.eu/wp-content/uploads/BioConSepT). Succinic acid market is expected to reach 599,449 MT in 2020, as its demand is increasing annually by 33% from 2010 to 2020. Moreover, its market value is expected to reach $ 539 million in 2020, from approximately $ 63 million that was estimated to be in 2011 (www.bioconsept.eu/wp-content/uploads/BioConSepT).

Bio-based succinic acid has the potential to become a platform intermediate for the production of various specialty chemicals. As a platform chemical, succinic acid could replace many commodities based on petrochemicals, such as maleic anhydride for the production of bulk chemicals such as 1,4-butanediol, succimide, *γ*-butyrolactone, tetrahydrofuran, *N*-methyl-2-pyrrolidone, 2-pyrrolidone, adipic acid, linear aliphatic esters and many others (Pateraki et al., 2016a; Zeikus et al., 1999). Mainly the market for the production of polybutylene succinate and polyethanes is expected to grow (Pateraki et al., 2016a).

To produce bio-based succinic acid as a platform chemical, the production cost should be reduced to $ 1 per kg. The biotechnological production of succinic acid presents many advantages over the chemical one, as it can be produced through the utilisation of numerous

carbon sources- and as a consequence from various renewable feedstocks-it presents high fermentation efficiency and furthermore, it significantly contributes to the reduction of greenhouse gas emissions as $CO_2$ fixation is necessary for its production through the reductive TCA cycle (Cok et al., 2014). Industrial interest for succinic acid is a Technology Readiness Level of 8, as many facilities are established in Europe and in North America (E4tech et al., 2015). Some examples of biotechnological production of succinic acid in Europe are the facilities of Bioamber in France, with an annual capacity of 3000 t, Reverdia in Italy with a capacity of 10,000 t per year and Succinity in Spain that produces about 10,000 t of bio-succinic acid per year (Pateraki et al., 2016b).

In order to establish a cost-competitive bio-process, both upstream and downstream processes should be well-designed. The upstream process is closely depended to the selection of the feedstock, and subsequently the pre-treatment steps needed for the production of a nutrient-rich substrate. Downstream process is still an important drawback for the establishment of bio-succinic acid, as especially for the production of biopolymers, high purity grade is required. Downstream process will be discussed in *Chapter 4*. The selection of the microorganism and the fermentation parameters employed, highly affect both stages. The use of facultative anaerobes, such as *Actinobacillus succinogenes* and *Basfia succiniciproducens*, is advantageous as no aeration is necessary, reducing significantly both capital and operating costs. Currently, the industrial facilities producing succinic acid are operating using either pure sugars or glucose derived from corn. The utilization of industrial waste streams and agricultural residues is crucial for cost-competitive and sustainable production of succinic acid. In general, succinic acid producing microorganisms can utilise various carbon sources, but in many cases (e.g. lignocellulosic hydrolysates), the development of appropriate detoxification/pre-treatment stages is necessary in order to achieve high final product concentrations.

There are many studies regarding succinic acid production from renewable resources, using wild type strains, or genetically modified ones. The production of succinic acid using hydrolysates rich in pentoses (generated form agricultural and industrial waste streams) will be the focus of this chapter, emphasizing in two wild-type strains, namely *A. succinogenes* and *B. succiniciproducens*.

### 3.2. Succinic acid production from wild type strains

Succinic acid is the end-product of the metabolism of several wild type microorganisms, and is produced through the TCA cycle. The most well-known succinic acid producers belong to the family *Pasteurellaceae* and are isolated from the bovine rumen. These strains are *Actinobacillus succinogenes* (McKinlay et al., 2007), *Mannheimia succiniciproducens* (Lee et al., 2003) and the newly isolated *Basfia succiniciproducens* (Becker et al., 2015). Other important succinic acid producer is *Anaerobiospirillum succiniciproducens* (Meynial-Salles et al., 2007).

Among these strains, *A. succinogenes* is well-studied in the literature (Jiang et al., 2017; Pateraki et al., 2016b), as it is able to grow and produce succinic acid utilising a variety of carbon sources like glucose, xylose, arabinose, mannose, fructose, lactose, cellobiose and other reducing sugars (J. Li et al., 2010). The efficiency of this strain has been tested using various renewable resources and industrial side streams as fermentation substrate such as whey (Wan et al., 2008), cane molasses (Liu et al., 2008), straw (Zheng et al., 2009), corn fiber (Chen et al., 2010), crop stalk wastes (Li et al., 2010), wheat (Dorado et al., 2009), Jerusalmen artichoke tuber (Gunnarsson et al., 2014), rice husks (Bevilaqua et al., 2015), duckweed (Chen et al., 2016), corn stover (Salvachúa et al., 2016a) and many others.

***Actinobacillus succinogenes*** is a Gram-negative, facultative anaerobic, non-motile, caprnophilic, pleomorphic rod that belongs to the family of *Pasteurellaceae* based on its 16S rRNA sequence analysis (Song and Lee, 2006). The key enzymes in *A. succinogenes*' metabolism responsible for succinic acid production are proved to be PEP carboxykinase (*pepck*), malate dehydrogenase (*mdh*), malic enzyme (*sfc*), fumarase (*fum*) and fumarate reductase (*frd*) (Song and Lee, 2006).

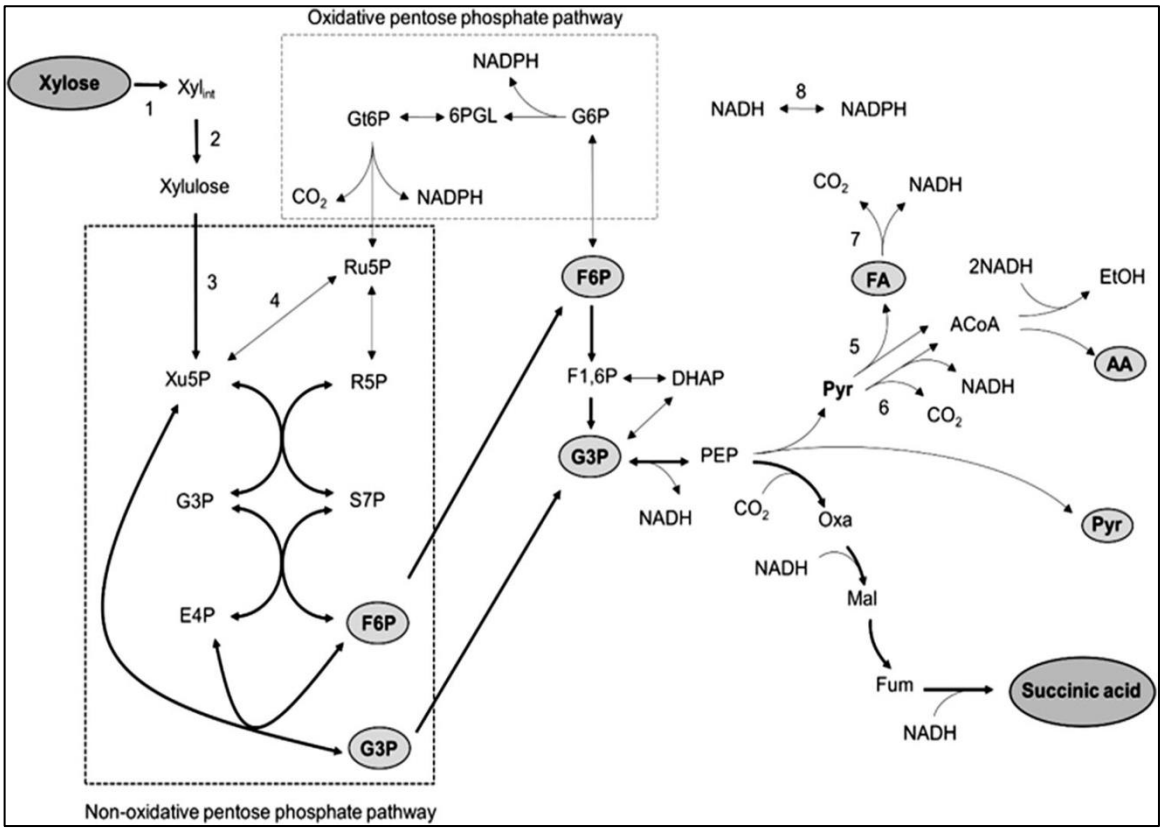



**Figure 3.1** Metabolic pathway of *A. succiniciproducens*, showing xylose consumption and formation of succinic acid and by-products. The numbers correspond to the enzymes involved in each reaction (Scheme adapted by Bradfield and Nicol, 2016).

$CO_2$ fixation is crucial for succinic acid production as PEP carboxylation is controlled by the levels of $CO_2$ in the microbial environment. In theory, 1 mol of $CO_2$ is required for the production of 1 mol of succinic acid through the TCA cycle. It is observed that in high $CO_2$ levels succinic acid formation is favored, over formic acid and ethanol (Song and Lee, 2006). Succinic acid production is also favored in the presence of electron donors such as hydrogen and electrically reduced neutral red (Park and Zeikus, 1999; Van Der Werf et al., 1997). As a consequence, the utilization of more reduced carbon sources such as mannitol, arabitol and sorbitol yields in enhanced succinic acid concentrations in comparison to glucose (Van Der Werf et al., 1997). Besides succinic acid, formic acid and ethanol, this strain also produces acetic acid.

The maximum theoretical yield of succinic acid production from glucose is 1.12 g/g, but of course it cannot be accomplished due to biomass and by-product formation. When 1 mol of succinic acid is produced, 2 moles of NADH are oxidized for the conversion of oxaloacetate (OAA) to malate and fumarate to succinate (Pateraki et al., 2016b). As a result, by-product formation is necessary for the cell in order to regenerate both NAD(P)H and ATP.

The production of 1 mol of acetic acid leads to the regeneration of 1 mol of ATP, whilst 1 mol of formate is formed together with 1 mol acetyl-CoA, an important intermediate for biomass production. It is obvious that the strain uses the C3 pathway for the production of NADH that would subsequently be used for the formation of succinic acid through the C4 pathway.

In order to achieve succinic acid yields close to the theoretical value, the reduction of by-product formation is crucial. The mutant strains *A. succinogenes* FZ6 and FZ53 are able to produce only succinic acid (Guettler et al., 1996). Besides genetic modification, the development of continuous processes and cell immobilization could lead to enhanced product yields and productivities (Bradfield and Nicol, 2014; Yan et al., 2014). It is also observed that *A. succinogenes* has the tendency to form biofilm during continuous cultivation, leading to reduced requirements of acetyl-CoA- that serves as precursor for biomass formation- hence to low by-product formation (Bradfield and Nicol, 2014).

***Basfia succiniciproducens*** is also a Gram-negative rod and facultative anaerobic, isolated recently from the rumen of Holstein cow (Kuhnert et al., 2010). As its environment contains high levels of $CO_2$, the fixation of carbon dioxide is necessary for succinic acid production. According to Becker et al., (2013), in this strain TCA cycle functions in two branches and not really as a cycle. The formation of succinate is carried out mainly from the reductive branch, whereas the oxidative one is mainly used for anabolism. As in case of *A. succinogenes*, besides succinic acid it also produces lactic, formic and acetic acids (Becker et al., 2013). Recently, there are some publications dealing with succinic acid production using lignocellulosic hydrolysates with *B. succiniciproducens* like *Arundo donax* hydrolysate (Cimini et al., 2016; Ventorino et al., 2017) and corn stover hydrolysate (Salvachúa et al., 2016b), but yet this train has not been evaluated to its full potential.

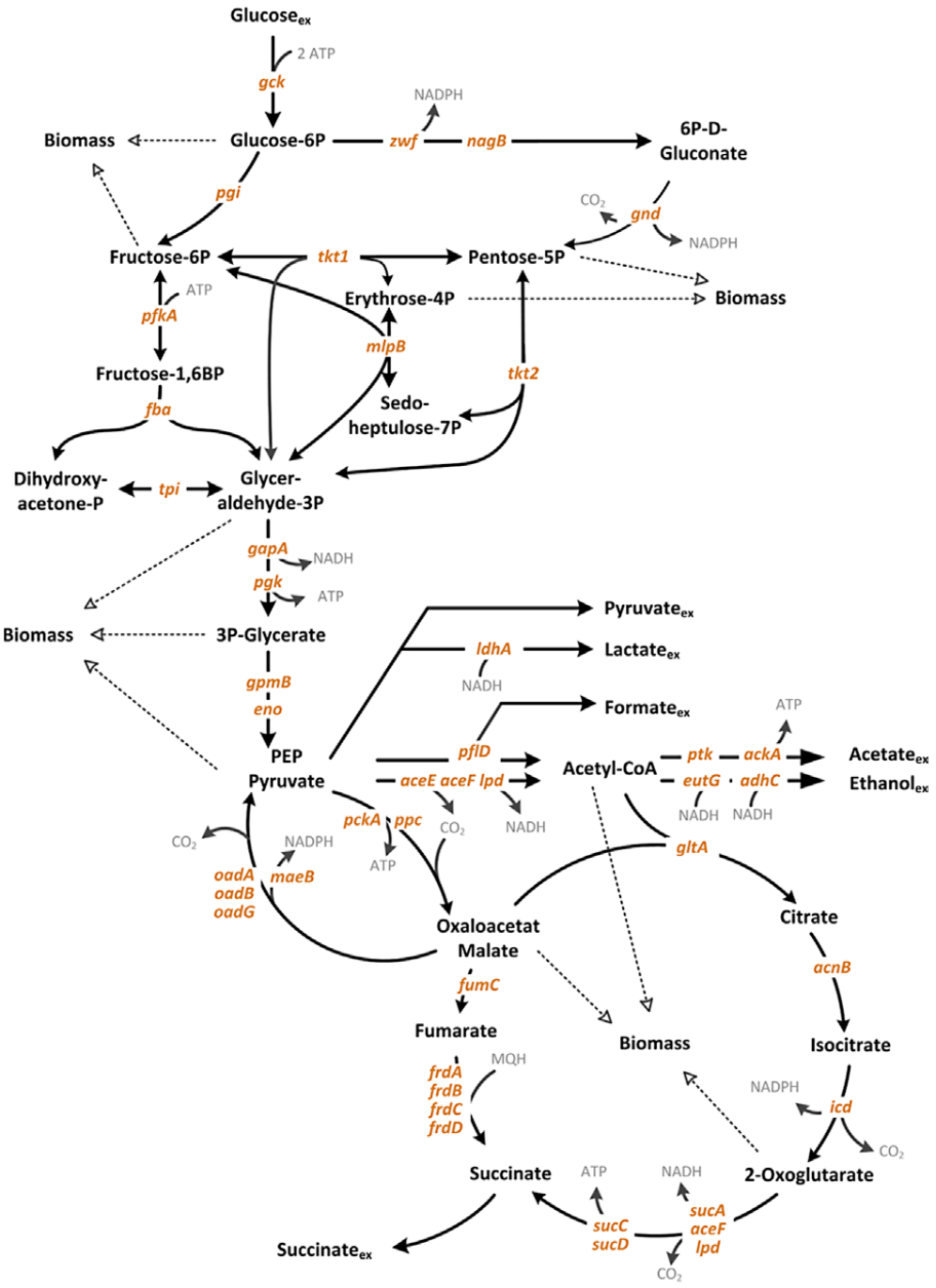



**Figure 3.2** Metabolic network of *B. succiniciproducens* as proposed by Becker et al., (2013)

***Mannheimia succiniciproducens*** is another capnophilic, Gram-negative strain belonging to the family of *Pasteurellaceae* and as *A. succinogenes*, is well-studied for succinic acid production using various substrates coming from renewable resources (Jiang et al., 2017). The major product of its metabolic activity is succinic acid, whilst acetic and formic acids are also formed, always with $CO_2$ fixation and at pH values of 6-7.5 (Song and Lee, 2006). As in case of *A. succinogenes* and *B. succiniciproducens*, presence of $CO_2$ highly increases both PEP carboxylation and the reductive TCA cycle flux in *M. succiniciproducens*.

Theoretically, taking into account of the metabolic pathway of this bacterium, it is able to produce 1.71 and 1.86 mol of succinic acid utilizing 1 mol of glucose under $CO_2$ and $CO_2$-$H_2$ fixation, respectively (Song and Lee, 2006).

***Anaerobiospirillum succiniciproducens*** is a strictly anaerobic, motile, Gram negative bacterium, member of the family of *Succinivibrionaceae*, that was isolated form the throat and feces of beagle dog. This strain is able to produce succinic and acetic acids as major products of its metabolism and ethanol and lactic acid as by-products under strictly anaerobic conditions. Like the strains of *Pasteurellaceae*, also *A. succiniciproducens* produces succinic acid through the PEP carboxylation pathway, requiring high $CO_2$ levels in its environment (Song and Lee, 2006). *A. succiniciproducens* is able to utilize various carbon sources like glucose, glycerol, maltose, sucrose, fructose and lactose. In the study of Lee et al., (2003), it was also proven that this strain is able to utilize wood hydrolysates for succinic acid production.

### 3.3. Succinic acid production using genetically engineered strains

The need for higher succinic acid final concentrations and productivities together with the need for elimination of by-products, led to the development of several metabolic engineered strains. The most important strains, which present also significant potential for commercialization, are *Escherichia coli*, *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, *M. succiniciproducens*, *B. succiniciproducens*, *A. succinogenes* and *Corynebacterium glutamicum* (Ahn et al., 2016).

*S. cerevisiae* engineered strains present the advantage of growing at low pH (range of 3-6), that is a huge advantage over the wild type strains. This strain is used by Reverdia for succinic acid production in a capacity plant of 10,000 ton that operates since 2011 (Ahn et al., 2016). *P. kudriavzevii* can also grow efficiently at low pH values. Bioamber uses this engineered strain for succinic acid production at a plant operating since 2014 and with a capacity of 30,000 tons. The wild-type *E. coli* strains are able to produce succinic acid, but as a by-product. Since this strain has the advantage of fast growth and methods of metabolic engineering are already well-established, several strategies have been employed in order to enhance its ability for succinic acid production. The strain AFP111, in a dual-phase fermentation was capable of producing 99.2 g/L of succinic acid utilizing glucose, with a yield of 1.74 mol/mol and productivity of 1.3 g/L/h (Vemuri et al., 2002). Engineered *E. coli* is employed by Myriant for succinic acid production in a capacity plant of 15,000 tons, operating since 2013 (Myriant: Succinic Acid & Derivatives SBU. http://www.myriant. com/pdf/myriant-succinic-acid-customer-presentation-english. pdf).

Mutants of wild-type strains have also been developed, mainly aiming in elimination of by-product formation. In the mutant strain *M. succiniciproducens* LPK7 the genes enconding the enzymes lactate dehydrogenase (*ldhA*), pyruvate formate lyase (*pflB*), phosphate acetyltransferase (*pta*) and acetate kinase (*ackA*) were deleted (Ahn et al., 2016). The best performance regarding both succinic acid production and low by-product formation were achieved using the mutant strain PALK (Ahn et al., 2016). The deletion of the genes responsible for *ldhA* and *pta-ackA* lead to the production of 45.8 g/L of succinic acid from glucose at a yield of 1.32 mol/mol and productivity of 2.36 g/L/h, with an insignificant co-formation of acetic and pyruvic acids (Ahn et al., 2016).

By deleting the genes responsible for formic acid production in *B. succiniciproducens* genome, reduced lactic acid formation was also achieved (Becker et al., 2013). The mutant strain presented enhanced succinic acid production as yield on glucose was 1.08 mol/mol (in comparison to 0.75 mol/mol obtained from the wild type one). This strain is employed by Succinity that produces succinic acid in a 10,000 ton capacity plant since 2013 (Alhn et al., 2016). The mutant strains *A. succinogenes* FZ6 and FZ21 have been developed from the deletion of genes enconding formate lyase and formate dehydrogenase. Even though succinic acid production was significantly enhanced, by-product formation was though not eliminated (Guettler et al,1996)Finally, genetically engineered *C. glutamicum* is utilized for succinic acid production by Ajinomoto and Mitsubishi Chemical since 2006 (Nishi et al., 2011).

### 3.4. Succinic acid production from renewable resources that main contain pentoses

The utilization of renewable resources would not only reduce the waste materials disposed in the environment, but also it will reduce significantly the upstream cost for bio-succinate. Lignocellulosic biomass is abundant and produced at high quantities worldwide. As it is already discussed in previous chapters, the main sugar derived from hemicellulose hydrolysis is xylose. The bioconversion of these hydrolysates towards ethanol is problematic, since most yeast are not able to assimilate pentoses. Succinic acid producing bacteria are proved to be able to consume pentoses and produce succinic acid, even though yield on xylose is much lower than glucose (Pateraki et al., 2016b). Table 3.1 presents a literature review on recent publications regarding succinic acid production from pentose-rich hydrolysates.

It is evident, that the strain that is more tested is *A. succinogenes*. Recently, *B. succiniciproducens* has also been employed for the valorization of pentose-rich streams, such as corn stover hydrolysates (Salvachúa et al., 2016b), and giving very promising results. The

fermentation efficiency is highly related to the inhibitory compounds (acetic acid, furans, phenolic compounds) present in the hydrolysates (Palmqvist and Hahn-Hägerdal, 2000). Various pretreatment methods have been evaluated in order to enhance succinic acid production, like deacetylation (Salvachúa et al., 2016a; 2016b), dilute alkali pre-treatment (Zheng et al., 2009), ultrasonication and activated charcoal (Xi et al., 2013). The cost-competiveness of bio-succinate is directly dependent on the efficiency of both upstream and downstream processes. The utilization of cheap or waste feedstocks like sugarcane bagasse, corn stover or SSL lower significantly the overall of the fermentation process.

### 3.5. Conclusions

Succinic acid is an important key intermediate with an increasing market potential. The biotechnological production of succinic acid using renewable resources could replace not one its own production from petrochemicals, but it could also work as precursor for the production of various other chemicals and bio-polymers. The selection of the adequate microbial strain together with the appropriate substrate pretreatment methods could result in high fermentation efficiency and high product yields. Lignocellulosic biomass could be employed for succinic acid production since the most common wild-type strains are able to consume pentoses. More research should be carried out in order to achieve product yields as efficient as in case of glucose-containing hydrolysates.

**Table 3.1** Succinic acid production from various strains utilizing mainly pentose-containing hydrolysates

| Substrate | Strain | Type of fermentation | Final Succinic acid (g/L) | Succinic acid yield[a] (g/g) | Succinic acid productivity (g/L/h) | Reference |
|---|---|---|---|---|---|---|
| Corn straw [b] | *A.succinogenes* CGMCC1593 | Batch | 45.5 | 0.78 | 0.95 | Zheng et al., (2009) |
| Corn straw [b] | *A.succinogenes* CGMCC1593 | Fed-batch | 53.2 | 0.83[c] | 1.21 | Zheng et al., (2009) |
| Corncob | *A.succinogenes* CICC 11014 | Batch | 23.6 | 0.58[c] | 0.49 | Yu et al., (2010) |
| Sugarcane baggasse | *A.succinogenes* CIP 106512 | Batch | 19.0 | 0.37 | 0.90 | Borges and Pereira, (2011) |
| Sugarcane bagasse | *A.succinogenes* NJ113 | Batch | 23.7 | 0.79 | 0.99 | Xi et al., (2013) |
| Corn stalk | *E.coli* BA408 | Batch | 23.1 | 0.70 | 0.24 | Bao et al., (2014) |
| Corn stover | *A.succinogenes* 130Z | Batch | 42.8 | 0.82 | 0.30 | Salvachúa et al., (2016a) |
| Corn stover | *B.succiniciproducens* | Batch | 30.0 | 0.50 | 0.43 | Salvachúa et al., (2016b) |
| Corn fiber hydrolysate | *E.coli* AFP184 | Batch | 26.3 | 0.49 | 0.55 | Yoo et al., (2016) |

[a] yield on total sugars present in the substrate
[b] 35 g/L glucose, 20.8 g/L xylose

# CHAPTER 4
# DOWNSTREAM SEPARATION AND PURIFICATION OF SUCCINIC ACID

The development of a cost-competitive biotechnological succinic acid production process is dependent not only on the upstream but also on the downstream process. Bechthold et al., (2008) reported that about 60% on total production costs are attributed to the separation and purification of the end product. For that reason, many studies are focusing on the establishment of an efficient downstream separation scheme for the recovery of bio-based succinate that not only would lead to low operational costs, but also to high product yield and purity.

Almost in all separation schemes proposed in the literature, there are some standard preliminary steps, such as centrifugation or microfiltration aiming to separate the biomass. Ultrafiltration is also employed in order to remove proteins or cell debris and other components from the fermentation broth (Kurzrock and Weuster-Botz, 2010). The isolation of succinic acid from the supernatant and/or permeate stream can be carried out using many different unit operations. The most well-studied methods for separation of succinic acid involve its precipitation using ammonia or calcium hydroxide, electrodialysis (monopolar and/or bipolar) and liquid-liquid extraction (such as reactive extraction). Chromatography and crystallisation are in most cases the final steps to obtain the purified succinic acid crystals.

An overview of the downstream processes reported in the literature so far will be briefly discussed regarding their potential industrial application as well as their effect on final yield and purity of succinic acid crystals.

## 4.1. Precipitation with calcium hydroxide

The industrial method for the recovery of carboxylic acids is based on precipitation using calcium hydroxide or calcium oxide. This method is well-employed for the recovery of lactic and citric acids in the industry (Kurzrock and Weuster-Botz, 2010). There are also some patents reporting the use of this method for the downstream separation of succinic acid, though only in laboratory scale (Berglund, 1991; Datta, 1992).

During this process, calcium hydroxide or calcium oxide is added to the fermentation broth and calcium salts of succinic acid precipitate and then filtered. The precipitate is then treated with excess of concentrated sulphuric acid. Succinic acid is further purified using

widely applied methods, such as activated carbon or ion exchange resins, followed by crystallisation. This method results in low succinic acid recovery yield and low crystal purity (Luque et al., 2009). Moreover, calcium sulphate ($CaSO_4$), the so-called gypsum is produced as a by-product.

### 4.2. Precipitation with ammonia

In this process, ammonia is used as neutralising agent during succinic acid production by fermentation, resulting in the precipitation of diammonium succinate. As in the case of calcium precipitation method, succinate salt is filtered from the fermentation broth and is subsequently treated via sulphuric acid. The produced ammonium sulphate can be thermally broken down to ammonia and ammonium bisulphate. Succinic acid is purified via dissolution to methanol and treated via crystallization. Yedur et al., (2001) reported a recovery yield of 93.3 %. The advantage of this method is the generation of lower quantities of by-products as both the base and the acid can be recycled and re-used. The disadvantage of the method is the co-precipitation of by-products present in the fermentation medium (Yedur et al., 2001).

### 4.3. Electrodialysis

Electrodialysis has already been industrially employed since 1966 for the separation of citric acid from fruit juice (Zang et al., 1966). Glassner et al., (1992) first reported the combination of desalting and water-splitting electrodialysis for succinic acid recovery. Conventional electrodialysis (CED) bears a repeating unit of a cation-selective membrane, an anion-selective membrane and two compartments. With this method, a solution containing an organic acid or organic acid salts is concentrated, as the ions are forced due to electric potential difference to pass through the ion-exchange membranes (Huang et al., 2008). Finally, two streams are generated called the concentrate and the diluate. In bipolar (BEM) or water-splitting electrodialysis, water is also broken down to $H^+$ and $OH^-$.

In the study of Glassner et al. (1992), the desalting electrodialysis was used in order to separate the organic acids (ionic form) from the uncharged molecules such as sugars and proteins. After this step a succinic acid separation yield equal to 77% was achieved. The next step involved the treatment with bipolar electrodialysis membranes is order to convert succinate salts to succinic acid. The overall purification yield of succinic acid was 60%. Treatment with anion and cation exchange membranes is then required in order to remove the

ionic impurities, aiming to achieve SA purity of > 99%. Succinic acid crystals are finally produced after evaporation and crystallization.

Electrodialysis is an environmental friendly method, as no waste streams are generated and the base stream can be recovered and re-used in the upstream process. The high energy cost and the high cost of the membranes are still an impediment of large scale utilization of this process (Kurzrock and Weuster-Botz, 2010).

### 4.4. Liquid-liquid extraction

Liquid-liquid extraction involves either the direct addition of an organic solvent into the fermentation medium, which selectively removes the desired product, or the recycling of the fermentation broth through an extraction unit in which the desired product is separated by a continuous flow of the solvent while the remaining fermentation broth is recycled back into the reactor. This method has been intensively studied for the past 30 years for the separation of biotechnologically produced carboxylic acids (Kurzrock and Weuster-Botz, 2010). The main drawback for the industrial application of liquid-liquid extraction is the low distribution coefficients of the most commonly used extraction solvents for carboxylic acids.

### 4.5. Reactive extraction

Reactive extraction is a liquid-liquid extraction method, where amines are employed as extractants, dissolved in non-water soluble organic solvents. Amines tend to react with carboxylic acids and they are suitable for the extraction of organic acids, such as succinic acid from aqueous solutions (Kurzrock and Weuster-Botz, 2010). The reaction between the selected amine and succinic acid is carried out at the interface of the aqueous and the organic phase, resulting in the formation of an amine-acid complex. This complex is not water-soluble and partitions to the organic phase. The main chemical mechanism involves proton transfer or ion pair formation, depending on the type of amine used and the organic solvent employed (Hong et al., 2001).

Long-chain aliphatic primary, secondary and tertiary amines have mainly beeb used in the literature for the extraction of succinic acid (Kurzrock and Weuster-Botz, 2010; Song et al., 2007). Primary amines present high water-solubility, whilst secondary amines are characterised by high distribution coefficients but they form amides during regeneration. Primary, secondary and tertiary amines are able to extract only the undissociated form of

succinic acid. Quaternary amines are able to extract both the dissociated and the undissociated form of succinic acid, but their regeneration by back extraction is not efficient. Hence, tertiary amines are more promising for the extraction of organic acids (Lee et al., 2008)

Important parameters affecting the extraction efficiency when aliphatic amines are employed include the pH of the fermentation broth, the temperature, the type and the properties of both the amine and the organic solvent used, the loading ratio, the distribution coefficient, the equilibrium constant and the concentration of succinic acid (Yun et al., 2004). According to Jun et al., (2007) for efficient extraction, the pH of the fermentation broth should be far below the $pKa_1$ of succinic acid (with pH value of 4.2), so that the acid exists mainly at its undissociated form.

Song et al., (2007) used reactive extraction with trioctylamine in 1-octanol for the separation of succinic acid from fermentation broth. Reactive extraction was coupled with vacuum evaporation and crystallization resulting in succinic acid yield of 67% and purity more than 99.5%. The presence of impurities like other organic acids and salts decrease the extraction yield. In order to overcome this problem, Huh et al., (2006) used reactive extraction with trioctylamine as a primary separation step, in order to improve the by-products and the salts of the fermentation broth. After this step, 97.1% of succinic acid remained in the broth. Finally, succinic acid crystals were obtained with an evaporative crystallizer, resulting to a purity of 99.8% and a total yield of 71.3%. Kurzrock and Weuster-Botz, (2011), studied the effect of 16 amines and 16 solvents for the reactive extraction of succinic acid from fermentation broths. Using a complex reactive extraction system consisted of diisoctylamine, dihexylamine, 1-octanol and 1-octanol the extraction yield of succinic acid ranged to 84.4-85.1%.

The recovery of succinic acid and the amine is carried out with back-extraction using trimethylamine, pH-swing, diluent-swing or temperature swing (Hong et al., 2001). The total process could be carried out in liquid-liquid centrifugal extractors that are already used for industrial scale applications (Kurzrock and Weuster-Botz, 2010). Another advantage of this process is the possibility to develop an integrated product recovery during fermentation (Rüffer et al., 2004).

### 4.6. Ion exchange and sorption

Ion exchange chromatography is employed in order to remove residual ions from succinic acid solutions. Kushiku et al. (2006) applied and strongly acidic H-type cation-

exchange resins in order to convert succinate salts to succinic acid. Li et al. (2008) reported the use of an alkaline anion-exchange resin for simultaneous removal of succinic acid from the fermentation broth. Jun et al., (2007a) proposed the use of mesoporous silica for the removal of pyruvic and succinic acid from fermentation broths.

As the ion exchange resins show no specific selectivity towards succinic acid, this process can only be applied as an additional separation step (Pai et al., 2002).

### 4.7. Direct crystallization

The direct crystallization method was first proposed by Luque et al. (2009). The authors, after removal of biomass via centrifugation and treatment with activated carbon, applied vacuum evaporation and crystallization. Before vacuum evaporation, the pH of the broth was adjusted to 4.2. Vacuum distillation at 60 $^{o}$C resulted in the removal of the volatile by-products like formic and acetic acids. The final purification step involved crystallization at 4 $^{o}$C. Succinic acid yield and purity were 75% and 97%, respectively when the method was tested in simulated fermentation broth. However, when the method was employed in a real fermentation broth, both yield and purity decreased significantly (28% and 45%, respectively).

Lin et al., (2010) also evaluated the direct crystallization method for succinic acid recovery from fermentation broths. The authors tested the significance of pH of the fermentation broth and found that a pH value of 2 results in higher succinic acid recovery yields. The application of a cation-exchange resin (Amberlite IR 120H) for the conversion of succinate salts to succinic acid, followed by vacuum evaporation and crystallization led to succinic acid recovery of 89.5%, with a purity of 99%.

### 4.8. Ultra- and nanofiltration

Filtration methods (including micro-, ultra- and nanofiltration) have been evaluated, mainly as primary separation steps for succinic acid separation. Yao et al., (2008) applied microfiltration, nanofiltration and then activated charcoal in order to separate succinic acid from the fermentation broth. The pH of the permeate stream was then adjusted to 2-3.5 and subjected to vacuum evaporation in order to remove water and the volatile by-products. After crystallization, the purity of succinic acid was more than 99.5% and the recovery yield was higher than 75%.

In the study of Wu et al., (2011) fermentation broth from corn straw hydrolysate was treated with microfiltration, ultrafiltration and nanofiltration. The final permeate stream was vacuum evaporated and crystallized resulting to succinic acid crystals with a purity of more than 99.4%.

### 4.9. Simultaneous esterification

Esters of many carboxylic acids, such as succinic acid esters and lactic acid esters, present an industrial potential as they are considered to be valuable platform chemicals and intermediates for the production of biodegradable plastics (Benedict et al., 2006). López-Garzón et al., (2012) proposed a novel direct downstream process for succinic acid recovery from fermentation broths. The authors described a new process based on *O*- alkylation of succinate using a mild electrophilic alkylating agent. Succinate salt was split and the organic anion sorbed by a strong anion exchange resin with quaternary amine functional groups ($Q^+$). The sorbed succinate salts reacted with gaseous chloroethane, yielding the diethyl ester and the quaternary amine in the chloride form**.** The authors claim that this direct downstream catalysis the need for succinic acid purification is not necessary, since the first sorption step could be used as an isolation step from aqueous solutions (such as fermentation broths). The resin functional group is regenerated with the alkylation reaction and subsequently can be reused. Therefore, many intermediate processes such as precipitation, filtration, acidification and crystallization are reduced. Finally, sorption and reaction steps could be performed in a single process.

Orjuela et al., (2011) studied the recovery of succinic acid from fermentation broth via acidification and esterification in ethanol. With acidification, the succinate salt is converted into succinic acid. A stoichiometric excess of sulfuric acid was also added in succinic acid salts. With this process, the authors performed simultaneous acidification and esterification, while the inorganic sulfate salts precipitated out of the ethanol solution. The succinate was recovered as a solution of free succinic acid, monoethyl succinate and diethyl succinate in ethanol. More than 95% of succinate was recovered when pure succinate salts were used. Similar results were obtained from salts recovered from fermentation broths.

Benedict et al. (2006) coupled simultaneous esterification with pervaporation for lactic and succinic acids recovery. Esterification of lactic acid and succinic acid took place in well-mixed reactors with solid catalysts (Amberlyst XN-1010 and Nafion NR50) and two pervaporation membranes (GFT-1005 and T1-b). Amberlyst XN-1010 was shown to be

compatible with the lactic acid and succinic acid reaction systems and therefore suitable as a catalyst for esterification of these acids. The best membrane for this process was proven experimentally to be GFT-1005. As ethyl lactate and diethyl succinate have different boiling points, they could be recovered via multistage distillation. This process is technically feasible but costs required for the membrane and the vacuum pump should be considered.

Budarin et al., (2007) proposed the use of Starbons, which are sulfonated forms of carbonaceous mesoporous materials. Starbons are active and reusable catalysts for the aqueous phase esterification of dicarboxylic acids. The authors studied the activity of these catalysts on the esterification of succinic, fumaric and itaconic acids. This process, however, was not applied to organic acids derived from fermentation broths.

## 4.10. Conclusions

There are many studies dealing with separation and purification of succinic acid. The major challenge is the transition of the laboratory scale methods to industrial large-scale process in a cost-competitive way. In order to achieve that, the traditional methods should be improved and combined with an efficient upstream process.

# CHAPTER 5
# OBJECTIVES

The acidic sulphite pulping process generates high amounts of spent sulphite liquor (SSL), whilst sugar beet pulp (SBP) is the by-product stream derived also in significant quantities from sugar production plants. The inevitable depletion of the fossil materials turns the attention towards renewable resources that not only help to preserve the environment but also assist the transition to the bio-economy era. In respect to the bio-economy era, the production of chemicals and biopolymers should be carried out via the exploitation of renewable carbon in a manner that enhances sustainability. The microbial bioconversion could be evolved into a key unit operation for the transition of petroleum refineries to biorefineries. In the future biorefinery, the conventional production of chemicals and plastics could be totally replaced from bio-based chemicals and bio-plastics generated completely from renewable resources. Succinic acid has been established as a key platform chemical and is currently produced mainly from petrochemicals. Its biotechnological production from industrial waste and by-products streams should be optimized in order to be cost-competitive.

Under this prospect, the present study is focused on the development of an advanced biorefinery concept based on both SSL and SBP. The conventional pulp and paper industry could be transformed into an integrated biorefinery, via the production of succinic acid, antioxidants and lignosulphonates, using SSL as feedstock. The valorization of SBP would add value to the crop and could also convert the conventional sugar production industry into an advanced biorefinery through the production of succinic acid, pectins and antioxidants. The main objectives of the experimental work are presented below (Figure 5.1).

- Optimisation of the extraction of phenolic compounds from SSL in order to produce an antioxidant-rich stream and at the same time a detoxified medium for succinic acid production
- Pretreatment of SSL in order to obtain high succinic acid concentrations and at the same time to produce value-added co-product streams (LS, antioxidants)
- Optimisation of succinic acid production using SSL by employing cell immobilization of the microbial strains *A. succinogenes* and B. *succiniciproducens* in two different supports
- Optimisation of pretreatment of SBP in order to produce a hydrolysate rich in fermentable sugars

- Development of an efficient bioprocess for succinic acid production via the utilisation of the SBP-derived hydrolysates
- Scale-up of the proposed bioprocess
- Efficient downstream separation of succinic acid produced from the two by-product streams in order to achieve high recovery yields and purity

The experimental processes that were followed in this study were planned so as all of the above objectives could be accomplished. For the optimization of the extraction of the phenolic compounds from SSL, the main parameters affecting solvent extraction were evaluated in detail. Finally, a factorial design was employed in order to assess the optimum parameters that lead to the highest extraction yield of the phenolic compounds. Subsequently the antioxidant activity of the obtained extracts was evaluated in order to propose potential commercial applications. The detoxified SSL was also evaluated as fermentation substrate for succinic acid production using two wild type strains, *A. succinogenes* and *B. succiniciproducens*.

Preliminary experiments revealed that SSL contains inhibitory compounds that hinder the growth of the two microbial strains. The extraction of phenolic compounds increased product yield, but final concentration was still very low. In order to increase succinic acid yield, other detoxification methods were evaluated. Subsequently, lab-scale bioreactor fermentations were employed in order to evaluate the effect of each detoxification method on succinic acid production. The combination of solvent extraction with ethyl acetate together with nanofiltration led to the production of antioxidants, LS and high succinic acid concentrations.

Cell immobilization was also employed in order to enhance further the fermentation efficiency. Two different supporting materials were tested: delignified cellulosic material (wood sawdust) and alginate beads. Both methods resulted in better fermentation performance, but immobilisation in alginate beads was proved to be a more efficient method for succinic acid production from SSL, especially with the strain *B. succiniciproducens.*

Hydrolysis of both hemicellulose and cellulose present in SBP was efficiently carried out by means of acid and enzymatic hydrolyses. Firstly, different liquid-to-solid ratios were evaluated as well as different acid concentrations and enzymatic loadings. The optimum conditions were determined by measuring the reducing sugars released in the hydrolysate. Subsequently, the hydrolysate was implemented as fermentation substrate for the microbial

production of succinic acid using both *A. succinogenes* and *B. succiniciproducens*. Different parameters were tested like sterilisation or pasteurisation of the substrate as well as the need for detoxification (via microfiltration). Finally, the bioprocess proposed was employed in a 50 L bioreactor, indicating the possibility for scale-up. The efficient fractionation of SBP resulted to the production of antioxidants, pectins and succinic acid.

The final objective of this study involved the separation and purification of succinic acid produced via fermentation of these waste streams. Four different methods were tested in the fermentation broths derived from SSL based media and a novel method based on bipolar electrodialysis membranes was employed using broths obtained from SBP fermentation. All this methods were evaluated according to the final succinic acid recovery yield as well as the crystal purity.

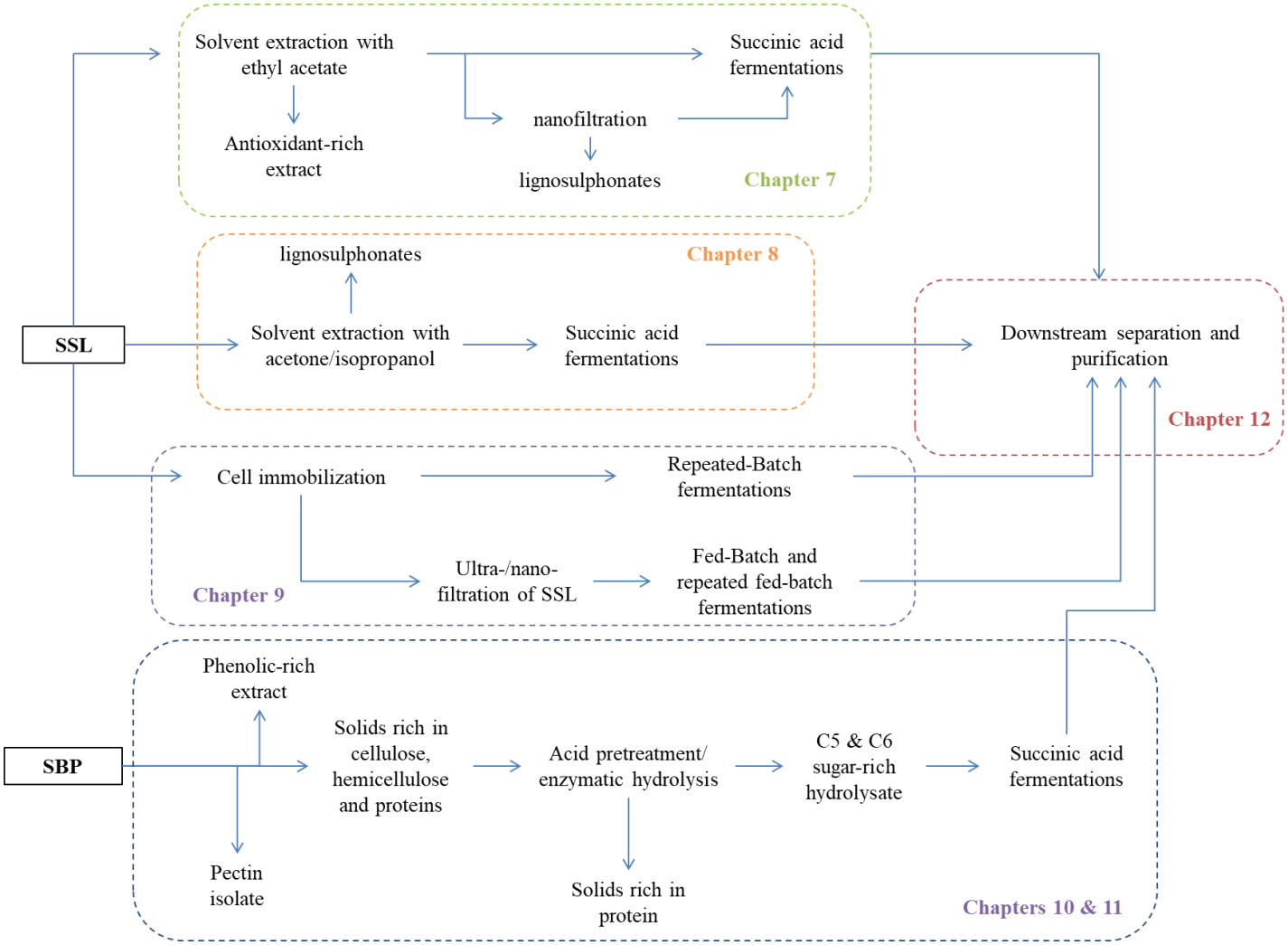



**Figure 5.1** Schematic diagram of the experiment processes designed during the present study for the valorisation of SSL and SBP

# CHAPTER 6
# MATERIALS & METHODS

## 6.1. Introduction

In this chapter, the processes and the methods applied for the development of the proposed bio-processes and for the extraction of value-added co-products are described. The cultivation conditions as well all the analytical and chemical methods followed are also presented. In the beginning of the study, SSL was evaluated as fermentation feedstock for microbial production of succinic acid. The extraction of the phenolic compounds from SSL was optimized by employing factorial design. The total phenolic content (TPC) as well as the antioxidant activity index (AAI) of the extract were determined. The individual phenolic compounds were also identified. The aqueous phase was then subjected to microbial fermentation for succinic acid production. In order to enhance the fermentation efficiency, different pretreatment methods and a combination of them was also evaluated. The best processing scheme could be also proposed for the development of an advanced biorefinery concept based on pulp and paper industry. Cell immobilization on two different supports was also tested in order to increase succinic acid from SSL. Besides SSL, SBP was also evaluated as renewable feedstock for bacterial production of succinic acid. The pretreatment methods required for the production of a nutrient-rich substrate are described in detail in this chapter. The sugar-rich hydrolysate produced was then employed as substrate for succinic acid production. In the end of this study, different downstream methods were evaluated for the separation and purification of succinic acid from fermentation broths.

## 6.2. Crude renewable feedstocks

### 6.2.1. Spent sulphite liquor

Spent sulphite liquor (SSL) was provided by the company Green Source SA (Spain) and was produced by the acidic sulphite pulping process of *Eucalyptus globulus*. The SSL contained 176.5 ± 4.85 g/L total sugars (128.1 ± 0.6 g/L xylose, 21.5 ± 2.5 g/L galactose, 19.3 ± 0.4 g/L glucose, 7.4 ± 1.3 g/L mannose and 0.2 ± 0.05 g/L arabinose), 458.8 ± 2.7 g/L LS, 12.4 ± 0.8 g/L phenolics, pH value of 2.7 and 64 ± 0.2% dry matter. All results are average values of triplicate samples.

**Image 6.1** Spent sulphite liquor (SSL)

### 6.2.2. Sugar beet pulp

Sugar Beet Pulp (SBP) was provided by the industry Pfeifer & Langen GmbH & Co. KG (Germany) in the form of dry pellets. The composition was the following (% w/w): 90.8 DM, 6.48 total sugars, 8.73 proteins, 0.14 fat, 20.92 cellulose, 15.22 hemicellulose, 2.34 lignin and 15.5 pectins.

## 6.3. Microorganisms

Succinic acid production was carried out using the bacterial strains *Actinobacillus succinogenes* 130Z (DSM 22257) and *Basfia succiniciproducens* JF 4016 (DSM 22022) that were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. The medium used for inocula preparation was 30 g/L Tryptic Soya Broth (TSB) and it was prepared at 37 $^{o}$C for 12 h.

## 6.4. Cell immobilization

### 6.4.1. Bacterial mass production

In order to produce adequate bacterial mass for the immobilization, 10% inoculum from the pre-culture was transferred into a synthetic medium that contained 20 g/L glucose, 5 g/L yeast extract, 15 g/L $MgCO_3$, 1.16 g/L $NaH_2PO_4 \cdot H_2O$, 0.31 g/L $Na_2HPO_4$, 1 g/L NaCl, 0.2 g/L $MgCl_2 \cdot 6H_2O$ and 0.2 g/L $CaCl_2 \cdot 2H_2O$. The production of bacterial mass for immobilization was incubated in Duran bottles in an orbital shaker at 37$^{o}$C and 150 rpm including continuous sparging of carbon dioxide (0.1 vvm).

### 6.4.2. Immobilization and support materials

Immobilization on delignified wood sawdust (DCM) was carried out based on literature-cited publications (Elezi et al., 2003). Delignification of wood sawdust was implemented by the addition of 1% (w/v) NaOH in the suspension of wood chips and the mixture was heated to 80 $^{o}$C for 3 h. The DCM was subsequently washed several times with hot water in order to remove the lignin residues. For the immobilization, 5 g of microbial biomass were added to a synthetic medium (same composition as above) with 10 g/L glucose and 55 g of DCM and left to ferment overnight.

For the preparation of the alginate beads, the bacterial culture was centrifuged in order to collect the microbial cells. After the centrifugation, 5 g of bacterial cells (on a wet basis) were suspended in 100 mL 0.9% NaCl solution and then added into 100 mL sodium alginate solution. The mixture was added dropwise in 100 mL calcium chloride solution as has been described by Brady et al., (1997). The sodium alginate solution was created by diluting the sodium alginate powder in distilled water and the mixture was stirred overnight. After the formation of the alginate beads, they were left for 1 h in the calcium chloride solution.

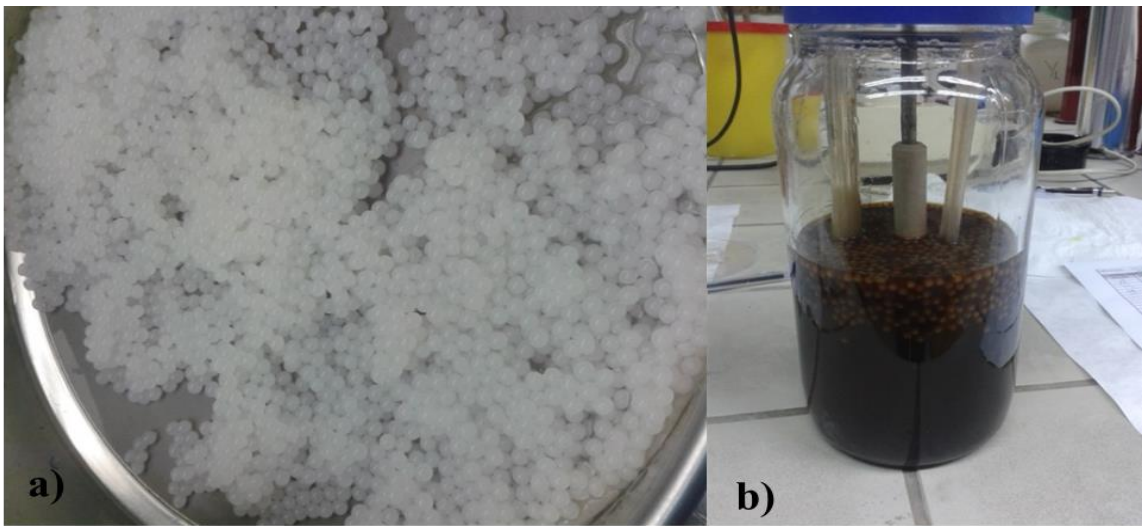



**Image 6.2** a) immobilized cells in alginate beads, b) bioreactor fermentation using immobilized cells in alginate beads

### 6.5. Pretreatment methods applied on SSL

#### 6.5.1. Extraction of phenolic compounds from SSL

Liquid-liquid extraction was performed in ten-times diluted SSL using ethyl-acetate as solvent (Faustino et al., 2010). SSL was mixed with the solvent in Erlenmeyer flasks, under stirring (300 rpm), for 30 min, at room temperature. The extraction was carried out at various

pH values and ethyl acetate-to-SSL ratios. The adjustment of the pH was carried out using either 5 M HCl or 5 M NaOH. The two phases were allowed to settle in a reparatory vessel for 10 min. The top phase (organic) was rich in phenolic compounds. The solvent was removed via vacuum evaporation and reused. The extract was then re-dissolved in methanol and stored at -20 $^{o}$C for further analysis. The bottom layer, containing mainly water soluble components, was concentrated to approximately 20% of its initial volume in order to eliminate ethyl-acetate residues. This phase was then used as fermentation substrate for succinic acid production.

**Image 6.3** Solvent extraction of SSL with ethyl acetate. Two phases are distinct: the aqueous phase (SSL) on the bottom and the phenolic-rich organic phase on the top

### 6.5.2. Experimental Design

The extraction of the phenolic compounds was subject to statistical optimization using the pH value of the extract and the ethyl acetate-to-SSL ratio as the most important parameters. A factorial design was initially implemented to observe the response in a wide range of pH values and ethyl acetate-to-SSL ratios followed by a more detailed design (Central Composite Design) on the optimum area, indicated by the factorial design.

*a) Factorial Design*

A $2^2$ factorial design was, initially, selected to evaluate a wide range of pH values and solvent-to-SSL ratios.The parameter ranges were based on similar studies found in the literature that have evaluated the extraction of phenolic compounds from various wood hydrolysates as well as according to preliminary experiments. Equation 1 was used in order to code the two examined variables.

$$x_i = \frac{X_i - X_i^{CP}}{\Delta X_i}$$ Eq. 1

Where: $i$ is the independent variable (pH or ethyl acetate-to-SSL ratio)

$x_i$ is the coded value

$X_i$ is the real value

$X_i^{CP}$ is the real value at the central point

$\Delta X_i$ is the step change value

The experimental responses were predicted by using a linear type equation:

$$Y_1 = b_1 + b_2x_1 + b_3x_2 + b_4x_1x_2$$ Eq.2

Where: $Y_1$ is the simulated response which in this case is the extracted phenolic concentration measured in g of gallic acid equivalents (g GAE) per L

$b_j$ is the vector parameter

$b_1$ is the intercept element

$b_2$ *and* $b_3$ are the linear effects of the two parameters

$b_4$ is the interaction parameter.

The parameters were obtained by linear regression minimizing the predicted with the experimental values.

*b) Central Composite Design (CCD)*

After the completion of the $2^2$ factorial design and according to the obtained results, a more detailed CCD was developed so as to introduce quadratic terms and hence allowing the estimation of curvature for the prediction of the extracted phenolic concentration. In the CCD, both the new central point and the new range of factors were indicated from the results obtained from the factorial design. Twelve runs were carried out for the CCD, four star points for the new extreme values, four at the corner of the design box and four runs (replicates) at the central point. Equation 1 was also used here for coding the two examined variables.

The new system was predicted by using the following $2^{nd}$ order polynomial equation:

$$Y_2 = bb_1 + bb_2 x_1 + bb_3 x_2 + bb_4 x_1 x_2 + bb_5 x_1^2 + bb_6 x_2^2 \quad \text{Eq.3}$$

Where: $Y_2$ is the simulated response (same as $Y_1$)

*bb(j)* is the parameter vector

$bb_1$ *to* $bb_4$ are the same as the corresponding parameters $b_1$ to $b_4$ of Eq.2

$bb_5$ and $bb_6$ are the quadratic parameters for pH and ethyl acetate-to-SSL ratio, respectively.

As in the factorial design, the parameters were obtained by linear regression minimizing the predicted with the experimental values. Matlab software (R2007b) was used for estimating the coefficients both in the factorial design and the CCD.

### 6.5.3. Solvent extraction of lignosulphonates from SSL

LS removal was carried out by liquid-liquid extraction using either 2-propanol or acetone as solvent. Before the extraction, SSL was diluted seven times with distilled water and the water-to-isopropanol ratio or water-to-acetone ratio was 12:88 according to Ikari et al. (1973). After the addition of the solvent, the mixture was agitated vigorously for five minutes and then left until the precipitation of the LS was observed. The LS were removed from the mixture by vacuum filtration and the solvent was evaporated under vacuum using a Büchi Rotavapor R-114 rotary evaporator at 60 $^{o}$C.

**Image 6.4** Solvent extraction of LS using isopropanol

### 6.5.4. Nanofiltration of SSL

Nanofiltration of SSL was carried out by AVECOM NV (Belgium) using a vibratory shear-enhanced processing filtration unit (V-SEP, New Logic Research, Emeryville, CA). A high shear at the surface of the filter membrane is achieved in a V-SEP filter via oscillatory vibration leading to significant improvement of the filter's resistance to fouling. This provides high throughputs and minimal reject volumes. The membranes used in the V-SEP filter for nanofiltration of SSL had MWCO of 500 Da (thin film non-polyamide, NF-500) and 800 Da (polyethersulfone, NF-PES-10). Before filtration, the SSL was diluted 7 times. The surface area of each membrane was 0.045 $m^2$. Each filtration run started with 28 - 35 L of the 7 times diluted SSL.


**Image 6.5** Set-up of the V-SEP nanofiltration module

*a) Membrane filtration with 800 Da MWCO*

The filtration unit was initially operated for 2 h in a closed circuit mode. The filtrate was returned to the influent vessel in order to avoid up-concentration of the influent. This preliminary membrane test was performed in order to determine the membrane flux under more or less steady-state conditions.

Around 28 L of 7 times diluted SSL were used in the nanofiltration run carried out with 800 Da MWCO membrane. During open circuit filtration mode, the flow rate of the

concentrate remained close to 450 L/h throughout filtration. In order to obtain an acceptable flux, the temperature and the pressure were increased during the filtration in the range of 38 – 54 $^{o}$C and 10.5 – 27.8 bar, respectively. The flux through the membrane was gradually decreased from 34.7 L/(m$^2$.h) to 14.7 L/(m$^2$.h) during filtration. The permeate obtained was analyzed for sugars, LS and phenolic compounds and the corresponding values were 27.5 g/L, 20 g/L and 1.8 g/L.

*b) Membrane filtration with 500 Da MWCO*

As in the previous case, the filtration unit was initially operated for 1 h in a closed circuit mode. Then, an open circuit filtration mode followed in order to collect the permeate. Around 32 L of 7 times diluted SSL were used in the nanofiltration run carried out with 500 Da MWCO membrane. The flow rate of the concentrate remained close to 680 L/h throughout filtration. The temperature and pressure during filtration were increased in the range of 54 – 57 $^{o}$C and 20.8 – 31 bar, respectively. The flux of the permeate through the membrane was decreased from 44 L/(m$^2$.h) to 10.7 L/(m$^2$.h) during filtration. The permeate obtained was analyzed for sugars, LS and phenolic compounds and the corresponding values were 22 g/L, 10 g/L and 1.6 g/L. More than 95% of LS separation is achieved with this membrane.

## 6.6. Pretreatment methods applied on SBP

### 6.6.1. Extraction of phenolic compounds from SBP

The extraction of the phenolic compounds present in SBP was carried out according to the procedure of Burniol-Figols et al., (2016). Briefly, 50 mL of acidified ethanol were added to 2 g of SBP (dry basis) and the mixture was added to a water bath at 70 $^{o}$C, at 150 rpm for 40 min. After incubation, the sample was filtrated and the solvent was vacuum evaporated. The extract was first weighted and then dissolved in 5 mL methanol for subsequent analysis.

### 6.6.2. Extraction of pectins

Pectins were extracted following the procedure of Hwang et al., (1998). More specifically, one liter of HCl (pH 1.8) was added in 50 g of dry sample, and the mixture was stirred at 85 $^{o}$C for 30 min. The supernatant was separated from the solids via centrifugation and filtered through Whatman No 1 filter papers. The next step involved the addition of 1N

NaOH, in order to adjust the pH to 4.5 and then 4 L of isopropanol were added. The precipitate was filtrated, washed with isopropanol and acetone and dried at room temperature.

### 6.6.3. Hydrolysis of SBP

SBP was subjected to both acidic and enzymatic hydrolysis, in order to hydrolyse hemicellulose and cellulose, respectively. Different parameters were studied in each case. The hydrolysis yield was evaluated according to the total amount of sugars released in comparison to the theoretical sugars derived from the cellulose and hemicellulose that are present in SBP. For cellulose the conversion factor used was 0.9 and for hemicellose 0.88 (Wyman et al., 2004).

A set of preliminary experiments was firstly carried out according to the study of Bellido et al. (2015). A suspension of 6% SBP (w/v) in water was placed in 250 mL Erlenmeyer flasks was pretreated in four different ways: autohydrolysis, autohydrolysis at pH 4, with dilute sulfuric acid and hydrochloric acid pretreatment (0.5% v/v in each case). Then, the hydrolysis of hemicellulose was carried out at 121 $^{o}$C for 15 min. Enzymatic hydrolysis was then followed, after adjusting the pH to 5, with the addition of 20% (w/w) NaOH using Accellerase 1500 (Du Pont). Enzyme loading was 0.1 mL Accellerase 1500 per gram of cellulose, and the hydrolysis was carried out at 50 $^{o}$C for 24 h, under stirring (150 rpm). These conditions were studied with and without mechanical pretreatment of SBP. When no mechanical pretreatment was used, the pellets were left in a small part of water in order to be decomposed. When mechanical pretreatment was used, SBP was ground with a cutting mill (Grindomix GM 200, Retsch, Germany) at a particle size of approximately 1 mm.

Four different solid-to-liquid ratios were subsequently studied, namely 6%, 7.5%, 10% and 15% (w/v). Acidic hydrolysis was carried out using 0.5% or 1% (v/v) $H_2SO_4$ at 121 $^{o}$C for 15 min or 30 min. Five militers of sample were taken in order to analyze the individual sugars that were released from hemicellulose degradation. Afterwards, the pH was adjusted to 5 as previously stated and Accellerase 1500 was added in a dosage varied from 0.1-0.5 mL per gram of cellulose. The reaction lasted for 24 h at 50 $^{o}$C at 150 rpm. The final slurry was centrifuged in order to remove the solids and the supernatant was analyzed for glucose.

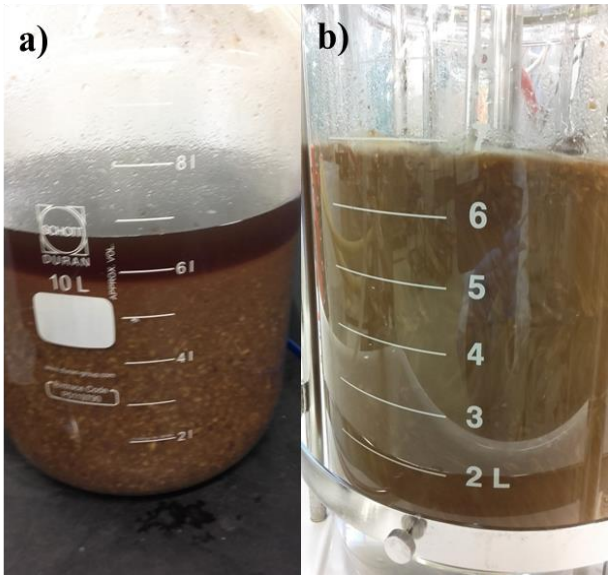



**Image 6.6** SBP pretreatment: a) after acid pretreatment and b) after enzymatic hydrolysis

### 6.6.4. Microfiltration of SBP-derived hydrolysates

SBP hydrolysate was microfiltrated using an UFI-TEC cross flow microfiltration system (UFI-TEC, Germany), equipped with 4 TAMI membranes (TAMI Industries, France) with 0.2 μm or 0.4 μm pore size. The system operated at 1.5 bar and 15 $^{o}$C.

## 6.7. Fermentation media

### 6.7.1. SSL-based media

Batch, fed-batch, repeated batch and repeated fed-batch fermentations were carried out in bench-top bioreactors with 0.5 L working volume. Dilution of SSL was carried out in order to achieve an initial sugar concentration of approximately 25 g/L. The medium also contained 5 g/L yeast extract, 15 g/L $MgCO_3$, 1.16 g/L $NaH_2PO_4 \bullet H_2O$, 0.31 g/L $Na_2HPO_4$, 1 g/L NaCl, 0.2 g/L $MgCl_2 \bullet 6H_2O$, 0.2 g/L $CaCl_2 \bullet 2H_2O$ and antifoam. The experiments were carried out under continuous sparging of $CO_2$ (0.5 vvm) at 37 $^{o}$C and 300 rpm agitation. The pH control was carried out by the continuous addition of 5 M NaOH in order to be maintained to 6.6-6.7. Fermentation medium was sterilized at 121 $^{o}$C for 20 min. The inoculum was 10% (v/v) in all fermentations.

When cell immobilization was employed, before their addition into the fermentation broth the immobilized cells were washed twice with 250 mL of synthetic medium with similar composition as described above, in order to remove the free cells. In repeated batch fermentations, before every batch run, the immobilized biocatalyst was also washed with

synthetic medium for the removal of residues from the previous batch run. In all cases the immobilized biocatalyst was introduced into the fermentation medium under aseptic conditions. As pH neutralizer, $Na_2CO_3$ was added in all fermentations. For the biomass formation, $Na_2CO_3$ was added at the same concentration as glucose. For the SSL fermentations, $Na_2CO_3$ was added at a concentration of 7.5 g/L.

Fed-batch fermentations were carried out either in pulses or continuously. When feeding pulses were performed, feeding occurred when sugars reached about 5 g/L. The volume added was calculated to yield a final sugar concentration of 15 g/L. In case feeding was carried out continuously, the pump rate was appropriately adjusted in order to keep the sugar concentration in the fermentor at about 10-15 g/L.

The calculation of succinic acid yield for the fed-batch fermentations was based on the following equation:

$$SA\ Yield = \frac{(g\ SA\ produced)+(g\ SA\ in\ samples)}{(g\ initial\ TS)+(g\ TS\ in\ feeding)-(g\ TS\ in\ samples)-(g\ final\ TS)} \quad \text{Eq.4}$$

where SA stands for succinic acid and TS stands for total sugars.

**Image 6.7** Bench-top bioreactor used for SSL fermentations

### 6.7.2. SBP-based media

*a) Bioreactor fermentations in laboratory scale*

Batch and fed-batch fermentations were conducted in 5 L bench-top BIOSTAT bioreactor (Sartorius AG, Germany), with 3 L working volume. Stirring was at 300 rpm and the pH was adjusted to 6.6-6.8 by adding 20 % (w/w) NaOH. The medium also contained 5 g/L yeast extract (Ohly KAT, Deutsche Hefewerke GmbH & Co. OHG, Germany) unless otherwise reported, 10 g/L $MgCO_3$, 1.16 g/L $NaH_2PO_4 \cdot H_2O$, 0.31 g/L $Na_2HPO_4$, 1 g/L NaCl, 0.2 g/L $MgCl_2 \cdot 6H_2O$, 0.2 g/L $CaCl_2 \cdot 2H_2O$ and antifoam. The experiments were carried out under continuous sparging of $CO_2$ (0.1 vvm) at 37 $^oC$ and 300 rpm agitation. Fermentation medium was either sterilized at 121 $^oC$ for 20 min or deactivated at 70 $^oC$ for 1h. The inoculum was 10 % (v/v) in all fermentations. Samples were taken regularly for the analysis of sugars, organic acids and biomass. Every sample was firstly inactivated by heating in a waterbath at 95 $^oC$ for 20 min, and stored at -20 $^oC$ until used for further analysis.

**Image 6.8** Biostat B-Plus bioreactor used for the fermentations using SBP (ATB, Germany)

*b) Pilot scale fermentations (72L)*

Pilot scale fermentation was performed in a 72 L BIOSTAT UD bioreactor (B-Braun Biotech, Germany) with working volume of 30 L. The bioreactor contained 23 L SBP hydrolysate, 5 g/L yeast extract, the aforementioned mineral solution and 10 g/L of $MgCO_3$. Fermentation conditions were as described for the laboratory scale tests. Inoculum was grown for 24 h in a TSB solution with 25 g/L glucose. Feeding was concentrated SBP hydrolysate. Samples were taken regularly and treated as previously described.

Feeding strategy and succinic acid yield calculations were carried out as described in paragraph **6.7.1**.

**Image 6.9** 72 L BIOSTAT UD bioreactor (ATB, Germany)

## 6.8. Analytical methods

### 6.8.1. HPLC analysis of sugars and organic acids

Analysis of sugars present in SSL and in the fermentations carried out using SSL-based media (xylose, glucose, galactose, mannose and arabinose) and carboxylic acids (succinic, formic, acetic and lactic acid) was carried out by a Shimadzu HPLC system using a Rezex ROA-Organic acid H+ column and a Shimadzu RI detector. The temperature of the column was 70 $^{o}$C and the mobile phase was a 10 mM $H_2SO_4$ aqueous solution with 0.6 mL/min flow rate.

Analysis of monosaccharide concentrations present in SBP-derived hydrolysates and fermentation media (glucose, xylose, and arabinose) and organic acids (succinic acid, lactic acid, formic acid and acetic acid) in both hydrolysates and fermentation samples were determined by high performance liquid chromatography (HPLC, DIONEX, USA). For the analysis, 10 μL of sample volume were injected in a Eurokat H column (300 mm x 8 mm x 10 μm, Knauer, Germany). Elution of the sample was carried out with 5 mM $H_2SO_4$, at a flow of 0.8 mL/min and detection was carried out by a refractive index detector (RI-71, Shodex, Japan). Each analysis was carried out in duplicate and peak areas and retention times were used in comparison to known concentrations of the pure substances. The analysis was carried

out by the department of Analytical Chemistry, in Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB), in Potsdam, Germany.

Ion concentrations present in the SBP-derived fermentation media were determined by ion chromatography (DIONEX, USA). For the measurement of cations, 25 μL of sample were injected to an IonPac CS 16 column (250 mm x 4 μm, DIONEX, USA). The eluent used was 30 mM $CH_3SO_3H$, at a flow rate of 1 mL/min at a temperature of 40 $^oC$. For the measurement of ions, 25 μL of sample were injected to an IonPac AS9-HC column (250 mm x 4 μm, DIONEX, USA). The eluent used was 9 mM $Na_2CO_3$ at a flow of 1.2 mL/min, operating at room temperature. In both cases, detection was carried out by a conductivity cell. For each analysis, the peak areas were compared to the ones of known concentrations of salt solutions. Analyses were carried out in duplicates and they were performed by the department of Analytical Chemistry, in Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB), in Potsdam, Germany.

### 6.8.2. Determination of individual phenolic compounds by HPLC-DAD

Chromatographic analysis was carried out on a Jasco liquid chromatography apparatus coupled to a diode array detector (MD-910 Jasco). The samples were injected using an autosampler Jasco AS-1555. The separation was achieved by a reversed phase C18 column, Waters Nova-Pack C18 column (3.9 mm x 150 mm, 4 μm) at ambient temperature. Gradient program was based on the modified method of Guendez et al., (2005). More specifically: eluent (A) was 0.1% aqueous solution of perchloric acid, and eluent (B) MeOH and the flow rate was 1 mL $min^{-1}$, with an injection volume of 20 μL. The elution program used was as follows: 100% A 0-5 min, 90% A/ 10% B 5-15 min, 82.5% A/ 17.5% B 15-25 min, 75% A / 25% B 25-45 min, 40%A/ 60% B 45-60 min, 100% B 60-80 min, 100% A 80-85 min. Chromatograms were monitored at 280, 320 and 360 nm and identification was based on retention times and on-line spectra data related to the standard compounds. Quantification was carried out based on calibration curves for each compound, using standard solutions.

### 6.8.3. Determination of phenolic content (% OH) and lignosulphonate content in SSL

Spectrophotometric determinations of the phenolic content (%OH) and LS content of the SSL were carried out using a double-beam UV-Vis spectrophotometer (Jasco V-530), according to UNE EN 16109:2012 protocol. This protocol involves the preparation of a solution containing 0.15 - 0.2 g SSL (on dry matter basis) diluted to 250 mL with distilled

water in order to acquire a clear solution with lignosulphonate content in the range of 0.6 – 0.8 g/L. The pH of this solution is then adjusted to 2.0-2.2 with a 5 M HCl solution. Five millilitres of this solution are transferred to a 50 mL volume flask and bring to volume with distilled water. Finally, the solution is scanned from 220 to 340 nm. The content of phenolic hydroxyl groups (g per 100 g dry SSL) of the SSL were calculated according to equation 1.

$$OH_{phenolics}(\%) = 0.192 \cdot \frac{B - C}{Conc(g/L)} \qquad \text{Eq.5}$$

Where: Conc (g/L) is the value of dry lignosulphonates concentration in 50 mL solution, B is the absorbance peak value at approximately 255 nm and C the minimum valley to either side.

### 6.8.4. Determination of trace elements and rare earth elements (REE) with ICP-MS

*a) Chemicals and standard solution preparations*

Chemicals used in the experimental work were nitric acid (suprapur, 65% w/v, Merck, Darmstadt, Germany), hydrogen peroxide (suprapur, 30% w/v, Merck, Darmstadt, Germany), ICP internal standards of Ge and In (Inorganic Ventures, New Jersey, USA), an ICP-MS certified multi-element standard for the trace elements (Inorganic Ventures, New Jersey, USA) and an ICP-MS rare earth multi-element standard (Inorganic Ventures, New Jersey, USA). Standard stock solutions were used to prepare calibration standard solutions after appropriate dilution with 1.0% v/v $HNO_3$ and reversed osmosis ultra-pure water with a resistance of 18.2 MΩ $cm^{-1}$ obtained from a MilliQ plus system (Millipore, Saint Quentin Yvelines, France).

*b) Sample treatment*

Two and a half milliliters of diluted SSL, nanofiltrated SSL, isopropanol treated SSL or the phenolic-rich extract were added into a microwave vessel. The appropriate dilution of the samples was carried out with reversed osmosis ultra-pure water. Five milliliters of 30% (v/v) $H_2O_2$ solution were added into each vessel and the mixture was left to stand for 1 h at room temperature. Then, 5 mL of 65% (v/v) $HNO_3$ solution were added in each vessel and microwave assisted acidic digestion was carried out in a closed CEM microwave-assisted

system (CEM, Mars X-Press, Matthews, NC, USA). The samples were heated in the microwave accelerated digestion system according to the following program: the power was ramped during 5 min from 0 to 960 W and held for 15 min. The temperature reached a maximum of 175 °C and followed by a cool-down cycle for 15 min. Losses of volatile element compounds did not occur as the tubes were sealed during heating. After the digestion, samples were again diluted with reversed osmosis ultra-pure water and trace elements, macro elements and REE were determined by inductively coupled plasma mass spectrometry (ICP-MS). Standard solutions used for calibration curves were prepared from standards of high purity.

*c) ICP-MS analysis*

ICP-MS is almost exclusively used for measuring REE providing high-throughput, ultra-trace level analysis down to ppt (Georgiou and Danezis, 2015). Elemental content was determined using a Perkin Elmer (SCIEX, Canada) 9000 Series, inductively coupled plasma mass spectrometry, ICP-MS. Operating conditions of the ICP-MS were as follows: nebuliser gas flow of 0.75 L $min^{-1}$, ICP RF power of 950 W, lens voltage of 7 V, pulse stage voltage of 950 V and sample uptake rate of 26 rpm. Rare earth element calibrations were obtained from 1 ppt to 10 ppb and trace element from 0.1 ppb to 1 ppm, respectively. The elements In and Ge were used as internal standards for REEs and trace elements, respectively. In order to assess the accuracy of the process the following standard reference materials were used: NIST-RM 8414 – bovine muscle powder (National Institute of Standards & Technology, USA), NIST-RM 8415 whole egg powder (National Institute of Standards & Technology, USA), ERM-BB 186 pig kindey (European Commision, Joint Research Center, institute for reference materials and measurements IRMM, Belgium) for trace elements and CRM-668 muscle tissue (European Commision, Joint Research Center, institute for reference materials and measurements IRMM, Belgium) for rare earth elements. The standard reference materials were subjected to the exact same analytical process and analyzed in triplicate. The limits of quantification are lower than the measured values for all the elements.

### 6.8.5. Spectrophotometric determination of TPC of the extracts obtained from both SSL and SBP

Determination of Total Phenolic Content (TPC) was carried out using the Folin-Ciocalteu colourimetric method as described by Faustino et al. (2010). Briefly, 50 μL of the methanolic extract were dissolved in 450 μL of distilled water. Then, 2.5 mL of 0.2 N Folin-

Ciocalteu reagent were added in the mixture. The solution was shaken and after 3 min, 2 mL of $Na_2CO_3$ (75 g/L) were added. The mixture was shaken for 1.5 h at 30 $^oC$. The absorbance was measured at 765 nm using a double-beam UV-Vis spectrophotometer (Jasco V-530) and compared to a gallic acid calibration curve. The concentration of total phenolic compounds in the SSL methanolic extract was expressed as grams of gallic acid equivalents per liter SSL. The concentration of total phenolic compounds in the SBP methanolic extract was expressed as milligrams of gallic acid equivalents per gram of dry SBP (mg GAE/g). All determinations were performed in triplicate.

### 6.8.6. Determination of antioxidant activity of the extracts obtained from SSL and SBP

Antioxidant activity of the extracts was determined according to the DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) scavenging radical method (Scherer and Godoy, 2009). Briefly, 0.1 mL of sample were added in 3.9 mL of DPPH$^{\bullet}$ solution (31.6 μg/mL). The mixture was shaken and then it was incubated at room temperature in the dark for 90 min. A solution of 0.1 mL aqueous methanol (70:30 v/v) in 3.9 mL DPPH$^{\bullet}$ was used as control. The decrease in absorbance was measured at 517 nm using a double-beam UV-Vis spectrophotometer (Jasco V-530, Tokyo, Japan).

The radical scavenging activities of the samples, expressed as percentage inhibition of DPPH$^{\bullet}$, were calculated according to the equation 6:

$$Inhibition(\%) = 100 \cdot \frac{A_B - A_A}{A_B} \qquad \text{Eq.6}$$

$A_B$ and $A_A$ are the absorbance values of the control and the test samples, respectively. Inhibition (%) or $IC_{50}$ corresponds to the minimum extract concentration required to decrease the initial absorbance of the DPPH• solution by 50%.

The antioxidant activity expressed as the antioxidant activity index (AAI) was calculated according to equation 7:

$$AAI = \frac{\left[ Final\_DPPH\left( \mu g / mL \right) \right]_{blank}}{IC_{50}\left( \mu g / mL \right)} \cdot 100 \qquad \text{Eq.7}$$

### 6.8.7. Dry matter (DM)

To determine the DM of the samples, a certain amount was weighted and dried until constant weight at 105 $^{o}$C. DM was then calculated according to the following equation:

$$DM(\%)\frac{m\ out}{m\ in} * 100 \qquad \text{Eq.8}$$

Where:

DM (%): dry matter

$m_{in}$: weight in grams of substrate before drying

$m_{out}$: weight in grams of substrate after drying

The results are presented as percentage of % (w/w).

### 6.8.8. Ash content

The ash content was determined by treating a defined portion of a sample at 550 $^{o}$C for 5 h in a muffle furnace. The calculation of the ash content was done according to the following equation:

$$Ash\ content(\%)\frac{m\ out}{m\ in} * 100 \qquad \text{Eq.9}$$

Where:

Ash content (%): ash content

$m_{in}$: weight in grams of substrate before drying

$m_{out}$: weight in grams of substrate after drying

The results are presented as percentage of % (w/w).

### 6.8.9. Living cells

Number of living cells was calculated as colony forming units counted on a plate containing Tryptic Soy Broth Agar (Merck, Germany), after 48 h of incubation at 37 $^{o}$C.

### 6.8.10. Fat analysis

Fat analysis was carried out in SBP using an ANKOM Technology (USA) according to the ANKOM Technology Method 2, 01-30-09: Determination of Oil/Fat Utilizing Temperature Solvent Extraction (ANKOM, 2009). The analysis was carried out by the department of Analytical Chemistry in ATB (Potsdam, Germany).

### 6.8.11. Determination of cellulose, hemicellulose, lignin and total Kjeldahl-nitrogen in SBP

Analyses of cellulose, hemicellulose and lignin content in SBP were carried out using an ANKOM fiber analyzer (ANKOM, 2014). Total Kjeldahl- nitrogen was determined according to the DIN-EN-25663 standard method. The analyses were carried out by the department of Analytical Chemistry in ATB (Potsdam, Germany).

## 6.9. Downstream Methods

### 6.9.1. Modified calcium precipitation method

The calcium precipitation method was performed with calcium hydroxide or calcium oxide and it is the classical industrial method employed for the separation of carboxylic acids from fermentation broths. The modified calcium precipitation method applied was based on the study of Luque et al. (2009). More specifically, 100mL of fermentation broth were centrifuged in order to remove the biomass. The supernatant was then filtrated through Whatman No. 1 paper. The filtrate was mixed with 12.5% (w/v) activated carbon (Sigma-Aldrich) for 1h in order to decolorize the sample. The suspension was then vacuum evaporated and the pH of the clear broth was adjusted to around 13.5 by adding approximately 20% (w/v) calcium hydroxide solution and the mixture was placed on a shaker at 200rpm at 39$^{o}$C for 20h. Distilled water was used and an excess of $H_2SO_4$ was added to form calcium sulfate. When the pH reached a value of around 2.5, the calcium sulfate precipitate was removed via filtration. The precipitate was washed twice with distilled water in order to remove the remaining SA. Vacuum distillation was then carried out in order to remove the residual volatile carboxylic acids. The final solution was concentrated to 20% of its original volume and the crystallization of SA was carried out at 4$^{o}$C for 24h. Final yield and purity were calculated according to the following equations:

$$Yield\ (\%) = \frac{Dry\ weight\ of\ the\ SA\ in\ crystals\ recovered\ (g)}{Initial\ dry\ weight\ of\ SA\ in\ the\ fermentation\ broth\ (g)} \quad \text{Eq.10}$$

$$Purity\ (\%) = \frac{Dry\ weight\ of\ the\ SA\ in\ crystals\ recovered\ (g)}{Dry\ weight\ of\ crystals\ recovered\ (g)} \quad \text{Eq.11}$$

### 6.9.2. Direct crystallization method

The fermentation broth was centrifuged for 15 min at 9,000 rpm and 4°C in order to separate the biomass. The supernatant was filtered through Whatman No.1 paper. Activated carbon was mixed with the filtrate for 1h to remove the organic impurities that are responsible for the color of the broth. The concentration of the activated carbon varied according to the medium used for fermentation (synthetic, untreated SSL or pretreated SSL). The suspension was then filtered and the clear fermentation broth was treated according to Process DC_1 or Process DC_2 in order to remove by-products and salts.

*a) Process DC_1: Direct crystallization method with acidification.*

The pH of the actual fermentation broth was adjusted to 2 using 98% sulfuric acid. The solution was vacuum evaporated at 60°C to remove the volatile carboxylic acids such as acetic and formic acids. Evaporation was terminated when the solution was concentrated to approximately 20% of its original volume and the crystallization of succinic acid was carried out at 4°C. The final slurry was filtered through Whatman No.1 paper and the crystals were carefully washed with a saturated solution of succinic acid to remove impurities. The SA crystals were dried at 70°C for 12h.

*b) Process DC_2: Direct crystallization using an ion-exchange resin*

In this process for the conversion of the sodium and magnesium salts into acids, a cation exchange resin was applied. Amberlite IR 120H (Sigma-Aldrich), a cationic resin of sulfonic ($SO_3H$) type based on a polystyrene-divinylbenzene copolymer, was selected according to the process developed by Lin et al. (2010). Approximately 100 mL of fermentation broth were passed through 50 g of resin. The pH of the effluent was 2.0. The acidified medium was then subjected to vacuum distillation and crystallization as previously described in Process DC_1.

### 6.9.3. Salting-out

The fermentation broth (100 mL) was treated with $H_2SO_4$ 76% until the pH reached a value of 3. Then, 30% (v/v) acetone and 20% (w/v) $(NH_4)_2SO_4$ were added and the mixture was shaken. Phase separation was carried out for 8 h to remove cell biomass, protein impurities, sugars, part of the by-products and part of the pigments. Succinic acid was extracted in the acetone phase. Activated carbon, 10% (w/v) was added to the succinic acid-acetone phase in order to remove the residual organic impurities. The next step includes vacuum filtration in order to remove the activated carbon and the filtrate was vacuum evaporated to recover acetone and to concentrate succinic acid approximately 5-fold. Subsequently, crystallization was carried out at a pH value of 2 and at $4^o$ C. Finally, succinic acid crystals were dried at $70^o$ C for 10h.

### 6.9.4. Reactive extraction

The list of the amines and the solvents that were used for the reactive extraction are shown in Table 6.1 together with some of their main properties. The amines and the solvents were purchased from Sigma-Aldrich. The amine concentration used in all cases was 0.5 mol/ kg solvent. Adjustment of pH was carried out either by using cation exchange resins or NaOH 10M. For the reactive extraction of the organic acids, 500 μL of succinic acid solution (aqueous succinic acid solution or fermentation broth) were mixed with 500 μL of a selected reactive extraction system. The two phases were mixed vigorously for 5 min. Subsequently, phase separation was carried out by centrifugation at 4,500 rpm for 5 min. The aqueous phase was used for analysis of the remaining organic acids, whereas the organic phase was kept for further processing. The selection of the various reactive extraction systems was decided after a thorough literature review on reactive extraction systems for the recovery of succinic acid (Kurzrock et al., 2011).

**Table 6.1** Properties of the different amines and solvents used for the reactive extraction of succinic acid

| Chemical | Type of amine/solvent | Boiling point ($^oC$) | Solubility in water (25$^oC$) |
|---|---|---|---|
| Octylamine | primary | 175-177 | 0.2 g/L |
| Dihexylamine | secondary | 192-195 | 0.3 g/L |
| Dioctylamine | secondary | 297-298 | < 0.1 mg/L |
| Diisooctylamine | secondary | 123 | 0.6766 mg/L |
| Tripentylamine | tertiary | 81-83/0.2 mmHg | insoluble |
| Trihexylamine | tertiary | 263-265 | No data available |
| Trioctylamine | tertiary | 365-367 | 0.05 mg/L |
| Methyldioctylamine | tertiary | 162-165/ 15 mmHg | No data available |
| 1-butanol | polar/proton donating | 117.4 | 73 g/L |
| 1-Hexanol | polar/proton donating | 155-159 | 5.9 g/L (20$^oC$) |
| 1-Octanol | polar/proton donating | 195 | 0.46 g/L |
| 2-Octanol | polar/proton donating | 178.5 | 1.12 g/L |
| MIBK (methylisobutylketone) | polar/aprotic | 117 | 19.1 g/L (20$^oC$) |

The back-extraction of the succinic acid was carried out either by pH-swing or by temperature-swing. For pH-swing, one milliliter of organic phase was vigorously mixed with 6 mL aqueous solution of NaOH (pH=13) for 12 h. Then, the two phases were separated via centrifugation and the aqueous phase was analyzed for organic acids. Temperature-swing was carried out in three different ways. One milliliter of organic phase was added to 6 mL of distilled water and then it was placed either in an ultrasonic bath for 9 h, or in a water bath for 5 h and 9 h at 50 $^oC$. In all cases, phase separation was achieved via centrifugation and the aqueous phase was analyzed for organic acids.

### 6.9.5. Downstream process based on bipolar electrodialysis membranes

The succinic acid produced using SBP-derived hydrolysates was separated from the fermentation broth using a novel downstream process based on bipolar electrodialysis membranes. The process followed was adapted from the one that ATB follows for lactic acid purification. The purification steps are the following:

*a) Filtration*

Fermentation broth was first inactivated and then microfiltrated using an UFI-TEC cross flow microfiltration system (UFI-TEC, Germany), equipped with 4 TAMI membranes (TAMI Industries, France) with 0.2 μm pore size. The system operated at 1.5 bar and 15 $^{o}$C.

*b) Softening*

Softening was carried out in order to remove cations (calcium, magnesium and sodium) that are present in the permeate stream after nano-filtration. Removal of the cations was carried out by using PUROLITE S950 acid chelating resin (Purolite, Germany). The pH of the permeate stream was first adjusted to 10 with the addition of 20 % (w/w) NaOH. Afterwards, the permeate was introduced to the column from below in an expanded bed setting. The flow was 6 bed volumes/h. Finally, the column was rinsed with ultrapure water until conductivity had a value below 1 mS/cm.

*c) Electrodialysis*

Monopolar electrodialysis was carried out in order to concentrate the initial succinate solution. Bipolar electrodialysis followed in order to split succinate into free succinic acid and base (sodium hydroxide). Electrodialysis (mono- and bipolar) was carried out in batch mode, at 35 $^{o}$C and at constant polarity. Monopolar electrodialysis is consisted of 11 cation exchange membranes Type II (Fujifilm, the Netherlands) and 10 anion exchange membranes Type II (Fujifilm, the Netherlands) at 20 V and 3 A. This process was finished when the conductivity of the diluate was below 0.5 mS/cm.

The next step involved the processing of the concentrated salt-solution obtained by the monopolar electrodialysis with the bipolar one. This process was carried out at 30 V and 5 A and it was consisted of 11 cation exchange membranes Type II (Fujifilm, the Netherlands) and 10 anion exchange membranes Type II (Fujifilm, the Netherlands) at 20 V and 3 A. With the use of the electric potential, the anions pass through the membranes into the acid cycle, whereas the cations are transferred to the base cycle. The process was stopped when the conductivity of the retentate reached a value below 25 mS/cm. The stream from the acid cycle was subjected to further purification steps.

*d) Decolorization*

Decolorization was carried out using PUROLITE MN-502 (Purolite, Germany), in an expanded bed configuration, which was filled from below. The flow rate was 6 bed volumes/h. When all the sample was treated, purified water passed through the column until conductivity was below 1 mS/cm.

*e) An- and cation exchange chromatography*

The use of anion and cation exchange chromatographies were carried out in order to separate succinic acid from the salt-anions. The decolorized stream was passed first through the weak anion exchange resins RELITE EXA 133 (Resindion, S.R.L., Italy) and through the strong cation exchange resin RELITE EXC 08 (Resindion, S.R.L., Italy). For both columns, the volume was 1 L and the flow rate was 6 bed volumes/h. As previously stated, the columns were filled up from below. The columns were then washed with purified water, until the conductivity reached a value of 1 mS/cm.

*f) Spray dryer*

The final step in order to obtain succinic acid in powder form, was to introduce the purified stream produced after chromatography to a spray dryer GEA Miro, type GEA Mobile Minor. Maximum inlet temperature was 120 $^{o}$C. Hot air was introduced at a flow rate of 80 kg/h. The maximum water evaporation was approximately 5-6 L/h. Pressure employed was 6 bars. Succinic acid powder was collected, weighted and analyzed for impurities.

# CHAPTER 7
# EXTRACTION OF PHENOLIC COMPOUNDS FROM SSL AND EFFECT ON SUCCINIC ACID PRODUCTION VIA FERMENTATION

## 7.1. Introduction

The global economic crisis and the gradual decrease of fossil resources due to increasing population and economic development have led to the imperative need for use of renewable resources and development of novel industrial processes fully adapted to the bio-economy era. This can only be achieved via refining of renewable resources leading to the production of numerous products with diversified market outlets and reduced energy consumption and production of wastes. The waste and by-product streams produced by current industrial processes could be employed for the development of novel biorefining concepts. In this concept, spent sulphite liquor (SSL) could be further valorized via its fractionation to value-added products, like sugars, phenolic compounds and lignosulphonates (LS). The detoxified stream that is produced could be employed as fermentation feedstock for the production of succinic acid. In this chapter, the extraction of phenolic compounds was optimized and the phenolic-rich stream obtained was characterized in order to estimate its commercial potential. Subsequently, succinic acid fermentations using detoxified and non-detoxified SSL were carried out in order to assess the inhibition of phenolic compounds on succinic acid production.

## 7.2. Extraction of phenolic compounds from SSL

The compositional analysis of SSL revealed that it contains phenolic compounds, at a concentration of 12.4 ± 0.8 g/L. Their presence is attributed to lignin degradation during delignification of wood. Phenolic extracts obtained from wood hydrolysates are proved to have strong antioxidant activity that could find various applications as active ingredients (Willför et al., 2003). Their removal could enhance the fermentability of the feedstock and at the same time could result to the production of a value-added product.

Liquid-liquid extraction with ethyl acetate is a well-studied method for the treatment of various lignocellulosic hydrolysates (Conde et al., 2011; Cruz et al., 2005; Garrote et al., 2008; González et al., 2004), as sugars remain in the aqueous phase, whereas phenolics and various other compounds are transferred to the organic phase. Other advantages of liquid-liquid extraction in comparison to other separation methods involve the relatively easy solvent

recovery (result of their low boiling point) and subsequent easy product recovery as well as the production of a detoxified-media that could be employed as fermentation substrate (Cruz et al., 1999). The compounds present in ethyl acetate extracts of pretreated wood hydrolysates can be: a) sugar derived compounds, mainly aldehydes from dehydration reactions, b) phenolic compounds, such as phenolic acids (with hydroxybenzoic and hydroxycinnamic acid skeleton) and their derivatives like alcohols, aldehydes and ketones, c) aliphatic fatty acids and other compounds (Garrote et al., 2004).

When liquid-liquid extraction is applied, the most important parameters affecting extraction efficiency are the solvent, the pH of the aqueous phase, the extraction time and solvent-to-sample ratio (Llano et al., 2015).There are many studies highlighting the importance of the pH of the aqueous phase in solvent extraction of phenolic compounds as well as the solvent-to-sample ratio (Cruz et al., 1999; Faustino et al., 2010; Llano et al., 2015)(Faustino et al., 2010; Cruz et al., 1999, Llano et al., 2015). Low pH values resulted in higher extraction yields, whereas a solvent-to-sample ratio of 3:1 (v/v) resulted in sufficient extraction efficiency. This is mainly attributed to the acidic nature of the phenolic hydroxyl group. In general, phenolic compounds are considered as week acids. The $pK_a$ of each phenolic compound results from its structure as well as the nature of the substituents on the benzene ring(s). The solvent employed is another important parameter. Ethyl acetate is the most commonly applied solvent, followed by diethyl ether and chloroform. Among these organic solvents, ethyl acetate has proved to result in higher phenolic removal yields (Cruz et al., 2005, 1999; Mateo et al., 2013; Parajó et al., 1997). Another important operational factor that affects the removal of phenolic compounds is the extraction time. Previous studies have shown that 30 min extraction time are sufficient for the removal of phenolic compounds from *Eucalyptus globulus* wood extracts (Cruz et al., 1999) and Spent sulphite liquors (Faustino et al., 2010).

In this work, different pH values and solvent-to-SSL ratios were evaluated by applying an experimental design approach in order to identify the optimum extraction conditions leading to the highest removal of phenolic compounds from SSL. Ethyl acetate was used as extracting solvent as it has proved to be more appropriate for phenolic extraction from SSL. Extraction time was also set to 30 min according to literature- cited publications (Faustino et al., 2010; Llano et al., 2015).

## 7.3. Optimization of extraction of phenolic compounds-Experimental design

### 7.3.1. Factorial Design

The significance of the model was assessed using the F-test. The model described on Eq.2 is (*Chapter 6: Materials and Methods*) very significant as the F-value $F_{(3,4)}$ is equal to 25.8 which is higher than the value of 16.69 indicated by the Fisher's distribution table with a level of importance of 0.01. All the results in the form of analysis of variance (ANOVA) as well as the obtained parameter values are presented in Table 7.1 and Table 7.2. The prediction capability of the model is high as dictated by the $R^2$ and $R^2$ adjusted values of 0.95 and 0.91, respectively.

**Table 7.1** ANOVA for the $2^2$ factorial design and estimation of parameter values

| **Source** | **Degrees of Freedom** | **Sum of Squares** | **Mean Square** | **F-value** | **P>F** |
|---|---|---|---|---|---|
| Model | 3 | 1.947 | 0.649 | 25.802 | 0.005 |
| Error | 4 | 0.101 | 0.025 | | |
| Lack Of Fit | 1 | 0.017 | 0.017 | 0.615 | 0.490 |
| Pure Error | 3 | 0.084 | 0.028 | | |
| Total | 7 | 2.047 | | | |
| **parameters** | **estimate** | **standard error** | | **t Ratio** | **Prob>\|t\|** |
| $b_1$ (intercept) | 5.866 | 0.056 | | 104.63 | <.0001 |
| $b_2$ ($X_1$) | -0.505 | 0.079 | | -6.37 | 0.003 |
| $b_3$ ($X_2$) | 0.420 | 0.079 | | 5.30 | 0.006 |
| $b_4$ ($X_1X_2$) | -0.235 | 0.079 | | -2.96 | 0.0414 |

**$R^2$ = 0.951, $R^2$ Adjusted=0.914, RMSE=0.159, CV=9.22%**

According to the *t* value obtained, the most significant parameter is the pH followed by the ethyl acetate-to-SSL ratio and finally by their interaction. The results obtained by the factorial design were interpreted in a contour plot where model predictions were illustrated for the examined area (Figure 7.1). Better extraction of phenolic compounds (> 6.0 g GAE/L) are obtained at low pH values (< 3.9) and high ethyl acetate-to-SSL ratios (> 2.5:1).

**Table 7.2** ANOVA for the CCD and estimation of parameter values

| Source | Degree of Freedom | Sum of Squares | Mean Square | F-value | P>F |
|---|---|---|---|---|---|
| Model | 5 | 3.240 | 0.648 | 7.299 | 0.016 |
| Error | 6 | 0.533 | 0.089 | | |
| Lack Of Fit | 3 | 0.458 | 0.153 | 6.112 | 0.086 |
| Pure Error | 3 | 0.075 | 0.025 | | |
| Total | 11 | 3.773 | | | |
| **Parameters** | **estimate** | **standard error** | **t Ratio** | **Prob>\|t\|** | |
| $bb_1$ (intercept) | 7.1550 | 0.149 | 48.03 | <.0001 | |
| $bb_2$ ($X_1$) | -0.4987 | 0.105 | -4.73 | 0.003 | |
| $bb_3$ ($X_2$) | 0.0420 | 0.105 | 0.40 | 0.704 | |
| $bb_4$ ($X_1X_2$) | -0.1475 | 0.149 | -0.99 | 0.360 | |
| $bb_5$ $(X_1)^2$ | -0.4018 | 0.118 | -3.41 | 0.014 | |
| $bb_6$ $(X_2)^2$ | -0.2120 | 0.118 | -1.80 | 0.122 | |

**$R^2$ = 0.86, $R^2$ Adjusted = 0.74, RMSE = 0.298, CV =8.68%**

Results from the $2^2$ factorial design of experiments indicate that the new design should be carried out with higher ethyl acetate-to-SSL ratios and lower pH values. This conclusion can be also drawn due to the negative value of coefficient $b_2$ (-0.505) and the positive value of coefficient $b_3$ (0.42). Therefore, the extraction conditions were further optimised by conducting a more detailed design (CCD) in the optimum area indicated by the factorial design.

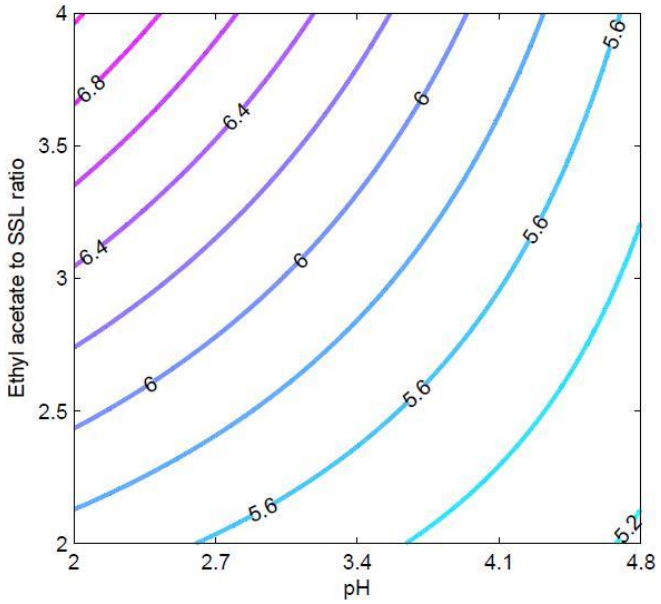



**Figure 7.1** Simulation results based on the preliminary experimental design

### 7.3.2. Central Composite Design

A CCD was further developed guided by the results obtained by the factorial design. The levels and the ranges of the two factors are presented in Table 7.3. pH values lower than 1.7 were not used in this experimental design because such low values required the addition of a higher quantity of acid solution resulting in significant precipitation of solids and loss of phenolic compounds. Furthermore, ethyl acetate-to-SSL ratios higher than 4.2 increased the required amount of solvent leading to significantly higher operating costs. For this reason, ethyl acetate-to-SSL ratios above 4.2 were also avoided.

**Table 7.3** The value ranges of the two factors for the CCD experiment

| Level | pH:$X_1$ | Ethyl acetate-to-SSL Ratio:$X_2$ (v/v) |
|---|---|---|
| +1.414 | 3.7 | 4.2 |
| +1 | 3.4 | 4.0 |
| 0 | 2.7 | 3.5 |
| -1 | 2.0 | 3.0 |
| -1.414 | 1.7 | 2.8 |

Experimental results and model predictions of the concentration of the extracted phenolic compounds (expressed as g GAE/L) from the CCD are presented in Table 7.4. According to the results obtained by the regression analysis, Eq. 3 becomes:

$$Y_2 = 7.1550 - 0.4987x_1 + 0.042x_2 - 0.1475x_1x_2 - 0.4018x_1^2 - 0.2120x_2^2$$

The model is significant according to the F-test as $F_{(5,6)}$ equals to 7.3 which is higher than the F-table value 5.9876 with level of importance 0.025. The prediction capability of the model is adequate as shown by the $R^2$ and adj $R^2$ values of 0.86 and 0.74, respectively. Moreover, the importance of the coefficients of Eq.3 was measured according to the student's *t*-distribution and their corresponding probability. As in the results obtained by the factorial design, the pH has a stronger linear effect than the ethyl acetate-to-SSL ratio as the $|t_{bb2(pH)}|$ value of 4.73 is higher than the $|t_{bb3(\text{ethyl acetate-to-SSL ratio})}|$ value of 0.40. The *t*-value of the linear interaction of the two factors is again low with an absolute value of 0.99. The quadratic coefficients seem to play an important role on the response as both of them have significant *t*-values, 3.41 for $|t_{bb5}|$ and 1.80 for $|t_{bb6}|$. In quadratic terms, as in the linear coefficients $bb_2$ and $bb_3$, the effect of pH is greater than the effect of the ethyl acetate-to-SSL ratio.

**Table 7.4** Experimental results and model predictions from the CCD

| | **Coded Values** | | **Real Values** | | **Concentration of extracted phenolic compounds (g-GAE/L)** | |
|---|---|---|---|---|---|---|
| Run | $x_1$ (pH) | $x_2$ (Ethyl acetate-to-SSL Ratio v/v) | $X_1$ (pH) | $X_2$ (Ethyl acetate-to-SSL Ratio v/v) | Experimental | Predicted |
| 1 | 1 | -1 | 3.4 | 3.0 | 5.92 | 6.15 |
| 2 | -1 | -1 | 2.0 | 3.0 | 6.63 | 6.85 |
| 3 | 1 | 1 | 3.4 | 4.0 | 5.68 | 5.94 |
| 4 | -1 | 1 | 2.0 | 4.0 | 6.98 | 7.23 |
| 5 | -1.414 | 0 | 1.7 | 3.5 | 7.29 | 7.06 |
| 6 | 1.414 | 0 | 3.7 | 3.5 | 5.89 | 5.65 |
| 7 | 0 | -1.414 | 2.7 | 2.8 | 6.89 | 6.67 |
| 8 | 0 | 1.414 | 2.7 | 4.2 | 7.05 | 6.79 |
| 9 | 0 | 0 | 2.7 | 3.5 | 7.22 | 7.16 |
| 10 | 0 | 0 | 2.7 | 3.5 | 7.33 | 7.16 |
| 11 | 0 | 0 | 2.7 | 3.5 | 6.96 | 7.16 |
| 12 | 0 | 0 | 2.7 | 3.5 | 7.11 | 7.16 |

Model predictions of the quadratic equation are presented in Figure 7.2a and Figure **7.2**b in a 3D plot and a contour plot, respectively. Both of them illustrate the concentration of the extracted phenolic compounds with respect to the two factors (pH and ethyl acetate-to-SSL ratio). In both cases, it is clear that there is a well-defined maximum response. High extraction values (>7.2 g GAE/L) occur at pH values of 1.8-2.6 and ethyl acetate-to-SSL ratios of 3.3-4.1, while low response values are mainly obtained at high pH values (>3.5). According to Eq.3, the optimum value of the extraction of the phenolic compounds is equal to 7.33 g GAE/L and occurs at pH value of 2.22 and ethyl acetate-to-SSL ratio of 3.67. Besides the corresponding *t*-value of $bb_4$, the small interaction of the two factors is also indicated by the weak elliptical shape of the contour plot.

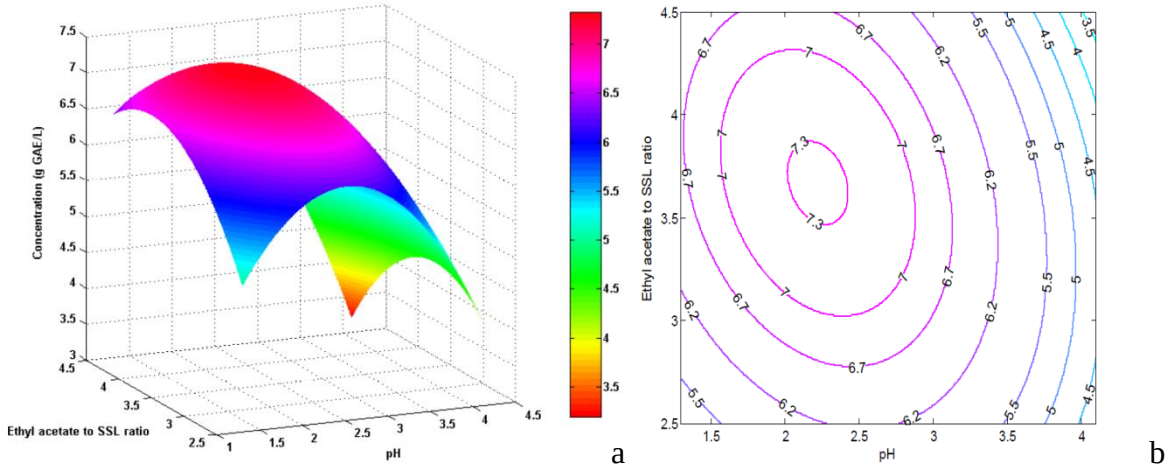



**Figure 7.2** A response surface 3-D plot of the predictions on the examined level range of the two factors (a) and contour plot of the model predictions from the CCD (b)

### 7.3.3. Liquid-liquid extraction of SSL-Validation of the model

According to the parameters obtained by the CCD, the model was validated at the optimum solvent extraction conditions (i.e. at pH value of 2.22 and ethyl acetate-to-SSL ratio of 3.67). The TPC value of the extract was 7.5 ± 0.75 g GAE/L, which is very close to the model prediction at the optimum point.

There are only a few literature-cited studies regarding the removal of phenolic compounds from SSL. Faustino et al. (2010) carried out solvent extraction with ethyl acetate at 5 different pH values. The extract separated at pH value of 4 showed the highest total phenolic content of 100 mg GAE/g but the extract obtained at pH value of 2 had the highest antioxidant activity (3.29). Llano et al. (2015) studied the extraction of phenolic compounds from SSL using different solvents (chloroform, diethyl ether, trichloroethylene, benzene and

hexane) at two pH values (3.65 and 2). The highest TPC value achieved was 6.31 g GAE/L, when diethyl ether was employed as extracting solvent at pH value of 3.65 and a 3:1 solvent-to-sample ratio.

The results of the present study is in agreement with the literature where *Eucalyptus globulus* wood hydrolysates were subjected to solvent extraction using acidic pH values and ethyl acetate as extracting solvent (Cruz et al., 2004; González et al., 2004). As phenolic compounds act as week acids, acidic pH values favor the dissolution of the protonated form in the organic solvent, in comparison to the non-protonated form that is more hydrophilic (Mateo et al., 2013). Cruz et al., (2001) removed the phenolic compounds from hydrolysates of different lignocellulosic resources (*Eucalyptus globulus* wood, barley bran, corn cobs and corn leaves) using ethyl acetate as extracting solvent. The extracts obtained from this process were tested for their bacteriogenic and bacteriostatic activities and *Eucalyptus* wood and barley bran acid hydrolysates presented the highest inhibition of both bacterial and yeast growth. Ethyl acetate was also evaluated for the extraction of bioactive compounds from brewery wastes in the study of (Barbosa-Pereira et al., 2013).

The extracts obtained when either a pH value of 2.22 or the original pH of the diluted SSL was used were analyzed via HPLC in order to identify and quantify the major phenolic compounds (Table 7.5). Ellagic acid is found to be the predominant phenolic compound (1165.5 mg/L), followed by gallic acid (1038 mg/L) in the extract obtained from pH 2.22. Other phenolic acids such as syringic acid and vanillic acid as well as the aldehydes syringaldehyde and vanillin were also detected. The flavonoids isorhamnetin and catechin were identified along with the lignans lariciresinol and acetosyringone. In the extracts obtained from the original pH of the SSL, ellagic (534 mg/L) and gallic acid (525 mg/L) were also the main phenolic compounds, followed by syringaldehyde (127 mg/L), vanillin (120 mg/L) and syringic acid (106 mg/L). Minor quantities of catechin, caffeic acid, vanillic acid and isorhamnetin were measured. Lariciresinol and acetosyringone were not detected in that case. The identification of the main phenolic compounds is directly related with the acidic pH values that prevail during the extraction. Gallic acid has four p$K_a$ values (as it bears four acidic protons). The most important one is the one of 4.0 from the –COOH group (Badhani et al., 2015). The original pH of the SSL is very close to the p$K_a$ value of gallic acid, which means that an important quantity of the acid exists also in its no-protonated form.

The presence of these compounds is due to the chemical composition of lignin. The structure and the monomers of this heterogenous polymer depend on several factors such as plant family, cultivation conditions and location. The main lignin precursors are trans-

coniferyl, trans-sinapyl and trans-*p*-coumaryl alcohols. Hardwood lignin contains guaiacyl-syrigyl groups in contrast to softwood lignin that contains mostly guaiacyl groups. The phenolic fraction derived by acidic treatment of hardwood is mainly composed of condensed tannins, ellagitannins (Cadahía et al., 2001), phenolic acids (ellagic, gallic, vanillic) and aldehydes (syringaldehyde) (Conde et al., 2011). Phenolic compounds such as vanillin, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, syringaldehyde and syringic acid are the main products of the acidic wood processing (Mitchell et al., 2014). Pereira et al. (2013) also reported the presence of pyrogallol and gallic acid in SSL produced via hydrolysis of tannins present in hardwoods. Gallic acid is found in *Eucalyptus globulus* wood hydrolysates derived either by acidic hydrolysis or autohydrolysis (Garrote et al., 2004). This phenolic acid is an important aromatic compound in hardwood hydrolysates, such as silver and red maple and chesnut, where Mitchell et al. (2014) reported concentrations of 108, 112 and 137 μg/mL, respectively. Xavier et al., (2014) detected ellagic and gallic acids as well as the flavonoid isorhamnetin in *Eucalyptus globulus* wood extracts. Ellagic and gallic acid were the main phenolics in extracts from *E. globulus* wood industrial wastes (Fernández-Agulló et al., 2015). Additionally, ellagic acid was the main phenolic compound detected in filtrates from *E. globulus* kraft pulp (Costa et al., 2014).

Ellagic acid is characterized by high thermodynamical stability as it is composed by two aromatic rings, four phenolic and two lactone groups (Figure 7.4). It is well studied in terms of its pharmacological and antioxidant properties (Costa et al., 2014). Ellagic acid is also reported to function as a cross-linking agent, interacting with polysaccharides without losing its antioxidant activity (Cho et al., 2009). Tirado-Gallegos et al., (2016) used ellagic acid as a cross-linking agent together with oxidized starches in order to produce biodegradable films having a potential use on food packaging and coating. Gallic acid finds an important application in tanning and in paper industry, as well as in inks, color developers and paints (Badhani et al., 2015). It has been also proved that gallic acid inhibits corrosion of steel surfaces as it is able to form insoluble complexes with ferric ions (Badhani et al., 2015)

The high concentration of phenolic acids could also be attributed to the severity of the hydrolytic process. The formation of aldehydes depends on the hydrolysis method as they are products of the acid-catalyzed degradation of the *β*-aryl-ether linkages of lignin and their subsequent oxidative degradation (Garrote et al., 2004). Syringic acid and syringaldehyde are formed from the degradation of syringyl propane units, whilst vanillic acid and vanillin are degradation products of the guaiacylpropane units (Cruz et al., 2004). High concentrations of ellagic, gallic and vanillic acids in *Eucalyptus globulus* wood hydrolysates were also

identified. The amount of ellagic acid increased due to the thermal degradation of ellagitannins. Faustino et al. (2010) studied the fractions of ethyl-acetate extracts obtained from SSL via NMR analysis. Syringic acid, syringadehyde, lariciresinol and acetosyringone were identified. Gallic acid, vanillic acid, ellagic acid and syringic acid were the main phenolics found in ethyl acetate extract of SSL in the study of Marques et al. (2009).

**Table 7.5** Main phenolic compounds identified via HPLC-DAD in the extracts of pH 2.22 and 3.4 at a SSL-to-solvent ratio 3.67:1 and 3:1 (v/v), respectively

| **Phenolic compound** | **Concentration (mg/L)** | | | |
|---|---|---|---|---|
| | **pH = 2.22 ratio 3.67:1 v/v** | **pH = 3.4 ratio 3:1 v/v** | **Measured nm** | **Type of compound** |
| Gallic acid | 1038 | 525 | 280 | Phenolic acid |
| Isorhamnetin | 41 | 21 | 280 | flavonoid |
| Syringic acid | 252 | 106 | 280 | Phenolic acid |
| Syringaldehyde | 32 | 127 | 320 | Aromatic aldehyde |
| Vanillic acid | 50 | 17.8 | 280 | Phenolic acid |
| Acetosyringone | 16 | - | 280 | lignan |
| Lariciresinol | 142 | - | 280 | lignan |
| Ellagic acid | 1165.5 | 534 | 280 | Phenolic acid |
| Caffeic acid | 3.2 | 4 | 320 | Phenolic acid |
| Vanillin | 115 | 120 | 254 | Phenolic aldehyde |
| Catechin | 127.6 | 53 | 280 | flavonoid |

The majority of the phenolic compounds identified in the ethyl acetate extract have also been identified in various agro-industrial wastes. Syringaldehyde has been identified in olive stone hydrolysates (Fernández-Bolaños et al., 1998), wheat straw (Klinke et al., 2004), sugarcane bagasse and wood (Martín et al.,2002; De Bari et al., 2002). Vanillic acid and vanillin were found in hydrolysates of steam- exploded olive stones (Fernández-Bolaños et al., 1998; Klinke et al., 2002), sugarcane bagasse (Martín et al.,2002), wheat straw, aspen wood hydrolysates and in cork extracts (De Bari et al., 2002; Santos et al., 2012). Gallic acid, ellagic acid and isorhamnetin have been identified in *Eucalyptus globulus* bark extracts and in cork extracts (Santos et al., 2013, 2012). Parajó et al. (2008) identified syrigaldehyde and vanillin in barley husk hydrolysates.

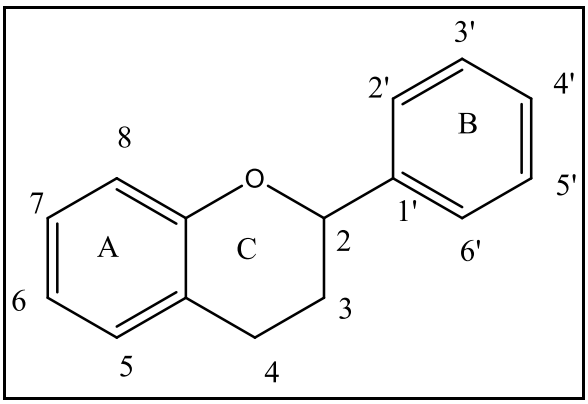



**Figure 7.3** General chemical structure of flavonoids

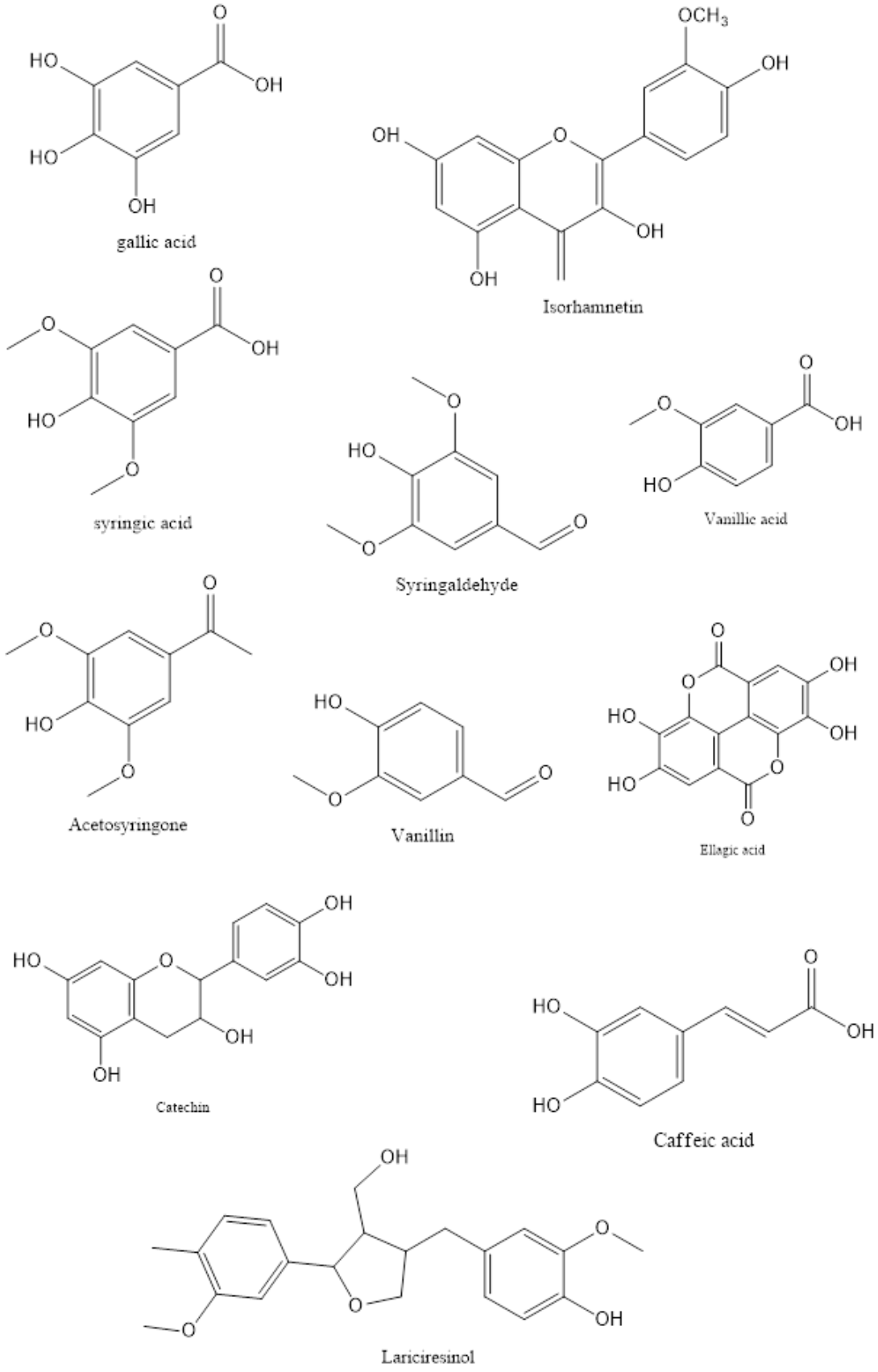



**Figure 7.4** Chemical structures of the main phenolic compounds identified in the SSL extracts

### 7.3.4. Antioxidant Activity

**Table 7.6** and **Table 7.7** present the results of the AAI and $IC_{50}$ of the extracts obtained at various pH values and solvent-to-SSL ratios, respectively. All the extracts showed very strong antioxidant activity as it is indicated by the scale proposed by Scherer and Godoy (2009), where poor antioxidants are considered those with an AAI < 0.5, moderate antioxidants when 0.5 < AAI < 1.0, strong antioxidants when 1.0 < AAI < 2.0 and very strong antioxidants when AAI > 2. The initial pH of the SSL slightly affects the AAI. The ratio of solvent-to-SSL, when liquid-liquid extraction was applied, did not affect the antioxidant strength of the extracts. The AAI of the extract obtained using the optimum extraction conditions was 3.64 ± 0.48, a value that classifies the extract as a strong antioxidant. For comparison reasons, the antioxidant activities of gallic acid, trolox and BHT are also presented in Figure 7.5. The high scavenging activity of the ethyl acetate extract makes it a potential source of natural antioxidant compounds that could be used as active ingredients in wood preservatives and biocides, as well as antioxidants in adhesives, lubricants and paper products to prevent yellowing (Willfoer et al., 2012). Pinto et al., (2013) proposed the application of extracts derived from *E. globulus* bark in the leather tanning industry.

Lignin derivatives are also reported to be used in rubber products, resins, oil-field products and as feed-stocks for the production of fine chemicals (Balakshin et al., 2010; Caillol et al., 2012). Further purification and safety analysis will increase the potential applications of this extract.

**Table 7.6** AAI and $IC_{50}$ values of the extracts obtained by four different combination of pH values and ethyl acetate- to- solvent ratios

| pH value | Ethyl acetate-to SSL ratio (v/v) | $IC_{50}$ (µg/mL) | AAI |
|---|---|---|---|
| 1 | 3:1 | 11.33 ± 0.23 | 2.70 ± 0.13 |
| 2.22 | 3.67:1 | 8.59 ± 1.08 | 3.64 ± 0.48 |
| 3.4 | 3:1 | 12.05 ± 2.17 | 2.62 ± 0.44 |
| 5 | 3:1 | 10.59 ± 1.81 | 3.07 ± 0.40 |

**Table 7.7** AAI and $IC_{50}$ values of the extracts obtained by initial pH values of 2 and 3.4 of the diluted SSL and at different ethyl acetate-to-SSL ratios

| pH value | Ethyl acetate-to SSL ratio (v/v) | IC $_{50}$ (µg/mL) | AAI |
|---|---|---|---|
| 2 | 1:1 | 9.47 ± 1.29 | 3.28 ± 0.45 |
| | 2:1 | 8.94 ± 0.54 | 3.30 ± 0.21 |
| | 3:1 | 8.29 ± 1.33 | 3.76 ± 0.60 |
| | 4:1 | 9.09 ± 0.96 | 3.40 ± 0.36 |
| 3.4 | 1:1 | 8.74 ± 0.50 | 3.53 ± 0.19 |
| | 2:1 | 10.99 ± 0.12 | 2.79 ± 0.02 |
| | 3:1 | 12.75 ± 2.55 | 2.46 ± 0.49 |
| | 4:1 | 12.07 ± 1.40 | 2.56 ± 0.30 |

The results obtained in this study comply with the results presented by Faustino et al. (2010), where the AAI of sulphite black liquors was 3.29 ± 0.35 at a pH value of 2 and the AAI of kraft liquors was 3.41 ± 0.33. Cruz et al. (1999) also reported that ethyl-acetate extracts of *E. globulus* wood hydrolysates showed important antioxidant activity. Aqueous extracts of *Eucalyptus* wood presented antimicrobial and anti-fungal properties (Fernández-Agulló et al., 2015).

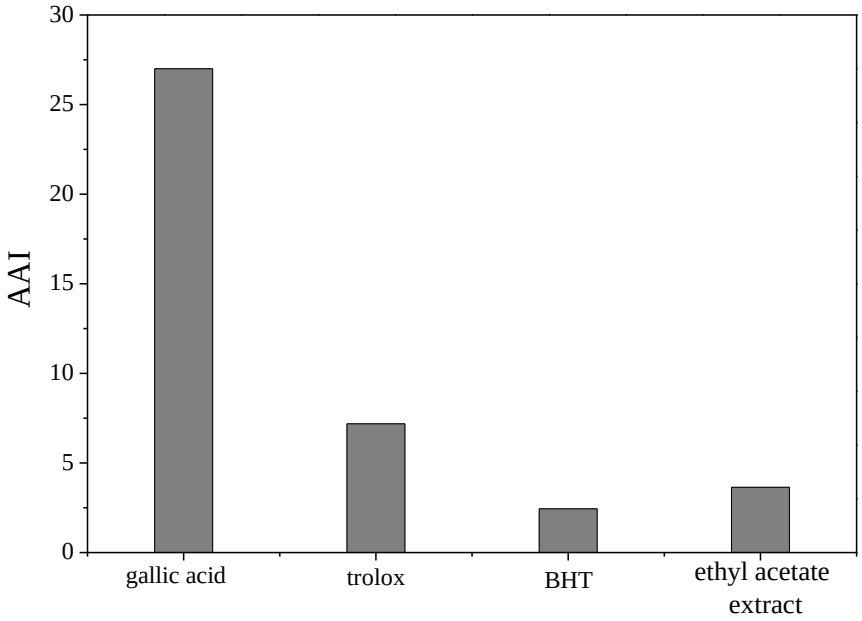



**Figure 7.5** Antioxidant activity indexes of standard phenolic compounds in comparison to ethyl acetate extract obtained at the optimum extraction conditions

The antioxidant activity of the phenolic compounds is directly related to their structure. As far as phenolic acids are concerned, their antioxidant activity is a result of the number and position of the hydroxyl groups attached to the aromatic ring, as well as the

carboxyl functional group (Balasundram et al., 2006). The higher the degree of hydrolxylation, the higher the antioxidant activity of the phenolic acid. On the other hand, when the hydroxyl groups are substituted by methoxyl groups at the 3- and 5- position, the antioxidant activity decreases (Proestos et al., 2006). This is the reason why, gallic acid acid exhibits higher antioxidant activity than syringic acid. Hydroxycinnamic acids are stronger antioxidants than hydroxybenzoic acids as the CH=CH-COOH group shows higher H-donating capacity than the –COOH group (Proestos et al., 2006).

The determination of the antioxidant activity of flavonoids is more complex, as they present many structural differences among its group. Isorhamnetin is an *O*-methylated flavon-ol, whereas catechin is a flavan-3-ol. The degree of hydrolyxylation as well as the positions of the hydroxyl (-OH) groups on the B ring of the flavonoid molecule, greatly affects the antioxidant activity (Figure 7.4). Catechin is one example and the fact that its –OH groups are in *ortho*-positions results in higher activity. Its structure leads to a stable aroxy radical due to electron delocalization. On the other hand, isorhamnetin bears a methoxyl group on the 3' position in B aromatic ring, which also affects its radical scavenging capacity (Balasundram et al., 2006). It has been proved that *ortho*-methoxy substitution increases the antioxidant activity, whereas the aldehyde group decreases it (as for example in vanillin) (Dizhbite et al., 2004). The same phenomena are also observed in case of lignans. Lignans are dimer or oligomer compounds derived from reaction of the monolignol radicals *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Llano et al., 2015). Dizhbite et al. (2004) studied the radical scavenging activity of lignin and lignans. They concluded that molecules with *ortho*-methoxy exhibit higher antioxidant activity than molecules with *a*-carbonyl substitution. Of course, in mixtures of different phenolic compounds, there are also synergistic effects of the various types of phenolics that could enhance the antioxidant activity of the extract (Parajó et al., 2008).

### 7.4. Succinic acid production from SSL pretreated with ethyl-acetate

SSL is a sugar rich waste stream that contains C5 and C6 monomeric sugars, with xylose being the predominant one. Glucose, mannose, galactose and arabinose are also present in this waste stream. This waste stream has been mainly used as fermentation substrate for the biotechnological production of ethanol (Chapter 2), but its high monosaccharide content renders it a potential substrate for succinic acid production. Two wild-type succinic acid producing bacteria were evaluated on their ability to grow and produce succinic acid using the sugars present in the SSL, namely *Basfia succiniciproducens*

and *Actinobacillus succinogenes*. *B. succiniciproducens* is able to consume all the sugars present in the SSL, whereas *A. succinogenes* cannot metabolize galactose (Pateraki et al., 2013). As the phenolic extract obtained via solvent extraction with ethyl acetate exhibited very strong antioxidant activity and specifically the phenolic compounds identified are known to have antioxidant and antimicrobial effects (de Campos et al., 2008), their removal is expected to enhance the succinic acid production via microbial fermentation. In order to evaluate the effect of phenolics removal on microbial growth, batch fermentations using both microbial strains were carried out in untreated SSL and in liquid-liquid extracted SSL, both diluted seven times. It should be mentioned, that liquid-liquid extraction with ethyl acetate removes only the phenolic compounds, while the lignosulphonates remain in the pretreated SSL.

Table 7.8 presents the results obtained by the fermentations carried out with *A. succinogenes* and *B. succiniciproducens*. The initial total sugar concentration of the pre-treated SSL in the case of *B. succiniciproducens* was 23 g/L that corresponds to 7 times dilution of the concentrated SSL. The total sugar consumption and the final succinic acid concentration achieved by *B. succiniciproducens* cultivated in pre-treated SSL were 19.9 g/L and 9.9 g/L, respectively. The succinic acid to initial sugar concentration (ISC) conversion yield was 0.43 g/g when solvent extracted SSL was used. Figure 7.6A shows that *B. succiniciproducens* cannot grow efficiently in the untreated SSL, as only 5.9 g/L of sugars are consumed, whereas when phenolic compounds are removed, the growth of *B. succiniciproducens* and the production of succinic acid were improved significantly. Regarding by-product formation, when solvent extracted SSL was used, the final concentrations of lactic, formic and acetic acids were 0.9 g/L, 2 g/L, and 4.9 g/L, respectively. The ratio of total by-products to succinic acid decreased from 1.84 in the case of non-treated SSL to 0.78 when solvent extracted SSL was used. This means that the presence of phenolic compounds increase the production of by-products during fermentation. The volumetric productivity was also significantly increased in the case of pre-treated SSL. In the study of Cimini et al. (2016), growth and succinic acid production were severely inhibited when 90 % of acidic hydrolysates from *Arundo donax* were employed. *A. donax* hydrolysates are reported to contain phenolic compounds such as 4-hydroxybenzoic acid and vanillin. The authors also observed that the concentration of vanillin decreased during the fermentation, indication that *B. succiniciproducens* is probably able to convert vanillin to vanillic alcohol (Salvachúa et al., 2016a, b). On the other hand, the concentration of 4-hydroxybenzoic acid remained invariable.

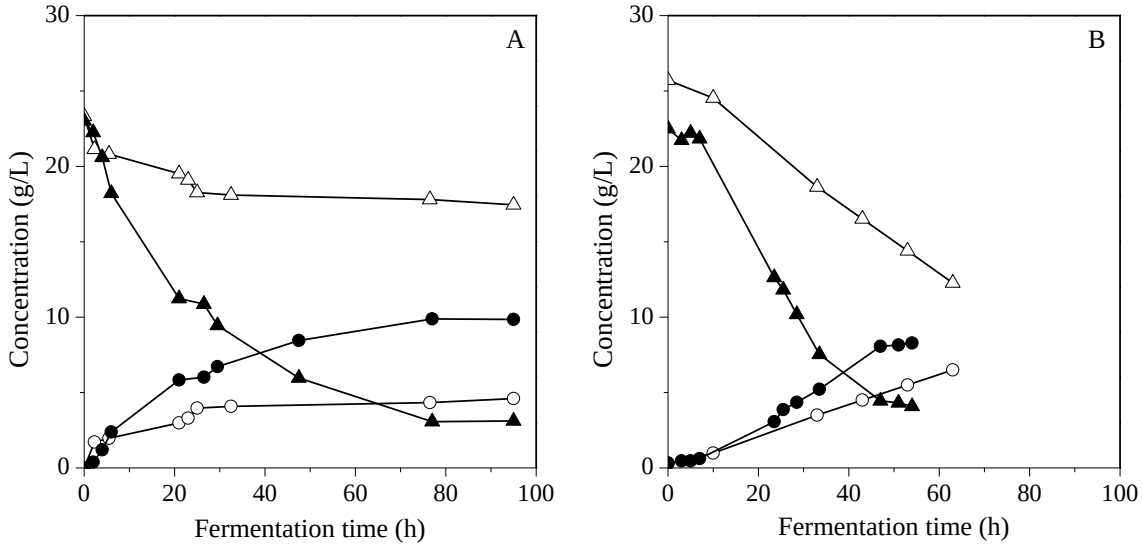



**Figure 7.6** Consumption of total sugars (▲,△) and production of succinic acid (●,○) in batch bioreactor cultures carried out with *B. succiniciproducens* (A) and *A. succinogenes* (B) using 7 times diluted SSL after extraction of phenolic compounds with ethyl acetate (filled symbols) and without any pre-treatment (unfilled symbols).

In the case of *A. succinogenes*, the removal of phenolic compounds did not result in the same improvement of succinic acid production as in the case of *B. succiniciproducens* (Figure 7.6B). The total sugars to succinic acid conversion yield was increased to 0.35 g/g from 0.24 g/g that was obtained when untreated SSL was employed. The ratio of total by-products to succinic acid was decreased in the case of solvent extracted SSL. More sugars were depleted in case of solvent extracted medium and productivity was also enhanced (Table 7.8). *A. succinogenes* seems to be more tolerant to phenolic compounds than *B. succiniciproducens*. These results are in agreement with the study of (Carvalho et al., 2014), where the concentration of 2 mg/mL of phenolic compounds in carob pod extracts did not affect the final succinic acid production by *A. succinogenes* 130Z. Carvalho et al. (2014) also tested various concentrations of gallic acid and it was reported that concentrations bellow 2 mg/mL did not affect the specific growth rate. However, phenolic compounds pose synergistic effects so further study should be carried out in order to clarify the minimum inhibitory concentration of combined phenolic compounds. It should be mentioned that both bacteria strains used are Gram-negative. These strains are less tolerant to phenolic acids than Gram-positive bacterial strains (Campos et al., 2009; Borges and Pereira, 2011). Xi et al. (2013) also observed that *A. succinogenes* NJ113 was resistant to a specific amount of phenolic compounds present in acid hemicellulose hydrolysates of sugarcane bagasse. The

authors also noticed an overall increase of total sugar consumption when detoxified medium was employed. Nevertheless, by product formation seems to be related to the presence of inhibitors, as also in case of *A. succinogenes* total by-product ratio to succinic acid was reduced when solvent extracted SSL was employed. More specifically, when non-treated SSL was used as fermentation substrate the total by-product to succinic acid ratio was 1.41, whilst when solvent extracted SSL was used, the ratio reduced to 0.97. Guo and Olsson, (2014) observed complete inhibition of *Saccharomyces cerevisiae* growth on various crude SSL streams. Only after detoxification the yeast was able to grow and produce ethanol. The authors also noticed that the fermentability of the medium was significantly improved when methods that mainly removed phenolics were applied.

Selective removal of inhibitors from hydrolysates of renewable resources has been carried out via ethyl acetate pretreatment (Zhuang et al., 2009; Cruz et al., 1999). Among activated charcoal treatment, overliming and solvent extraction, the use of ethyl acetate as solvent was the most effective for the removal of furans and phenolics, but also resulted in the most expensive method due to the 3:1 solvent-to-sample ratio used (Mateo et al., 2013). An important advantage of solvent extraction in comparison to overliming is that sugars are not lost during processing. Treatment with activated charcoal also leads to partial sugar loss and also the used charcoal is not easily regenerated (Mussatto and Roberto, 2004). Ion-exchange resins have also been applied for the detoxification of SSL, but even though they separated both acetic acid and phenolic compounds, the cost and the operation conditions employed render their industrial application a challenging endeavor (Fernandez et al., 2012).

The lignin derived compounds present in SSL have an adverse effect on cell growth. These inhibitory compounds include polyaromatic compounds, phenolics and aldehydes. Their toxicity is in close correlation with their molecular weights. Low molecular weight phenolics are considered to be more toxic to the microorganisms than the high molecular weight ones (Zhuang et al., 2009). Phenolic compounds inhibit cell growth by causing loss of the integrity of the biological membranes, thus by negatively influencing their ability as selective barriers and enzyme matrices (Heipieper et al., 1994). Luís et al., (2016) studied the antioxidant and antimicrobial activity of *E. globulus* stump wood methanolic extracts. The main phenolic compounds identified in the extracts were also gallic and ellagic acid and they presented a strong antimicrobial effect when tested on various human pathogens.

**Table 7.8** Fermentation results using SSL with and without treatment with ethyl acetate

| Microorganism | Process | Consumed sugars (g/L) | SA (g/L) | SA yield (g-SA/g-ISC*) | Yield (g-SA/g-SSL) | Productivity (g-SA/L/h) | Lactic acid (g/L) | Formic acid (g/L) | Acetic acid (g/L) |
|---|---|---|---|---|---|---|---|---|---|
| *A. succinogenes* | Solvent Extraction | 18.4 ± 2.2 | 8.0 ± 0.2 | 0.35 ± 0.05 | 0.043 ± 0.001 | 0.150 ± 0.004 | - | 3.2 ± 0.2 | 4.5 ± 0.1 |
| *B. succiniciproducens* | Solvent Extraction | 19.9 ± 1 | 9.9 ± 0.1 | 0.43 ± 0.03 | 0.054 ± 0.001 | 0.130 ± 0.002 | 0.9 ± 0.1 | 2.0 ± 0.2 | 4.9 ± 0.3 |
| *A. succinogenes*** | Non-treated SSL | 13.4 ± 2.6 | 6.2 ± 0.6 | 0.24 ± 0.01 | 0.035 ± 0.003 | 0.090 ± 0.009 | - | 3.5 ± 0.8 | 5.3 ± 0.9 |
| *B. succiniciproducens* | Non-treated SSL | 5.9 ± 1.9 | 4.6 ± 0.1 | 0.20 ± 0.02 | 0.026 ± 0.001 | 0.050 ± 0.001 | 1.3 ± 0.1 | 2.8 ± 0.3 | 4.4 ± 0.1 |

* Initial sugar concentration

**The initial sugar concentration in this fermentation was 25.7 g/L

The inhibitory effect of different phenolic compounds both on microbial growth and product formation is well studied in the literature, mainly towards yeast growth. Hu et al. (2009) reported that the growth of *Rhodosporidium toruloides* was inhibited by the presence of 10 mM p-hydroxybenzaldehyde and vanillin in the fermentation medium. The negative effect of vanillin and syringaldehyde on xylitol production by the yeast strain *Candida guilliermondii* was reported by Cortez and Roberto,( 2010). Their results showed that both phenolic compounds (with concentrations up to 2.0 g/L) inhibited cell growth and a complete inhibition was observed by the presence of both compounds in the fermentation medium. Furthermore, vanillin did not significantly affect xylose to xylitol conversion yield in contrast to syringaldehyde that significantly reduced the production of xylitol. The synergistically negative effect of 4 different phenolic compounds (phenol, syringaldehyde, 4-hydroxybenzaldehyde and vanillin) was also highlighted by Zhang et al., (2012) on xylitol production by *Candida athensensis* SB18. The presence of each compound (concentrations used were up to 1.0 g/L) led to a prolonged lag phase and reduced cell growth, with the intensity of inhibition being analogous to increasing initial concentrations. The combination of the four phenolic compounds increased the overall inhibition, with phenol presenting the highest toxicity in terms of cell growth and xylitol production. It was also shown that these inhibitors affected mainly xylose reductase activity, concluding that their negative effect was concentrated mainly on xylitol productivity rather than xylitol final concentration and yield. The use of SSL from *E. globulus* is reported to inhibit yeast growth and bioethanol production (Xavier et al., 2010). Only after detoxification using filamentous fungi that led to the removal of phenolics, the yeast *Scheffersomyces stipitis* was able to produce ethanol (Zhang et al., 2012). Syringaldehyde and vanillic acid are also reported to inhibit microbial growth and ethanol production by various microorganisms (Delgenes et al., 1996).

### 7.5. Conclusions

These results of this chapter indicate that liquid-liquid extraction with ethyl acetate as extracting solvent is a process that provides an extract rich in phenolic compounds possessing strong antioxidant activity and also a detoxified aqueous phase for enhanced succinic acid production via microbial fermentation. The pH of diluted SSL and the solvent-to-liquid ratio are the most important parameters affecting the extraction of phenolic compounds from SSL. These parameters were optimized by employing experimental design. At the optimum conditions (pH value of 2.22 and ethyl acetate-to-SSL ratio of 3.67) the TPC value was 7.5 ± 0.75 g GAE/L, thus validating the model. HPLC-DAD analysis revealed that the main

phenolic compound present in the SSL extracts was ellagic acid at a concentration of 1165.5 mg/L, followed by gallic acid with a concentration of 1038 mg/L. Other phenolic compounds identified were syringic and vanillic acids, syringaldehyde, vanillin, isorhamnetin, catechin, lariciresinol and acetosyringone. The fact that the extract also possesses high antioxidant activity (AAI = 3.64 ± 0.48 at optimal extraction conditions), makes it a potential value-added product with various applications. The removal of the phenolic compounds led to enhanced succinic acid production from both *A. succinogenes* and *B. succiniciproducens*, especially in case of the latter. In the following chapters, fermentation efficiency will be optimized by employing different detoxification methods as well as cell immobilization.

*The results presented in Chapter 7 have been published in Journal of Chemical Technology and Biotechnology:*

Alexandri M., Papapostolou H., Vlysidis A., Gardeli Chr., Komaitis M., Papanikolaou S., Koutinas A.A. (2016). Extraction of phenolic compounds and succinci acid production from spent sulphite liquor. J Chem Technol and Biot, 94(11): 2751-2760

# CHAPTER 8
# BIOREFINERY DEVELOPMENT FOR PRODUCTION OF SUCCINIC ACID, LIGNOSULPHONATES AND ANTIOXIDANTS

## 8.1. Introduction

In the previous chapter it was shown that SSL contains phenolic compounds with high antioxidant activity that could be a potential value- added product. Moreover their removal from SSL enhanced significantly the production of succinic acid using the two microbial strains *A. succinogenes* and *B. succiniciproducens*, especially in the case of the latter. Besides phenolics compounds, SSL also contains an important amount of lignosuphonates (LS) that are also considered a co-product of the pulping process, holding already a place in the market. The LS inhibit microbial growth and their removal from SSL will not only lead to the production of a commercial product, but also to improved succinic acid production. In this chapter, two separation methods of LS were evaluated and then the produced stream was subjected to microbial fermentation for succinic acid production. The development of the optimum separation scheme, including the removal of both LS and phenolic compounds, together with the biotechnological production of succinic acid, could transform the conventional pulp and paper industry into a novel biorefinery.

The main challenge is to develop a method that efficiently removes the LS, leaving sugars intact. There many methods dealing with the separation of LS from SSL, that are mostly based on changing their solubility in the solution or by membrane filtration. The use of solvent extraction with 2-propanol and acetone was firstly evaluated as the first biorefinery strategy. The second biorefinery strategy involved the combination of solvent extraction with ethyl-acetate for phenolic compounds removal, followed by nanofiltration for LS separation. Both streams were used as fermentation substrates for succinic acid production with *A. succinogenes* and *B. succiniciproducens*.

## 8.2. First biorefining strategy

### 8.2.1. Separation of lignosulphonates by solvent extraction with 2-propanol

As a first step for separation of LS from SSL, solvent extraction of SSL was carried out using two different extracting solvents, isopropanol and acetone. Both solvents were initially tested at ten times diluted SSL after pH adjustment to 5.5. These organic solvents were chosen based on experimental data reported by Ikari and Yokoyama, (1973). Figure 8.1

presents the percentage of LS that was separated and the amount of total sugars that were precipitated when isopropanol and acetone were used as solvents. When both solvents were applied, the solubility of the LS was altered and they almost instantly started precipitating in the bottom of the separation funnel. Sulphuric groups in LS appear in the form pf sulphuric acid. Therefore, the low molecular weight LS are more polar, than the high molecular weight ones (Leger et al., 2010). After their separation via vacuum filtration, the liquid stream was subjected to vacuum evaporation in order to remove the extracting solvents. It is obvious that 2-propanol resulted in higher LS removal (76%) than acetone (47%), even though more sugars were also lost in the first case (39% sugar loss instead of 21% in case of extraction with acetone). The co-precipitation of sugars could be attributed to chemical reactions or bonding of LS with the monosaccharides. It has been found that LS fractions can also be consisted of sugar acids, sulphonated sugars, lignans, terpenes and various other compounds (Glennie 1971). Many researchers have claimed that the sugars in lignin containing streams (such as softwood and hardwood liquors) exist mainly in lignin-sugar complexes rather than as free sugars (Lawoko et al., 2005, 2006; Choi et al., 2007). More specifically, studies on various lignin-sugar complexes have shown that the sugar nature is strongly depended to the type of pulping process. Softwood lignin derived from kraft pulping was found to be bound to glucomannan or xylan, whilst sulphite pulp softwood lignins were found to have glucan, glucomannan and xylan-lignin-glucomannan complexes (Lawoko et al., 2005, 2006). Choi et al. (2007) found that sugars were connected to lignin via benzyl-ether bonds, based on their research on spruce unbleached pulps resulting from three different pulping processes. These finding justify the difficulty in separation of sugars from LS (Leger et al., 2010).

Based on the results obtained from the first set of experiments, isopropanol was selected for further experiments. The LS removal together with sugar losses were determined at two pH values (5.5 and 6.5), and at seven and ten times diluted SSL (Figure 8.1). At these pH values, LS (as well as the phenolic compounds) are in their sodium form, making the extraction feasible. The seven times dilution corresponds to “thin” SSL, meaning the stream before the multiple evaporation steps. In all cases, the percentage of LS removal was similar, ranging from 73 – 76%. The sugar precipitation observed was in the range of 25 – 39%. Although the percentage of LS removal was similar in all cases studied, the precipitation of sugars was lower when 7 times diluted SSL was employed. In addition, the pH did not affect significantly the precipitation of sugars when 7 times diluted SSL was used. Isopropanol was also used by Leger et al. (2010), in order to separate different fractions of an ammonium lignosulphonate sample. Fractionation was achieved by using different dilutions of

isopropanol solutions and finally separation was carried out by polarity. In most of the fractions, lignin was found in the form of soluble lignin, which is logical since it is sulphonated lignin.

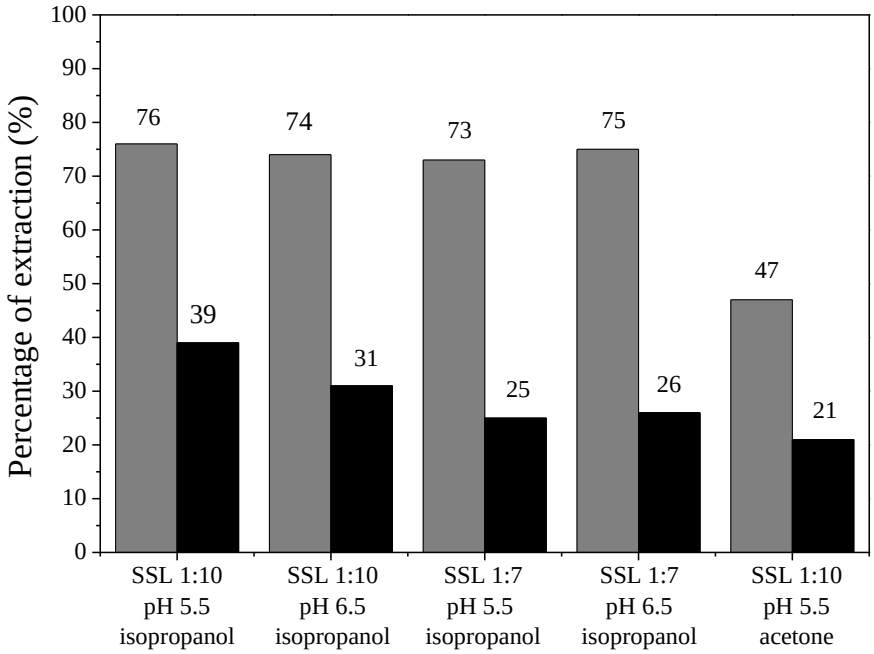



**Figure 8.1** Percentage of LS removal (grey bars) and sugar precipitation (black bars) after pretreatment of 7 and 10 times diluted SSL with isopropanol and acetone at pH values of 5.5 and 6.5

In order to reduce the sugar loss due to precipitation, a second set of experiments with two stages of extraction was carried out. In these experiments, the selected conditions were seven times diluted SSL with an SSL to solvent ratio of 1:7 (v/v) under the same pH values of 5.5 and 6.5. After the first treatment with isopropanol, the precipitated LS were re-diluted in water and the same amount of isopropanol was added. The concept of this experiment was to recover the sugars that were co-precipitated with the LS. When the same process was performed for a third time no further precipitation of LS was observed. The results presented in Figure 8.2 indicate that in the second solvent extraction stage the precipitation of LS and sugars is significant. The simultaneous precipitation of sugars and LS makes this process unfeasible.

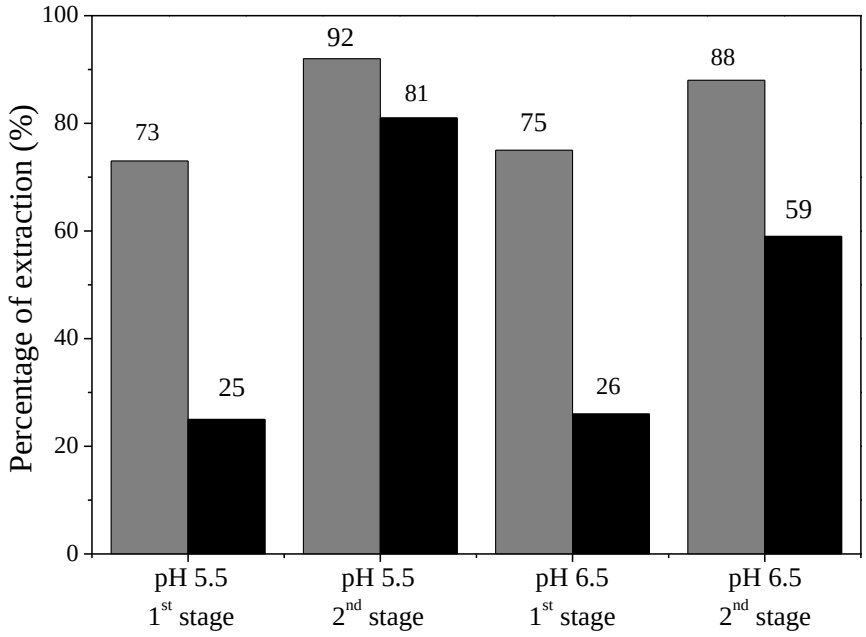



**Figure 8.2** Percentage of LS removal (grey bars) and sugar precipitation (black bars) after pre-treatment of 7 times diluted SSL with isopropanol at pH of 5.5 and 6.5 in two consecutive stages

### 8.2.2. Succinic acid production using SSL pretreated with isopropanol

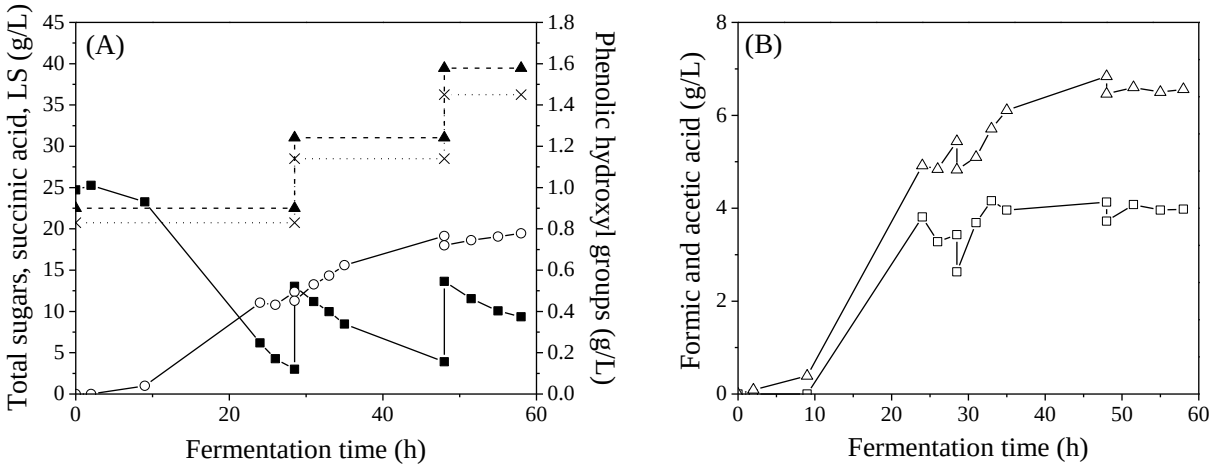
The sugar-rich stream produced via pretreatment with 2-propanol, at pH 6.5 and using seven times diluted SSL, was subsequently tested as fermentation substrate for succinic acid production using the wild-type strains *A. succinogenes* and *B. succiniciproducens*. Seven times dilution of SSL was preferred since with this dilution less sugar precipitation was observed and at the same time more sugars were present in the detoxified stream. Between the two microorganisms selected, *A. succinogenes* has been cultivated in many renewable resources, whereas the ability of *B. succiniciproducens* to grow in crude waste streams and to produce succinic acid has not been studied in great detail. The fermentations carried out were in fed-batch mode, aiming to succeed the highest possible succinic acid concentration.

When *A. succinogenes* was employed, the initial sugar and LS concentrations were 25 g/L and 22.5 g/L respectively. The concentration of total phenolic OH groups was 0.83 g/L. The feeding solution used was concentrated isopropanol extracted SSL with a total sugar concentration of approximately 170 g/L. The consumption of sugars started rapidly after inoculation and feeding started at around 29 h (Figure 8.3A) when the total sugar concentration reached 3 g/L. Two feeding pulses were carried out during this fermentation. At around 48 h, the second feeding pulse was carried out, but it did not lead to significant

increase in succinic acid concentration. Although sugar consumption by *A. succinogenes* continued, the low succinic acid production could be attributed to the inhibition caused by the accumulated LS and phenolic compounds. After each feeding pulse, the concentration of LS and phenolics increased and by the end of the fermentation their corresponding values were 39.4 g/L and 1.45 g/L, respectively. A succinic acid concentration of 19.1 g/L was achieved at 48 h. Succinic acid yield and productivity were 0.55 g succinic acid per g of total sugars (or 0.06 g per g SSL) and 0.4 g/L/h, respectively. Formic and acetic acids were also produced during fermentation with final concentrations of 4.1 g/L and 6.8 g/L, respectively (Figure 8.3B). After the second feeding pulse, the formation of by-products was equally low as in the case of succinic acid production. The final ratios of FA:SA, AA:SA and total by-products to SA were 0.22, 0.36 and 0.57, respectively and their corresponding molar ratios were 0.55, 0.70 and 1.26. The difference between this experiment and the batch fermentation with 1:7 diluted non-treated SSL is obvious (see Chapter 7). The ratio of each by-product to succinic acid was reduced more than half (FA:SA and AA:SA were 0.56g/g and 0.85 g/g). Interestingly, the ratio of each by-product to succinic acid was similar to the one reported by Pateraki et al. (2016b), when commercial mixed sugars (simulating SSL) were used in a fed-batch fermentation mode with the same strain. Moreover, FA:SA and AA:SA was not equimolar when solvent extracted SSL with 2-propanol was employed as more acetate was produced. The same findings were observed in the experiments of Amqvist et al. (2016). The authors found that more acetate is produced through the PDH route. In the end of the fermentation, 9 g/L of total sugars remained unconsumed, but 5.4 g/L were galactose. It is reported that this strain cannot metabolize galactose (Pateraki et al. 2016c). Besides the inhibition from LS and phenolic compounds accumulation, the total concentration of organic acids plays also an important role. At the end of the fermentation, total organic acid concentration was approximately 33 g/L, value reported to be critical for *A. succinogenes* growth (Pateraki et al. 2016c).

The initial sugar concentration used, achieved via dilution of the pre-treated SSL, in the fed-batch fermentations carried out with the two bacterial strains was lower in the case of *B. succiniciproducens*. Preliminary experiments showed that this strain is less tolerant than *A. succinogenes* to the inhibitors present in SSL (see *Chapter 7*). Taking this finding into account, the initial LS concentration was adjusted to 13.3 g/L, resulting to an initial sugar concentration of 15 g/L. Preliminary fermentations with the same medium used in the fermentation carried out with *A. succinogenes* resulted in poor growth and succinic acid production when *B. succiniciproducens* was used. The feeding solution used was the same

concentrated isopropanol extracted SSL used in the case on *A. succinogenes*. Figure 8.3C shows that the two feeding pulses were carried out at 11 h and 22 h, respectively. The fermentation finished after 29 h, resulting in 19.6 g/L of succinic acid concentration with final yield and productivity of 0.58 g per g sugars (or 0.06 g per g SSL) and 0.67 g/L/h, respectively. Besides succinic acid, *B. succiniciproducens* also produces lactic, formic and acetic acids (Figure 8.3D). The final concentration for each by-product was 1.6 g/L lactic acid, 1.7 g/L formic acid and 6.7 g/L acetic acid. The ratio of LA:SA, FA:SA, AA:SA and total by-products to SA were 0.08, 0.08, 0.34 and 0.51, respectively. Their corresponding molar ratios were 0.11, 0.22, 0.67 and 1. As in case of *A. succinogenes*, also this strain produced fewer by-products in the detoxified medium. Both lactic and formic acid formation was greatly lower and the main by-product was acetic acid. The ratio of each by-product to succinic acid is also significantly lower even in comparison to solvent extracted SSL with ethyl acetate. This could mean that by-product formation is highly associated to the inhibitory compounds present in the fermentation medium. It should be stresses that the final concentrations of LS and phenolic compounds in the medium were 32.4 g/L and 1.2 g/L, respectively. In comparison to fed-batch fermentations with mixed sugars reported by the literature, the final yield in the isopropanol treated SSL was higher. More specifically, in this study the yield on consumed sugars was 0.73 g/g, whereas in case of commercial mixed sugars the yield reported was 0.65 g/g (Pateraki et al., 2016b).



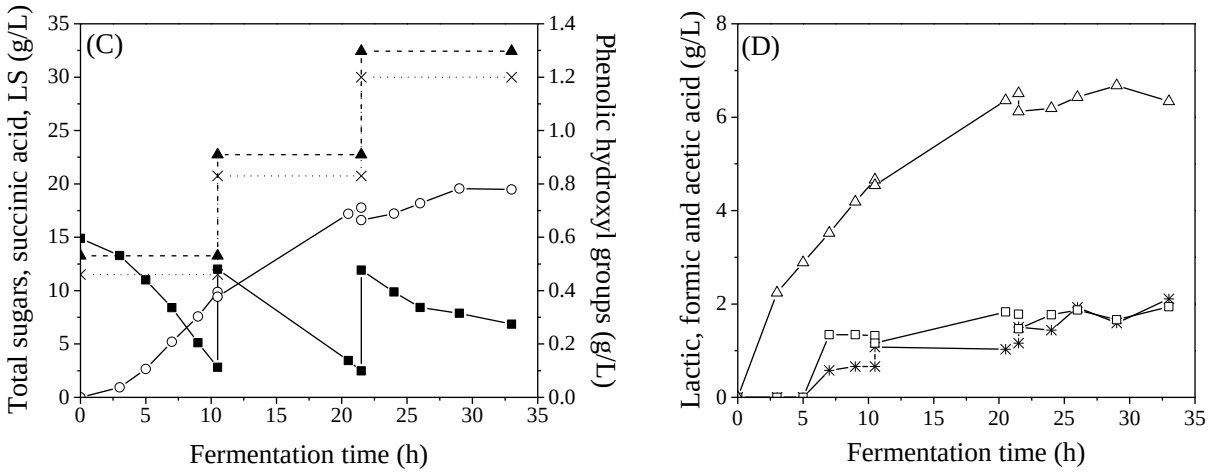



**Figure 8.3** Fed-batch fermentation using (A), (B) *A. succinogenes* and (C), (D) *B. succiniciproducens* using SSL pre-treated with isopropanol. Total sugar consumption (■), succinic acid (○), LS concentration (▲), phenolic hydroxyl groups concentration (×), acetic acid (△), formic acid (□) and lactic acid (∗).

These results indicate that *B. succiniciproducens* is a better candidate for succinic acid production from detoxified SSL streams, as this strain performed better on pretreated SSL than *A. succinogenes* regarding both yield and productivity. However, it required a more detoxified medium than *A. succinogenes*. Extraction with isopropanol leads to partial removal of LS, but an important amount of phenolic compounds remains in the fermentation broth. In *Chapter 7*, it was shown that the removal of phenolic compounds improved bacterial growth and succinic acid production especially in the case of *B. succiniciproducens*. Therefore, subsequent experiments were focused on the separation of both LS and phenolics from SSL.

## 8.3. Second biorefining strategy

### 8.3.1. Separation of lignosulphonates by nanofiltration

Extraction with ethyl acetate was previously reported that can efficiently remove the phenolic compounds from SSL (*Chapter 7*). The obtained extract rich in phenolic compounds contained 7.5 g GAE/ L SSL with an antioxidant activity index of 3.64. Scherer and Godoy (2009) proposed a scale in which extracts with an antioxidant activity index higher than 2 are considered as very strong antioxidants. Taking this scale into account, the phenolic extract derived from the SSL could be considered as a value added co-product that could be used in various applications.

Membrane separation, via ultrafiltration and nanofiltration, is commonly employed for the treatment of wastewaters and effluents. Ultrafiltration, nanofiltration and reverse osmosis have been used for the separation of lignosulphonates (Restolho et al., 2009). In this study, nanofiltration was employed in order to separate an LS-rich stream (retentate) from the sugar-rich stream (permeate). The permeate was then subjected to solvent extraction with ethyl acetate in order to separate the phenolic compounds. The combination of these two pre-treatment processes led to the production of a detoxified medium for succinic acid production and the production of two value-added co-products, the LS and the phenolic-rich extract.

Membranes of two different MWCO were tested and the process of nanofiltration is described in detail in *Chapter 6*. Seven times diluted SSL was selected for the filtration tests, simulating again the 'thin' SSL, from the pulping process. The diluted SSL was then filtrated from 500 Da and 800 Da MWCO membranes. The nanofiltration tests were carried out by AVECOM NV (Belgium). When the 800 Da membrane was employed, a removal of 69.5% of LS was achieved, whilst when the 500 Da was used the removal researched values of 84.7%. It should be noticed, that no loss of specific sugars was observed during nanofiltration and solvent extraction.

### 8.3.2. Succinic acid production using nanofiltrated and ethyl acetate extracted SSL

The permeate streams obtained via nanofiltration of SSL using both 500 Da and 800 Da, where then subjected to solvent extraction with ethyl acetate in order to remove the phenolic compounds. The stream from the 500 Da MWCO was used in batch fermentation with *B. succiniciproducens*, whereas the stream from 800 Da MWCO was used as substrate for *A. succinogenes*. The lowest MWCO membrane was chosen in the case of *B. succiniciproducens* because this strain is less tolerant to the presence of inhibitory compounds. Initial sugar concentration was approximately 27 g/L in both cases as it is shown is Figure 8.4. The initial concentration of LS was 20 g/L in the case of *A. succinogenes* fermentation and 10 g/L in the case of *B. succiniciproducens* fermentation.

When *A. succinogenes* was employed, the final succinic acid concentration reached a value of 14.2 g/L, with succinic acid yield of 0.51 g/g of total sugars and productivity of 0.29 g/L/h (Figure 8.4A). The sugars were not consumed completely at the end of fermentation (49h), as this strain cannot catabolize galactose. In comparison to the previous detoxification methods studied, nanofiltrated-extracted SSL seems to be a more suitable substrate for succinic acid production. By-product formation was also reduced, as the ratio of each by-

product to succinic acid was 1:0.27:0.28. The final concentration of formic acid and acetic acid were 3.9 g/L and 4 g/L respectively.

*B. succiniciproducens* consumed all sugars after 28 h (Figure 8.4B) and produced a final succinic acid concentration of 18.3 g/L. The succinic acid yield was 0.68 g succinic acid per g of total sugars and the productivity was 0.65 g/L/h. Lactic acid, formic acid and acetic acid were also produced at final concentrations of 1.3 g/L, 2.82 g/L and 3.8 g/L, respectively. The total by-product to succinic acid ratio was 0.44 g/g, which is lower than the ratio observed in the fermentation where isopropanol extracted SSL was used as substrate. Actually, the molar ratios of succinic acid to each by-product were 1:0.07:0.15:0.21, values significantly lower than the previous experiments carried out with *B. succiniciproducens*. The results obtained from these fermentations are promising as compared to the succinic acid production efficiency achieved when lignocellulosic hydrolysates are used (Table 8.1).

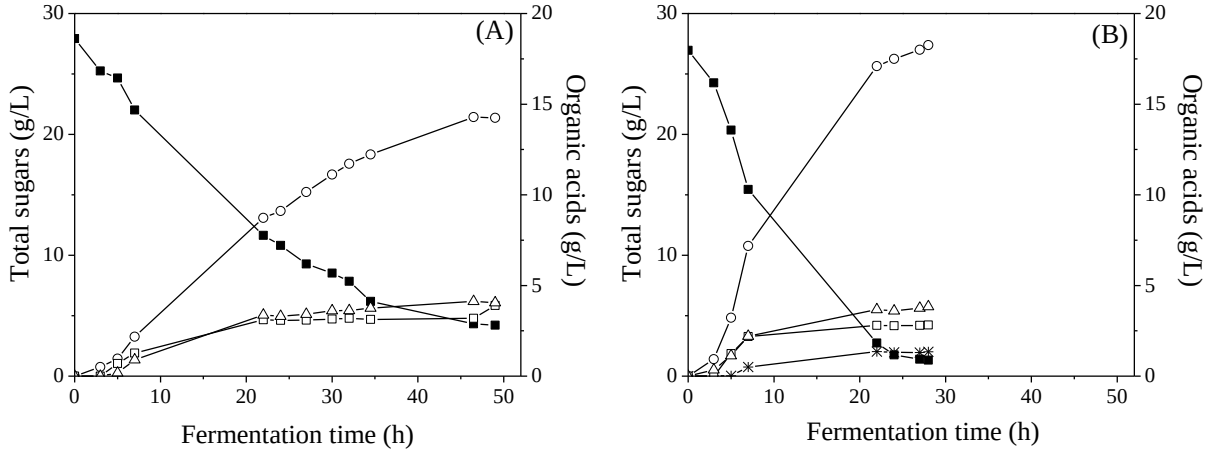



**Figure 8.4** Total sugar consumption (■) and production of succinic acid (○), acetic acid (△), formic acid (□) and lactic acid by (A) *A. succinogenes* and (B) *B. succiniciproducens*. The SSL was pre-treated by nanofiltration using membranes with 800 Da MWCO in the case of *A. succinogenes* and 500 Da MWCO in the case of *B. succiniciproducens*.

As *B. succiniciproducens* performed better than *A. succinogenes* in the SSL-derived medium via pre-treatment with nanofiltration and ethyl acetate extraction, a fed-batch fermentation was carried out with this strain. The initial conditions were the same as in the batch culture and the feeding medium used was concentrated filtrated-extracted SSL. Sugar consumption started without any lag phase and after about 30 h the first feeding pulse took place (Figure 8.5A). The added sugars were rapidly consumed and after 28 h of fermentation, more sugars were added to the medium. Until that time, the by-product formation was kept low in comparison to succinic acid, as it is shown by their individual yields (0.68 g/g, 0.05

g/g, 0.10 g/g and 0.14 g/g for SA, LA, FA and AA, respectively). Between, 28 h and 53 h of fermentation, SA yield kept high (0.68 g/g), LA yield increased (0.11 g/g) whereas the yield of both FA and AA decreased (yield of AA was 0.03 g/g). After the first feeding pulse, formic acid production ceased. This attitude was not observed in batch fermentations of *B. succiniciproducens*, neither when isopropanol treated SSL was tested. The fermentation ended after 127 h, where 39 g/L of succinic acid were produced, with a succinic acid yield of 0.51 g/g and productivity of 0.31 g/L/h. The yield of succinic acid produced per gram SSL used was 0.06 g/g. The final concentrations of lactic, formic and acetic acids were 10.7 g/L, 2 g/L and 3.5 g/L (Figure 8.5B). Interestingly, after the last feeding pulse (82 h), the concentration of formic and acetic acids remained constant and only lactic acid continued to increase. The ratio of LA:SA was 0.27, of FA:SA was 0.05, of AA:SA was 0.09 and finally the ratio of total by-products to SA was only 0.41, values much lower than in the case where SSL extracted with isopropanol was used as fermentation medium.

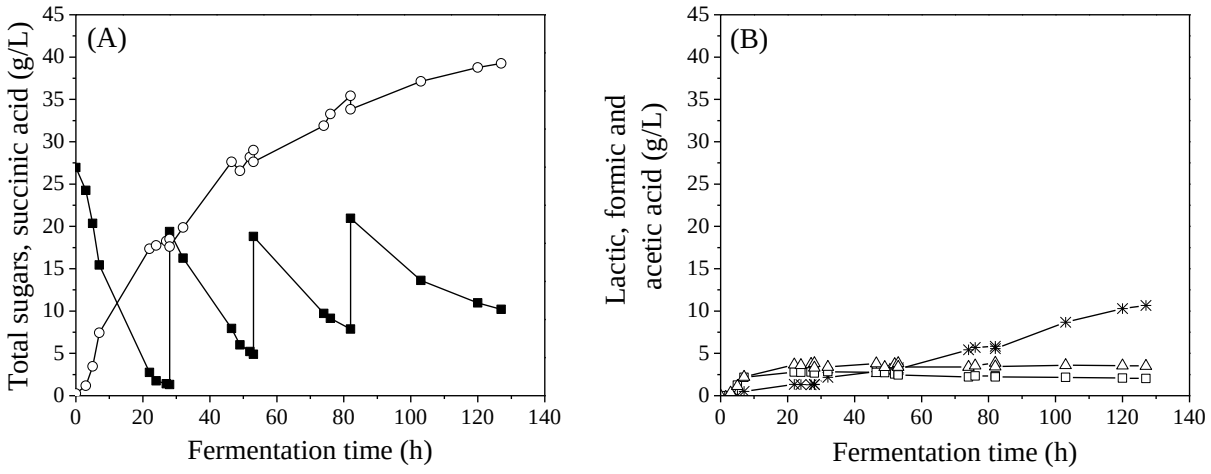



**Figure 8.5** Total sugar consumption (■) and production of succinic acid (○), acetic acid (△), formic acid (□) and lactic acid (∗) using *B. succiniciproducens* in filtrated-extracted SSL using the same medium as feeding solution after concentration via evaporation.

Table 8.1 presents literature-cited results on succinic acid production when crude hydrolysates from pre-treated lignocellulosic biomass were used, emphasizing on xylose-rich ones. Xi et al. (2013) reported the production of 23.7 g/L of succinic acid concentration by *A. succinogenes* NJ113 when xylose-based sugarcane bagasse hydrolysate was used. Salvachua et al. (2016) reported a succinic acid concentration of 42.8 g/L with high conversion yield (0.74 g/g) when xylose-based corn stover hydrolysates were employed using the strain *A. succinogenes* 130Z. It should be stressed that for this experiment the authors used a more rich substrate (6 g/L yeast extract and 10 g/L corn steep liquor). Pateraki et al. (2016b) achieved

27.4 g/L succinic acid with *A. succinogenes* in ultrafiltrated SSL treated with 3 kDa membranes, and 33.8 g/L when *B. succiniciproducens* was employed and SSL from 500 Da membranes was used as substrate. Recently, *B. succiniciproducens* was studied on corn stover deacetylated hydrolysate (Salvachua et al. 2016b) and on *Arundo donax* hydrolysate (Cimini et al., 2016). This strain seems to perform better in xylose-based hydrolysates in comparison to *A. succinogenes*, even though it produces also lactic acid as by-product. Salvachua et al. (2016b) attributed the high production of lactic acid to energy excess in the media, as this pathway is not ATP-efficient. This could explain the fact that the main by-product in the isopropanol treated SSL was acetic acid, whereas the major one in the nanofiltrated-extracted SSL was lactic acid. Regarding the SSL-based media, nanofiltration coupled with solvent extraction with ethyl-acetate results in higher final succinic acid titrations and at the same time lower by-product formation. Taking into account these findings, this process could be employed in order to develop and advanced biorefinery, based on pulp and paper industry.

**Table 8.1** Production of succinic acid from lignocellulosic hydrolysates containing high xylose concentration

| Raw material | Pre-treatment | Major sugars (g/L) | Microorganism | Succinic acid (g/L) | Yield (g SA/g total sugars) | Productivity (g/L/h) | SA:LA:FA:AA (molar ratios) | Ref. |
|---|---|---|---|---|---|---|---|---|
| Pinewood extract | ionic liquid 1-allyl-3-methylimidazolium chloride | gluc[1] (~30), xyl[2] (~17) | *A. succinogenes* 130Z | 20.7 [6] | 0.65 | 0.9 | - | Wang et al., 2014 |
| Spruce hydrolyzates | overliming and/or activated carbon | gluc (30), xyl+man[3]+gal[4] (~44) | *Escherichia coli* AFP184 | 42.2 [7] | 0.57 | 1 | - | Hodge et al., 2009 |
| Corn stalk | $H_2SO_4$ and activated carbon | xyl (22.7), gluc (3), arab (2) | *E. coli* mutant DC115 derived from AFP111 | 21.1 | 0.76 | 0.19 | 1:0:0:0:0.76 | Jiang et al., 2014 |
| Sugarcane bagasse | ultrasonication, dilute $H_2SO_4$ and activated charcoal | xyl (22.4), gluc (3.6), arab[5] (3.9) | *A. succinogenes* NJ113 | 23.7 [8] | 0.79 | 0.99 | 1:0:0:0.37 | Xi et al., 2013 |
| Corn straw | dilute alkali treatment | gluc (22.4), xyl (13.7), feeding medium (200 g/L total sugars) | *A. succinogenes* CGMCC1593 | 53.2 [9] | 0.82 | 1.21 | 1:0:0:0.22 | Zheng et al., 2009 |
| Corn stover | alkaline wash and dilute acid treatment | xyl (58), gluc (8.7), arab (8.7), gal (4.6) | *A. succinogenes* 130Z | 42.8 [10] | 0.74 | 0.30 | 1:0:0:0.47 | Salvachua et al., 2016a |
| OPF bagasse | NaOH-AH | Glu (74 %), xyl (22%) | *A. succinogenes* 130Z | 36.6 | 0.61 | 0.61 | - | Luthfi et al. 2016 |
| Corn stover | alkaline wash and dilute acid treatment | xyl (43), gluc (6.6), arab (6.8), gal (3.5) | *B. succiniciproducens* | 30.0 [10] | 0.51 | 0.43 | 1:0.11:0.33:0.56 | Salvachua et al., 2016b |
| Arundo donax | No pretreatment | Gluc (~16), xyl (~9) | *B. succiniciproducens* | 17.0 | 0.68 | 0.35 | - | Cimini et al., 2016 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| hydrolysate | | | BPP7 | | | | | |
| SSL-based media | Ultrafiltration (3 kDa membrane) | Xyl (72.6 %), gal (12.2%), gluc (10.9%), mann (4.2%), arab (0.1) | *A. succinogenes* 130Z | 27.4 | - | 0.39 | 1:0:0.38:0.51 | Pateraki et al., 2016b |
| | Nanofiltration (500 Da membrane) | Xyl (72.6 %), gal (12.2%), gluc (10.9%), mann (4.2%), arab (0.1) | *B. succiniciproducens* | 33.8 | - | 0.48 | 1:0.46:0.31:0.52 | Pateraki et al., 2016b |
| | Solvent extraction with isopropanol | gluc (1.5), xyl+man+gal (22.6), arab (0.68) | *A. succinogenes* 130Z | 19.4 | 0.55 | 0.33 | 1:0:0.55:0.70 | |
| | Solvent extraction with isopropanol | gluc (0.74), xyl+man+gal (15), arab (0.68) | *B. succiniciproducens* | 19.5 | 0.58 | 0.60 | 1:0.11: 0.22:0.67 | This study |
| | Nanofiltration & solvent extraction with ethyl acetate | gluc (2.4), xyl+man+gal (24), arab (0.4) | *B. succiniciproducens* | 39 | 0.54 | 0.31 | 1:0.35:0.13:0.18 | |

[1] glucose; [2] xylose; [3] mannose; [4] galactose; [5] arabinose; [6] 10 g/L yeast extract; [7] 15 g/L yeast extract and 15 g/L corn steep liquor; [8] 4 g/L yeast extract and 5 g/L corn steep liquor; [9] 15 g/L yeast extract; [10] 6 g/L yeast extract and 10 g/L corn steep liquor

### 8.4. Analysis of metals and REE

An analysis of trace elements and rare earth elements (REE) was also carried out, as the elemental content could affect not only the valorization of the LS and the phenolic extract, but also the microbial fermentation.

Table **8.2** summarizes the results of the ICP-MS analysis. SSL contains many metals with Ca (50901062 ppb), Mg (9407642 ppb), Zn (2444856 ppb), Fe (9723440 ppb) being the predominant. The rare earth element La is also found at a considerable concentration (7648.3 ppb), followed by Ce (3092.6 ppb) and Dy (2318.9 ppb). Eu, Ho, Nd, Y and the actinide Th were also present at concentrations higher than 1000 ppb. Detoxification with both methods led to significant metal removal. Important removal of trace and rare earth elements was observed in the sample of the SSL extracted with isopropanol. Nanofiltration also led to large removal of metals, probably of those connected to the LS. The phenolic extract also contained metals and heavy metals but in lower quantities. The macro element Ca was found at a concentration of 35798 ppb in the ethyl acetate extract, followed by Zn (2826 ppb), Ti (389 ppb) and Be (309.9 ppb). The concentration of the heavy metals Cu and Fe is lower than the initial sample, but yet high (160.3 and 300.4 ppb respectively). The presence of calcium and magnesium is mainly attributed to the use of dolomite during the pulping process.

The presence of some metals could influence the market potential of the lignosulphonates. For example, iron enhances the properties of LS as fertilizers (Rueda et al. 2014). It is the first study that ICP-MS is used for the identification of trace elements and REE in SSL. Rueda et al. (2014) used ICP-MS only for the heavy metal analysis, whereas atomic absorption was used for the identification of Ca, Mg, Na, K and Fe of various SSL samples. Rueda et al. (2014) also reported significant amounts of Ca and Mg in the samples (18 and 5.3 g/L respectively with 58.5% dry matter and 1.3 g/mL density of the SSL used, values quite similar to the ones of this study, but much lower than the concentrations identified in this study.

**Table 8.2** Concentrations of minerals and REE in SSL, in solvent extracted SSL, in nanofiltrated SSL and in the phenolic-rich extract

| Trace elements | SSL | SSL extracted with isopropanol | Permeate after SSL nanofiltration | Phenolic extract |
|---|---|---|---|---|
| | | µg/L | | |
| Se | 9540.3 | 9.3 | 93.9 | 9.4 |
| Zn | 2444856 | 2922 | 47775 | 2826 |
| Mn | 14442 | 3.2 | 691.5 | 0.9 |
| Mg | 9407642 | 5151.9 | 736266 | 290.8 |
| Pb | 13790 | 7.5 | 128.1 | 11.7 |
| Fe | 9723440 | 5324.8 | 760990 | 300.4 |
| Cu | 75476 | 107.3 | 650.2 | 160.3 |
| Co | 1934.5 | 2.4 | 32.3 | 1.5 |
| Ca | 50901062 | 39394 | 1192689 | 35798 |
| As | 305.4 | 0.1 | 3.0 | 0.2 |
| Sb | 1310.4 | 1.6 | 14.7 | 0.8 |
| Mo | 5516.2 | 7.4 | 63.4 | 4.2 |
| Ni | 11506 | 11.2 | 142.9 | 7.8 |
| Cd | 650.4 | 0.7 | 10.1 | 0.2 |
| Cr | 417.3 | 1.0 | 7.2 | 12.6 |
| V | 153.0 | 0.1 | 1.4 | 0.1 |
| Sr | 30899 | 23.9 | 710.4 | 21.0 |
| Li | 700.0 | 0.7 | 7.6 | 1.1 |
| Be | 47790 | 48.2 | 532.8 | 309.9 |
| Ti | 398092 | 388.9 | 3970.8 | 389.0 |
| Tl | 60.9 | n.d.* | 0.6 | n.d. |
| **Rare Earth Elements** | | | | |
| Ce | 3092.6 | 3.5 | 75.3 | 2.7 |
| Dy | 2318.9 | 2.3 | 24.7 | 2.3 |
| Er | 181.5 | 0.2 | 2.9 | 0.2 |
| Eu | 1661.9 | 1.6 | 16.9 | 1.7 |
| Gd | 568.2 | 0.5 | 7.8 | 0.5 |
| Ho | 1775.9 | 1.8 | 18.0 | 1.8 |
| La | 7648.3 | 8.1 | 106.1 | 7.4 |
| Lu | 306.0 | 0.3 | 3.2 | 0.3 |
| Nd | 1003.4 | 1.0 | 21.0 | 1.0 |
| Pr | 246.6 | 0.2 | 5.7 | 0.2 |
| Sc | 60.4 | 0.1 | 0.8 | 0.3 |
| Sm | 335.6 | 0.3 | 5.3 | 0.4 |
| Tb | 160.0 | 0.1 | 1.8 | 0.1 |
| Tm | 35.1 | n.d. | 0.5 | n.d. |
| Y | 1645.5 | 1.4 | 29.3 | 2.0 |
| Yb | 167.2 | 0.2 | 2.4 | 0.2 |
| **Actinides** | | | | |
| Th | 3754.3 | 3.8 | 38.8 | 4.0 |
| U | 466.6 | 0.4 | 7.4 | 0.3 |

*n.d: not detected

The presence of heavy metal ions (such as chromium, copper, nickel and iron) is mainly attributed to the corrosion of the equipment of the pulp industry. Their toxicity is correlated to inhibition of key enzymes in the microbial metabolism (Mussatto and Roberto, 2004). Heavy metals tend to bind with thiol and other groups of proteins or replace the metals present in enzyme prosthetic groups (Soldatkin et al. 2012). It is shown that the presence of nickel ions at a concentration of 100 ppm caused 60% inhibition on *Pachysolen tannophilus* as it highly affected the enzymes associated to xylose metabolism (Mussatto and Roberto, 2004). Heavy metals are inhibitory to many anaerobic microorganisms such as acetogenic, acidogenic and methanogenic bacteria. It is reported that concentrations above 70 mg/L of Cu, 200 mg/L of Zn and 10 of Ni are toxic to the microorganisms of anaerobic digestion (Chen et al. 2014).

The toxicity of some REE towards fermenting microorganisms has been widely studied in the literature. Ruming et al. (2002) reported that cerium ions present an inhibitory effect on *E. coli* at concentrations above 400 μg/mL. Peng et al. (2006) studied the toxicity of $La^{3+}$ on *E. coli* and they concluded that its inhibitory effect is correlated to the permeability of the cell membrane towards different concentrations of $La^{3+}$. More specifically, low concentrations of $La^{3+}$ increase the membrane permeability, so as more nutrients can be transported into the cell, whereas high concentrations of $La^{3+}$ are toxic as they are accumulated in the cells.

To identify the inhibitory effect of each metal and heavy metal on *A. succinogenes* and *B. succiniciproducens* separate studies should be carried out.

Table **8.2** shows that both pretreatment methods reduced the concentration of metals and heavy metals in the pre-treated fermentation broths and therefore a lower inhibitory effect should be expected during fermentation.

### 8.5. Proposed integrated biorefinery based on SSL

In this study, two different pretreatment processes of SSL, derived from the acidic sulphite pulping process of *Eucalyptus globulus* wood, were evaluated in order to produce value-added products. The combination of nanofiltration and solvent extraction resulted in the production of three value-added products, namely LS (more than 95% recovery), phenolic compounds (1.15 g of extracted mass per 100 g SSL containing around 43% phenolic compounds) and succinic acid (4.3 g per 100 g SSL). This process is considered more suitable as an integrated biorefining strategy as more products are produced than in the case that isopropanol extraction was employed. More specifically, LS are already commercialized in various applications such as concrete additives, placticizers, adhesives, industrial detergents, cement dispersants, compressed material binders, glue, stabilizers or as feedstock for chemicals (Restolho et al. 2009). In Norway and Finland, there are pulp and paper industries operating ultrafiltration plants that produce high molecular weight LS (Fernández-Rodríguez et al., 2015). Membrane separation of LS has some important benefits, such as the easy handling of the waste, as no pH or temperature adjustment is required, and the possibility to produce LS with different molecular weights based on the MWCO of the membrane used (Jönsson et al. 2008). The price of LS varies from 200-700 $ per t (source: www.alibaba.com).

The high antioxidant activity of the phenolic extract makes it a potential natural source of antioxidants that could be applied as active ingredients in biocides and wood preservatives, adhesives, resins, plastics, rubber products, oil-field products, agricultural products, lubricants and in paper products in order to prevent yellowing (Willfoer et al. 2012; Balakshin et al. 2013). The analysis of the individual phenolic compounds of the ethyl acetate extract showed that its is rich in gallic and ellagic acids as well as in vanillin and catechin. Gallic acid is used for the synthesis of trimethoprim, gallamine triethiodide and trimetazidine. It is also used for paper manufacturing (Badhani et al., 2015) and for collagen stabilization in leather making (Jackson et al., 2010). Propyl gallate in used as antioxidant by the food industry. The annual market of gallic acid is 170,000 kg (source: www.usda.gov/oce/reports/). Recently, ellagic acid was used in order to enhance the mechanical and barrier properties in starch-based films (Tirado-Gallegos et al., 2016). Phenolic extracts derived from natural resources containing

gallic and ellagic acids are commercialized products with market price in the range of 30-100 $/kg (source: www.alibaba.com). The annual market of vanillin is 12 million kg and is mainly used as a flavor additive but also in the pharmaceutical industry (source www.usda.gov/oce/reports/). It is estimated that 60% of vanillin is used by the food industry, 33% is used in perfumes and cosmetics and 7% is used in pharmaceuticals (Priefert et al. 2001). Lignin-derived vanillin, which is considered as a premium product, is priced in the range of 100-200 $/kg (Borges da Silva et al., 2009). Lately, there is a growing interest on vanillin-based polymers such as polyesters, methacrylate and acrylate polymers and phenolic, benzoxazine and epoxy resins (Fache et al., 2015).

Finally, succinic acid is an important platform chemical with a wide range of applications, from the production of food additives, to plasticizers, bioplastics and as intermediate for the production of other chemicals. Bio-based succinic acid has a market potential of $7-10 billion, but its worldwide capacity is estimated to reach 600,000 t, giving a profit of $539 million (Taylor et al., 2015). The high market potential has already led to the construction of industrial facilities for the production of bio-succinic acid, such as BioAmber with annual capacity of 3,000 t and Succinity with 10,000 t per year (Pateraki et al., 2016a). Moreover, Succinity (Basf and Cordion) uses the strain *B. succiniciproducens*, but glycerol and sugars as feedstocks (Becker et al., 2015).

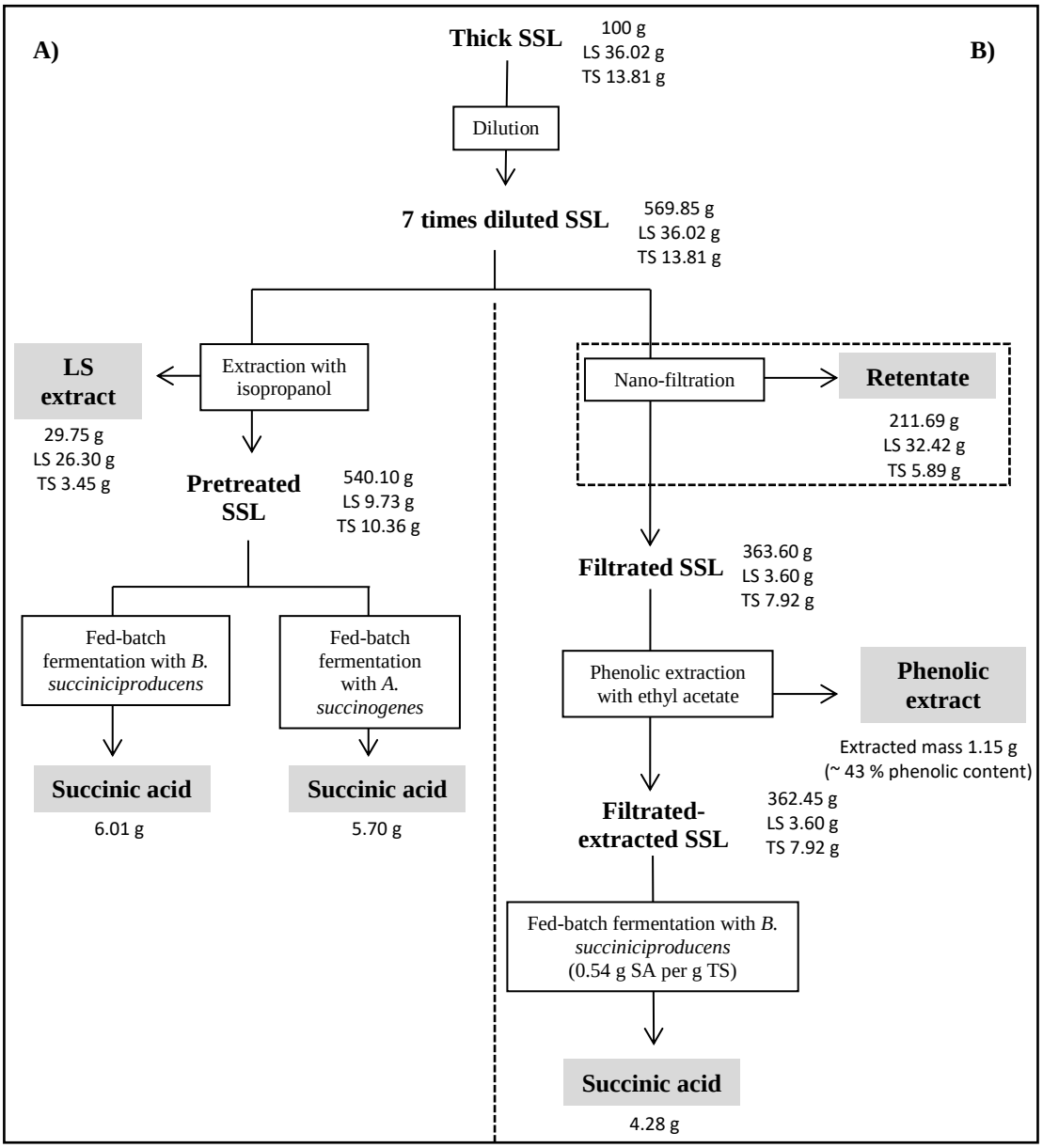



**Figure 8.6** Schematic representation of the studied biorefinery strategies

## 8.6. Conclusions

The results presented in this Chapter indicate that conventional pulp and paper mills could be converted into integrated biorefineries through the exploitation of the sugars for the production of bio-based succinic acid and the separation of LS and phenolic compounds as value–added co-products. Nanofiltration coupled with solvent extraction using ethyl acetate could lead to sufficient detoxification of the SSL leading to high succinic acid production efficiency by *B. succiniciproducens*. The proposed biorefinery scheme could lead to valorisation of SSL for the production of LS, phenolics and succinic acid. Future research should focus on the optimization of succinic acid production via fermentation, the purification of the phenolic compounds as well as a techno-economic evaluation of the proposed biorefinery scheme.

*The results presented in Chapter 8 have been published in Bioresource Technology:*

Alexandri M., Papapostolou H., Komaitis M., Stragier L., Verstraete W., Danezis GP., Georgiou CA, Papanikolaou S., Koutinas AA. (2016). Evaluation of an integrated biorefinery based on fractionation of spent sulphite liquor for the production of an antioxidant-rich extract, lignosulphonates and succinic acid. Bioresour Technol, 214: 504-513

# CHAPTER 9
# EVALUATION OF IMMOBILIZED CULTURES FOR ENHANCED SUCCINIC ACID PRODUCTION FROM SSL

## 9.1. Introduction

The development of an advanced biorefinery based on pulp and paper industry was demonstrated in the previous chapter. From SSL it is possible to recover the LS as well as an antioxidant-rich extract. The produced detoxified stream led to a high concentration of succinic acid (39 g/L), using the bacterium *B. succiniciproducens*. These results are considered promising in comparison to literature-cited publications, but further improvement of the process should be achieved. In order to make the process of bio-succinic acid cost-efficient, it is crucial to achieve higher final titrations and higher productivities. The reduction of the by-products is also critical, not only due to competitive effects towards succinic acid, but also for the separation and purification of succinic acid. The presence of lactic acid in the fermentations of *B. succiniciproducens* could be problematic during the downstream process, as this organic acid presents similar properties to succinic acid. An efficient upstream process with *A. succinogenes* would overcome this issue.

These drawbacks could be addressed by employing cell immobilization. Immobilization of microbial cells has been well studied mainly for the production of bioethanol, wine, butyric acid and hydrogen (Nikolić et al., 2009; Pachero et al., 2010; Mallouchos et al., 2003; Jiang et al., 2010; Kumar et al., 2001). This technic has been reported to enhance both fermentation efficiency and tolerance of the microorganism to various inhibitory compounds that are present in the medium. Another advantage of cell immobilization lies on the possibility of re-utilization of the biocatalyst, eliminating the need for biomass separation during the downstream process, facilitating the industrial application of the biotechnological production of chemicals using renewable resources.

Two different immobilization methods were applied by using different support materials. Firstly, immobilization on delignified cellulosic material (DCM) was studied using the strain *A. succinogenes*. The material applied was nothing more but wood sawdust, which is abundant in the pulp and paper industry, it is cheap and studies have proved its operational stability (Elezi et al., 2003). Cell immobilization occurs either via physical adsorption due to electrostatic forces, or via covalent binding between the cell membrane and the support (carrier). The other support studied, using both strains, was calcium alginate beads.

Immobilization in alginates is based on entrapment of microbial cells and it is one of the most commonly used supports. The process of cell immobilization in alginate beads is also cheap and easily applicable (Jiang et al., 2010). In this chapter, cell immobilization was evaluated in terms of succinic acid production using crude, ultra- and nano-filtrated SSL. Repeated batch, fed-batch and repeated fed-batch fermentations were carried out in order to evaluate the possibility of re-using the biocatalyst for more fermentation cycles.

### 9.2. Immobilization of *A. succinogenes* on DCM

In the previous chapters, it was shown that even though *A. succinogenes* is a more robust strain towards SSL's inhibitors in comparison to *B. succiniciproducens*, the final succinic acid titrations achieved with the latter are much higher. However, *A. succinogenes* does not produce lactic acid and it is also able to grow in untreated SSL much more efficiently than *B. succiniciproducens* (Chapter 7). Taking into account the results of previous experiments, immobilized cells of *A. succinogenes* on DCM were used in repeated batch fermentations using 7 times diluted SSL as substrate. Figure 9.1 illustrates immobilized *A. succinogenes* cells on DCM as were monitored by scanning electron microscopy.

In the first batch (Figure 9.2), it is obvious that sugar consumption started after a lag phase that lasted approximately 10-12 h. The fermentation was terminated after 33 h. The residual sugar (2.1 g/L) was mainly galactose, the monosaccharide that the strain is not able to consume. The final succinic acid concentration was 10.2 g/L, with yield on total sugars equal to 0.51 g/g and total volumetric productivity equal to 0.31 g/L/h. The improvement of the fermentation in comparison to free cells is evident. In the corresponding fermentation with free cells of *A. succinogenes* (Chapter 7, Figure 7.6B), more than half of the sugars present in the medium remained unconsumed, resulting in a final yield of only 0.24 g/g (on total sugars) after 63 h. Regarding by-products, their formation started at the same time as the production of succinic acid. Interestingly, in this case the production of lactic acid was also observed (1.43 g/L). Low lactic acid concentrations were also observed by Almqvist et al. (Almqvist et al., 2016) when SSL was used as fermentation substrate. The production of lactic acid is not much reported in *A. succinogenes* cultures, although the gene responsible for lactate dehydrogenase (LDH) expression has been found in its genome and LDH activity has been previously measure in low, yet detectable amounts (Van der Werf, 1997; McKinlay et al., 2010). The main by-product was acetic acid with final concentration of 5.51 g/L, followed by formic acid with final concentration equal to 4.1 g/L. Even though lactic acid was produced,

the ratio of total by-products to succinic acid was 1.08 g/g that is a lower value in comparison to free cells (1.41 g/g).

This experiment showed that immobilized cells are more tolerant to the inhibitory compounds of SSL, so the possibility of re-utilization should also be tested. For this reason, three more batch fermentations were carried out using the same biocatalyst (Figure 9.2). In the second batch, the final succinic acid concentration reached 13.4 g/L, the highest value achieved among the fourth batch runs. Product yield on total sugars and volumetric productivity were also slightly higher in the second batch in comparison to the first run (0.57 g/g and 0.39 g/L/h). By-product formation was lower, with the main one being acetic acid (4.41 g/L), followed by formic acid (2.84 g/L). A very low concentration of lactic acid was also observed (0.12 g/L). It is evident that during the second batch run the immobilized cells were also adapted to the inhibitors, resulting in lower by-products and higher product yields (Table 9.1). The by-product to succinic acid ratio decreased in the second batch run, but it was gradually increased in the third and fourth batch runs (Table 9.1). Final succinic acid concentration and yield also gradually decreased in the third and fourth batch. Lactic acid was not produced in the third batch, but it was formed again in the fourth run. Even though the immobilized cells are still able to ferment the sugars of the SSL, the molar ratio of succinic acid to total by-products increased notably in the last batch run (2.18 mol/mol). Chen et al. (2016) carried out three repeated batch fermentations using sugarcane bagasse hydrolysates as carbon source and sugarcane bagasse as immobilization support with the strain *A. succinogenes* CCTCC M2012036 leading to a high average yield (80.5%) and productivity (1.65 g/L/h) but a gradually reducing productivity between the first and the third batch run.

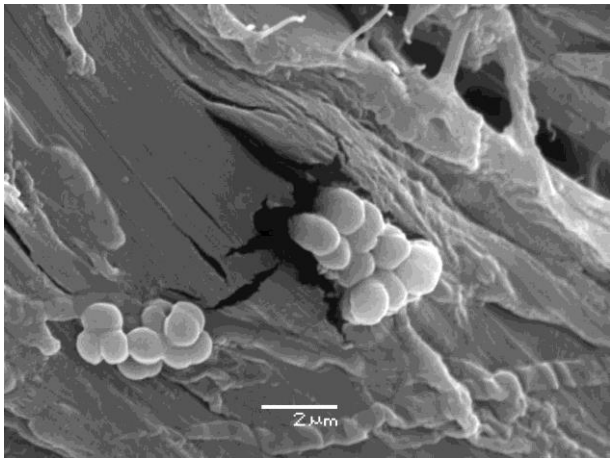



**Figure 9.1** Scanning Electron microscopy with immobilised cells of *A. succinogenes* on DCM

The accumulation of the inhibitory compounds of SSL (especially lignosulphonates) onto the DCM resulted in a significant reduction of succinic acid concentration and yield in the fourth repeated batch fermentation. The accumulation of LS on the pores of DCM may have blocked the access of $CO_2$ to the microorganism. This assumption could be justified by the increased by-product formation in the fourth repeated batch (Table 9.1). $CO_2$ availability is crucial for SA production as it is directly correlated with the activity of the enzyme PEP carboxykinase and the reductive TCA flux in the microorganism (Herselman et al., 2017). Herselman et al. (2017) studied the importance of $CO_2$ on continuous fermentations with *A. succinogenes*. Their research revealed that below a critical concentration of $CO_2$, the production of SA decreases significantly, whilst the concentrations of both acetic and formic acids remained rather stable. The authors claimed that this trend shows that the carbon flux turned to the $C_3$ pathway (leading to by-product formation) instead of $C_4$ (leading to SA production). This switch in carbon flux takes place at the phosphoenolpyruvic acid (PEP) branch, between the $C_3$ and $C_4$ pathways.

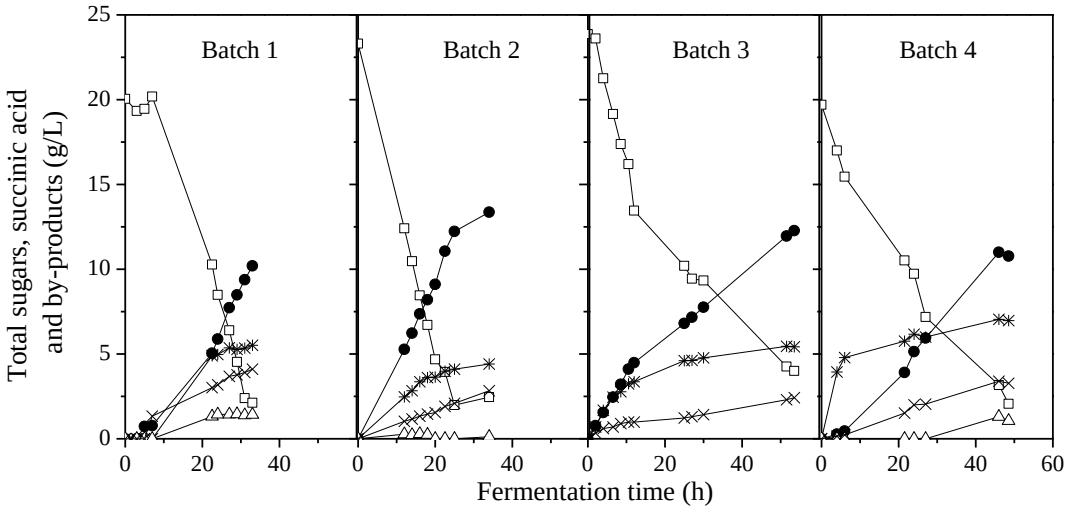



**Figure 9.2** Total sugar consumption (□) and production of succinic acid (●), lactic acid (△), formic acid (×) and acetic acid (∗) during repeated batch fermentations of SSL with immobilized *A. succinogenes* cells on DCM

In Chapter 9, it was shown that nanofiltration of SSL led to improved succinic acid production. Ultrafiltration has been also employed for succinic acid production from SSL, resulting to enhanced final product concentration with strains *A. succinogenes* and *B. succiniciproducens* (Pateraki et al., 2016b). Moreover, by applying a filtration process, lignosulphonates are also recovered, which are considered a co-product and their recovery contribute revenue to the pulp and paper industry. Therefore, the separation of

lignosulphonates prior to succinic acid fermentation is necessary. For this reason, the subsequent fed-batch fermentation with the immobilized biocatalyst was carried out with ultrafiltrated SSL using a membrane of 10 kDa MWCO (Figure 9.3). The SSL was diluted 10 times prior to ultrafiltration. The concentration of lignosulphonates in ten times diluted SSL was 39.2 g/L, whereas the permeate produced during ultrafiltration of SSL had a lignosulphonate concentration of 27.4 g/L. As the initial sugar concentration of the permeate was relatively low (about 18.4 g/L), the permeate stream was concentrated to a sugar concentration of approximately 25 g/L. Lignosulphonate concentration also increased to about 35 g/L. Figure 9.3 presents the consumption of total sugars as well as the production of succinic acid and by-products during fermentation carried out with immobilized *A. succinogenes* cells in DCM. The fermentation started with the permeate from ultrafiltrated SSL and the feeding solution used was concentrated ultrafiltrated SSL, with a sugar concentration of approximately 200 g/L. Sugar consumption started after a long lag phase that lasted about 20 h. During the next 20 h of fermentation, sugars were depleted rapidly and the first batch was carried out (Figure 9.3). One more feeding pulse was carried out after 60 h of fermentation. The final succinic acid concentration achieved was 27.3 g/L and the total sugar to succinic acid conversion yield was 0.65 g/g. Total volumetric productivity achieved was 0.32 g/L/h. The residual sugars contained mainly galactose as this strain is not able to consume this sugar (Pateraki et al., 2016b). The main by-product was acetic acid, with final concentration 7.4 g/L, followed by formic acid with 1.6 g/L. Lactic acid was also observed with concentration at the end of the fermentation equal to 1.1 g/L. The produced succinic acid concentration is similar to the one achieved by free cells of *A. succinogenes* cultivated on ultrafiltrated SSL using 3 kDa MWCO, while the yield achieved with free cells (0.41 g/g) was much lower (Pateraki et al., 2016b). The ratio of formic acid and acetic acid to succinic acid as well as the ratio of total by-products to succinic acid (Table 9.2) are lower than those achieved in fed-batch fermentations using free cells cultivated on ultrafiltrated SSL using 3 kDa MWCO membrane as was reported by Pateraki et al. (2016b).

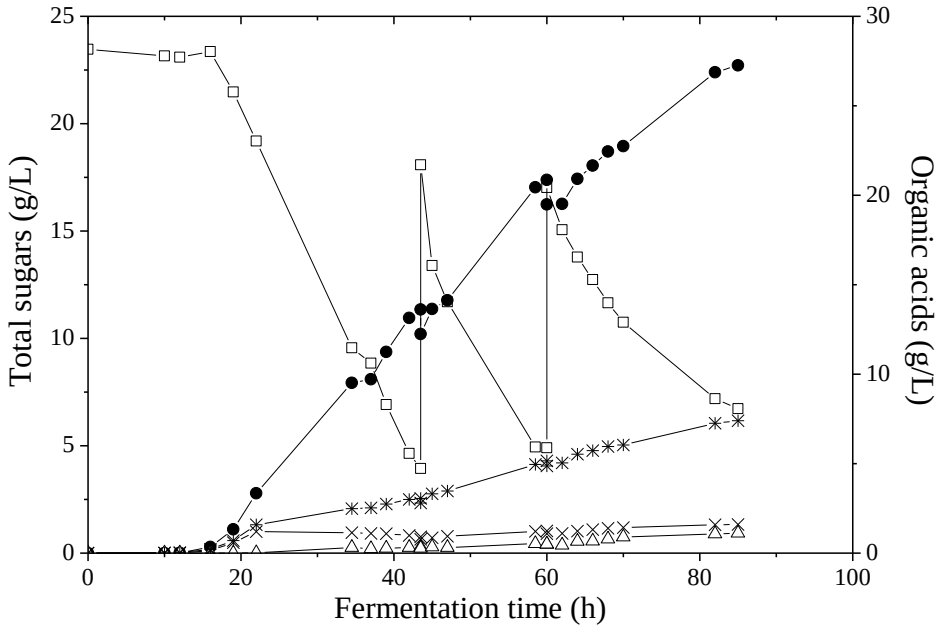



**Figure 9.3** Total sugar consumption (□) and production of succinic acid (●), lactic acid (△), formic acid (×) and acetic acid (∗) during fed-batch fermentation of ultrafiltrated SSL with immobilised *A. succinogenes* cells on DCM

### 9.3. Immobilization of *A. succinogenes* in alginate beads

Immobilization on DCM gave promising results regarding final succinic acid concentration, but inhibitors' accumulation, especially LS pose another obstacle. Moreover, with this type of immobilization technique, free cells also exist in the medium, leading to additional operating costs for biomass removal during downstream process. In order to eliminate these problems alginate beads were also tested as a support. The process of cell encapsulation is described in detail in Chapter 6. The diameter of the alginate beads was also measured before fermentation and their average diameter was 5.0 – 5.5 mm fermentation (Figure 9.4).

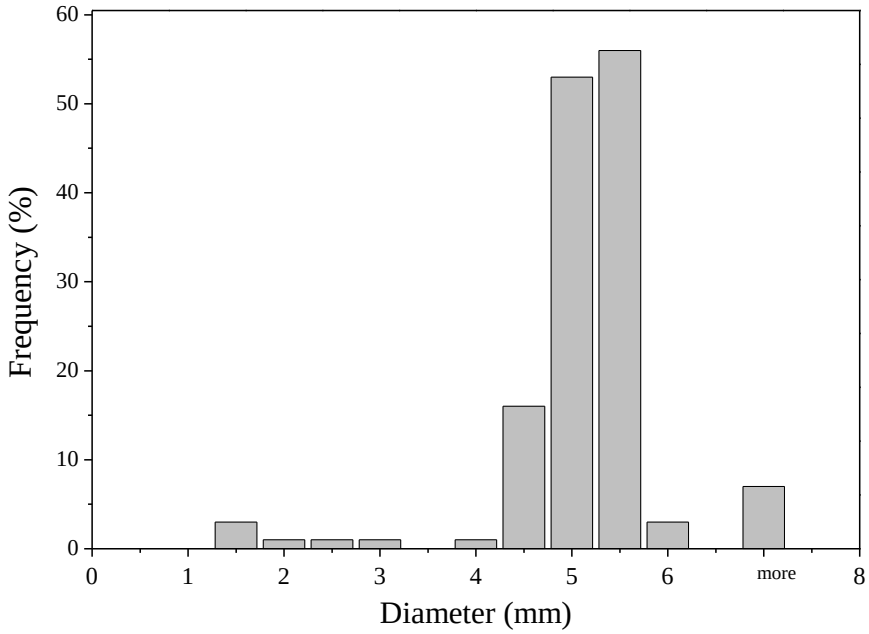



**Figure 9.4** Distribution of alginate beads diameters

Five repeated batch fermentations were carried out using 7 times diluted and untreated SSL (Figure 9.5) under the same conditions used in the repeated batch fermentations carried out with *A. succinogenes* immobilized cells in DCM. Table 9.1 shows that the final succinic acid concentration and yield were gradually increased from the first to the fifth batch fermentation. In the first batch fermentation, the final succinic acid concentration was 9.1 g/L and the total sugar to succinic acid conversion yield was 0.58 g/g. These values were increased to 12.3 g/L and 0.71 g/g in the fifth batch fermentation. These results indicate that lignosulphonates as well as other inhibitors present in SSL did not affect the efficiency of immobilized culture alginate beads to produce succinic acid. In the case of repeated batch fermentations carried out in DCM, the fermentation efficiency was reduced after 3 batch cycles.

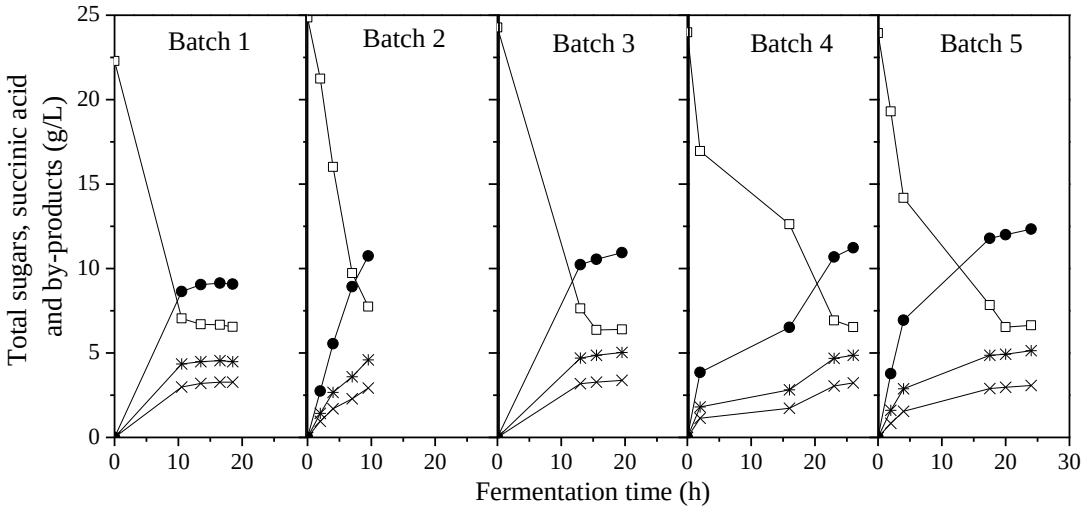



**Figure 9.5** Total sugar consumption (□) and production of succinic acid (●), lactic acid (△), formic acid (×) and acetic acid (✳) during repeated batch fermentations of SSL with immobilised *A. succinogenes* cells in alginate beads.

Although succinic acid concentration and yield were gradually increased when immobilization in alginate beads was carried out (Figure 9.5 and Table 9.1), the productivity was decreased from 1.13 g/L/h in the second batch to 0.51 g/L/h in the fifth batch fermentation. Regarding by-product formation, lactic acid production was not observed and the main by-product was acetic acid. Figure 9.5 shows that the final concentration of both by-products remained stable and only succinic acid concentration increased from the first to the fifth batch run. The final formic acid concentration was around 3 g/L in every batch, whilst acetic acid had an average concentration of 5 g/L. The ratio of total by-products to succinic acid decreased from 0.85 to 0.67 from the first to fifth batch, values that are lower than the ones observed when DCM was used as support material. No lactic acid production was observed. The by-product formation was also much lower than the fermentation where free cells were used (Table 9.1). Immobilization by entrapment seems to provide a better environment for the cells to produce succinic acid. The same observation was also reported by Corona-González et al. (2014) where immobilization of *A. succinogenes* cells by entrapment (especially when agar beads were used as support material) proved a better immobilization methodology than adhesion. Corona-González et al. (2014) conducted five repeated batch fermentations using glucose as carbon source leading to increased productivity from the first (1.05 g/L/h) to the fifth (2.72 g/L/h) batch run with a conversion yield within the range of 0.57 – 0.64 g/g. Corona-González et al. (2016) employed immobilized *A. succinogenes* cells in agar beads cultivated in five repeated batches using agave bagasse hydrolysates as carbon

source leading to increased productivity (0.49 – 1.33 g/L/h) and constant yield (around 0.44 g/g) during sequential batches. Corona-González et al. (2016) also observed that the concentration of acetic acid increased in each batch, whereas formic acid was stable.

In this study, productivity increased in the second batch for both support materials (Table 9.1) probably due to cell adaptation to the inhibitory compounds of the SSL. A decreasing trend of total volumetric productivity was observed in subsequent batches that could be attributed to the presence of lignosulphonates. The yield was relatively stable for every batch run in both studied supports. In the case of alginate beads, even after five repeated batches, the cells continued to be viable and able to produce succinic acid from crude SSL. The molar ratios of succinic acid to each by-product SA:FA:AA decreased from 1:1.46:1.69 in case of the free cell fermentation, to 1:0.51:0.87 in the third batch on DCM and 1:0.64:0.83 in the fifth batch run of immobilized cells in alginates.

**Table 9.1** Fermentation results with immobilized *A. succinogenes* cells on DCM and alginate beads with 7 times diluted SSL in repeated batch cultures in comparison to free cells.

| Batch run | Total sugar consumed (g/L) | Succinic acid (g/L) | Succinic acid yield (g SA / g TS) | Productivity (g/L/h) | LA/SA (g/g) | FA/SA (g/g) | AA/SA (g/g) | Tot/SA (g/g) |
|---|---|---|---|---|---|---|---|---|
| Repeated batch fermentation with DCM | | | | | | | | |
| 1 | 17.9 | 10.2 | 0.51 | 0.31 | 0.14 (0.20)* | 0.40 (1.00) | 0.54 (1.10) | 1.08 (2.30) |
| 2 | 20.8 | 13.4 | 0.57 | 0.39 | 0.01 (0.01) | 0.21 (0.55) | 0.33 (0.65) | 0.55 (1.21) |
| 3 | 19.9 | 12.3 | 0.51 | 0.23 | 0.00 | 0.20 (0.51) | 0.44 (0.87) | 0.64 (1.38) |
| 4 | 17.6 | 10.8 | 0.55 | 0.22 | 0.10 (0.13) | 0.30 (0.78) | 0.65 (1.27) | 1.05 (2.18) |
| Repeated batch fermentation with alginate beads | | | | | | | | |
| 1 | 15.7 | 9.1 | 0.58 | 0.49 | 0.00 | 0.36 (0.92) | 0.49 (0.96) | 0.85 (1.89) |
| 2 | 17.1 | 10.7 | 0.63 | 1.13 | 0.00 | 0.27 (0.69) | 0.43 (0.85) | 0.70 (1.54) |
| 3 | 17.9 | 10.9 | 0.61 | 0.56 | 0.00 | 0.31 (0.80) | 0.46 (0.90) | 0.77 (1.70) |
| 4 | 17.5 | 11.2 | 0.64 | 0.43 | 0.00 | 0.29 (0.74) | 0.43 (0.85) | 0.72 (1.59) |
| 5 | 17.3 | 12.3 | 0.71 | 0.51 | 0.00 | 0.25 (0.64) | 0.42 (0.83) | 0.67 (1.47) |
| Fermentation using free cells of *A. succinogenes* | | | | | | | | |
| | 13.4 | 6.2 | 0.24 | 0.09 | - | 0.57 (1.46) | 0.86 (1.69) | (3.15) |

*The numbers in parenthesis represent the molar ratio (mol/mol)

Ultrafiltrated SSL was subsequently evaluated as fermentation substrate using immobilized cells of *A. succinogenes* in alginate beads. The results of this fed-batch fermentation are presented in Figure 9.6. The feeding solution used was ultrafiltrated concentrated SSL, with a sugar concentration of about 200 g/L as in case of DCM immobilization technique. Sugar consumption started rapidly, without any lag phase. First feeding pulse was carried out after 22.5 h of fermentation and after approximately 10 h another feeding pulse was necessary. Six feeding pulses were carried out in total and 26.4 g of sugars were consumed. The final succinic acid concentration achieved was 36.8 g/L, while the total by-product concentration was around 10 g/L. Succinic acid production was almost 77% higher when the microorganism was immobilized in alginate beads, as product yield was 0.81 g/g. The yield of total by-products to succinic acid was lower than the fed-batch fermentation carried out using DCM immobilized cultures (Table 9.2). Bradfield et al. (2014) also reported low formic and acetic acid formation but high succinic acid production in a fermentation carried out in a continuous biofilm reactor, working with nearly growing cells. This state could be related to cell immobilization and the findings of this study, where mainly formic acid production is very low. Almqvist et al. (2016) also observed low formate production during the stationary phase of *A. succinogenes* fermentation. The authors also proposed that during the stationay phase, the carbon redox switches from the $C_3$ branch to the oxidative pentose phosphate pathway (OPPP). The fermentation efficiency was also improved when compared to fed-batch fermentations carried out with free cells cultivated in ultrafiltrated SSL with 3 kDa MWCO membrane (Pateraki et al., 2016b) both in terms of final succinic acid concentration and yield. The total sugar to succinic acid conversion yield was the highest (0.81 g/g) observed among the fermentations presented in Table 9.2.

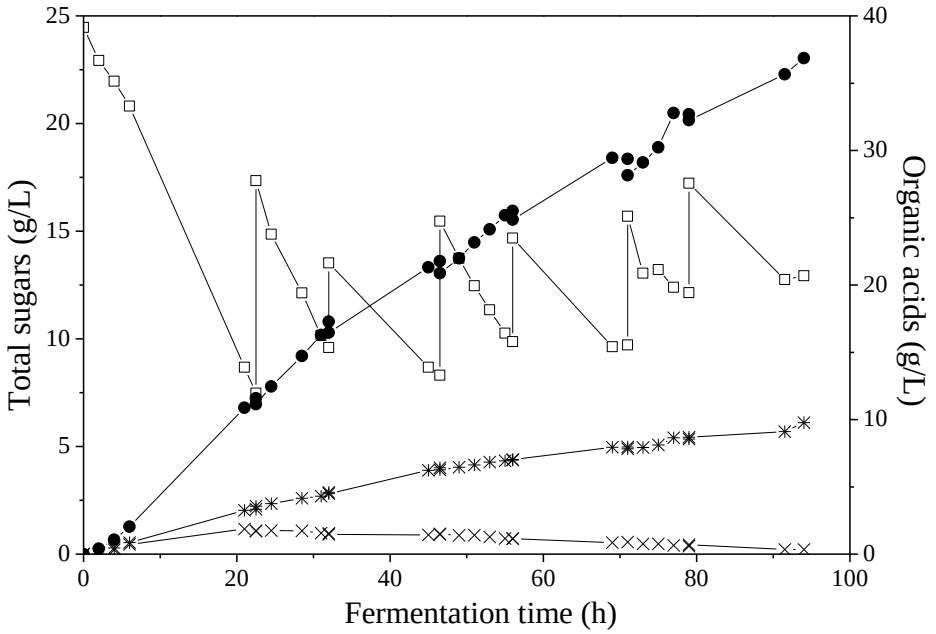



**Figure 9.6** Total sugar consumption (□) and production of succinic acid (●), formic acid (×) and acetic acid (∗) during fed-batch fermentation of ultrafiltrated SSL with entrapped *A. succinogenes* cells in alginate beads

Based on these results, it can be concluded that calcium alginates is a better immobilization support material for succinic acid production when *A. sucinogenes* is employed. The repeated batch fermentations demonstrated that cultures immobilized on alginate beads could be used in more repeated batches. As reported by Corona-González et al. (2014) in the case of immobilization of *A. succinogenes* cells in agar beads, a slight pH drop within the alginate beads, attributed to the increased proton permeability of the cytoplasmic membrane, may induce enhanced ATP consumption and increased cellular metabolism. These findings could be correlated to the results of this study and the enhanced succinic acid production when cells were immobilized in alginates instead of DCM.

### 9.4. Fermentations with nano-filtrated SSL and immobilized cells in alginate beads

Nanofiltration has been tested for succinic acid production from SSL with free cells of *A. succinogenes* and *B. succiniciproducens* and the results were promising. Actually, the fed-batch fermentation when *B. succiniciproducens* was employed, reached a final succinic acid concentration equal to 39 g/L. Taking these results into account, immobilized cultures in alginate beads using both *A. succinogenes* and *B. succiniciproducens* were evaluated in fed-batch cultures using nanofiltrated SSL. The concentration of lignosulphonates in the permeate

produced during the filtration of 7 times diluted SSL was only 5 g/L. Continuous feeding of concentrated nanofiltrated SSL with approximately 400 g/L total sugar concentration was carried out in both fed-batch fermentations. Fermentation kinetics for both strains are presented in Figure 9.7.

As when ultrafiltrated SSL was employed, no lag phase was observed when *A. succinogenes* was applied and feeding started after 23h of fermentation. *B. succiniciproducens* started consuming the sugars present in the SSL more rapidly and the consumption rate was higher than *A. succinogenes*. Feeding started only after 5.5 h of fermentation in comparison to 28 h in case of free cell fermentation. It is obvious that under identical fermentation conditions with *A. succinogenes, B. succiniciproducens* produced almost 46% more succinic acid by consuming 34% more sugars. The final succinic acid concentration was almost 45 g/L in the case of *B. succiniciproducens* and 35.4 g/L in the case of *A. succinogenes*. The yield and productivities achieved were 0.61 g/g and 0.38 g/L/h for *A. succinogenes* and 0.66 g/g and 0.58 g/L/h for *B. succiniciproducens*. Furthermore, the by-product formation was lower when *B. succiniciproducens* was used as it is observed from the ratio of individual by-products to succinic acid as well as the ratio of total by-products to succinic acid (Table 9.2). When free cells were cultivated in nanofiltrated SSL pretreated with 500 Da membrane (with lignosulphonate concentration of 4.1 g/L), the final succinic acid concentration was only 25.2 g/L for *A. succinogenes* and 33.8 g/L (Pateraki et al., 2016b). In Chapter 8, it was shown that 39 g/L of succinic acid were produced in SSL pretreated sequentially by nanofiltration and solvent extraction with *B. succiniciproducens*.

By-product formation was also lower in comparison to free cell cultures for both strains, especially in the case of *B. succiniciproducens* (Table 9.2). More specifically, in *A. succinogenes* fermentation final concentrations of formic acid and acetic acid were 3.6 g/L and 9.5 g/L, respectively. The mol/mol ratio of SA:FA:AA was 1:0.28:0.53, instead of 1:0.77:0.83 in the free cell fermentation reported by Pateraki et al. (2016b). When *B. succiniciproducens* was applied, at the end of the fermentation 6.1 g/L of lactic acid, 2.1 g/L of formic acid and 6 g/L of acetic acid were also produced. The ratio of SA:LA:FA:AA (mol/mol) was 1:0.18:0.05:0.02 instead of 1: 0.35:0.13:0.18 in case of free cells cultivated in nanofiltrated and solvent extracted SSL. It should also be mentioned that the fermentation of *B. succiniciproducens* actually finished after 141.5 h with a final succinic acid concentration of 52.4 g/L. However, these results are not presented because the productivity was very low.

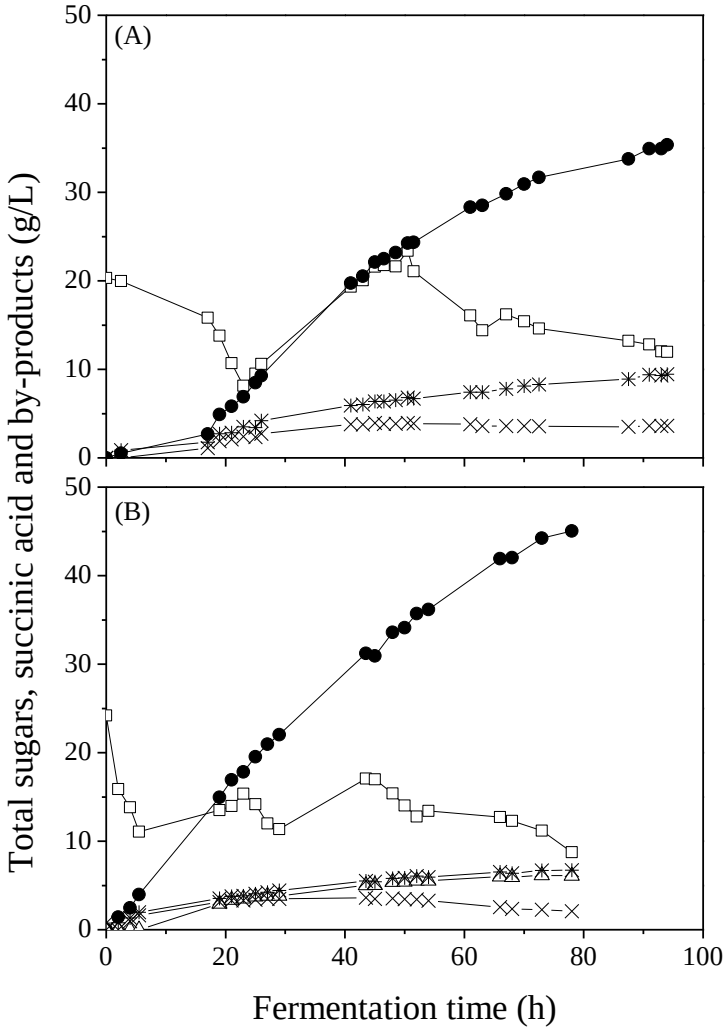



**Figure 9.7** Total sugar consumption (□) and production of succinic acid (●), lactic acid (△), formic acid (×) and acetic acid (∗) during fed-batch fermentations with continuous feeding of nano-filtrated SSL using entrapped cells of A) *A. succinogenes* and B) *B. succiniciproducens* in alginate beads

*B. succiniciproducens* is a newly isolated strain and there are a few publications available for its capability of producing succinic acid from xylose-based feedstocks. Recently, Salvachua et al. (2016) used high xylose-content hydrolysates from corn stover in free cell cultures of *B. succiniciproducens* leading to the production of 30 g/L succinic acid concentration with 0.69 g/g conversion yield and 0.43 g/L/h productivity. Among all fed-batch fermentations presented in Table 9.2, the highest succinic acid production was achieved when immobilized cells of *B. succiniciproducens* in alginate beads and nanofiltrated SSL were used. For this reason, these conditions were used for repeated fed-batch fermentations.

**Table 9.2** Experimental results of fed-batch fermentations with immobilized cells of *A. succinogenes* and *B. succiniciproducens* on DCM and alginate beads with ultra- and nano-filtrated SSL as fermentation substrate in comparison to free cells

| Bacterial strain (free cells or immobilized cultures) | Total sugar consumed (g) | Succinic acid produced (g/L) | Succinic acid yield (g SA / g TS) | Productivity (g/L/h) | LA/SA (g/g) | FA/SA (g/g) | AA/SA (g/g) | Tot/SA (g/g) | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| Fed-batch with ultrafiltrated SSL | | | | | | | | | |
| *A. succinogenes* on DCM | 18.73 | 27.3 (12.15)[a] | 0.65 | 0.32 | 0.04 (0.05)* | 0.11 (0.28)* | 0.24 (0.47)* | 0.39 (0.81)* | *This study* |
| *A. succinogenes* in alginate beads | 26.37 | 36.8 (21.30)[a] | 0.81 | 0.39 | 0.00 | 0.02 (0.05)* | 0.27 (0.53)* | 0.29 (0.58)* | |
| *A. succinogenes* free cells[c] | *n.m.*[b] | 27.4 | 0.52 | 0.39 | 0.00 | 0.15 (0.38)* | 0.26 (0.51)* | 0.41 (0.89)* | Pateraki et al., 2016b |
| Fed-batch with nanofiltrated SSL | | | | | | | | | |
| *A. succinogenes* in alginate beads | 38.71 | 35.4 (23.42)[a] | 0.61 | 0.38 | 0.00 | 0.11 (0.28)* | 0.27 (0.53)* | 0.38 (0.81)* | *This study* |
| *A. succinogenes* free cells[d] | *n.m.*[b] | 25.2 | 0.57 | 0.47 | 0.00 | 0.30 (0.77)* | 0.42 (0.83)* | 0.72 (1.60)* | Pateraki et al., 2016b |
| *B. succiniciproducens* in alginate beads | 51.79 | 45.0 (34.17)[a] | 0.66 | 0.58 | 0.14 (0.18)* | 0.02 (0.05)* | 0.01 (0.02)* | 0.17 (0.25)* | *This study* |
| *B. succiniciproducens* free cells[d] | *n.m* [b] | 33.8 | 0.58 | 0.48 | 0.35 (0.46)* | 0.12 (0.31)* | 0.26 (0.52)* | 0.74 (1.29)* | Pateraki et al., 2016b |
| *B. succiniciproducens* free cells[e] | *n.m* [b] | 39 | 0.54 | 0.31 | 0.27 (0.35)* | 0.05 (0.13)* | 0.09 (0.18)* | 0.41 (0.66)* | *This study* |

*mol/mol ratio
[a] total grams of succinic acid produced
[b] not mentioned
[c] ultrafiltration of SSL was carried out with 3 kDa membrane
[d] nanofiltration of SSL was carried out with 500 Da membrane
[e] nanofiltration of SSL was carried out with 500 Da membrane and phenolic compounds were removed with ethyl-acetate

### 9.5. Repeated fed-batch fermentation

The aim of the repeated fed-batch fermentation was to evaluate the re-use of the *B. succiniciproducens* immobilized biocatalyst in fed-batch fermentation mode. Thus, the immobilized cells were re-used in 4 sequential fed-batch fermentations with nanofiltrated SSL as substrate (9.8). The initial sugar concentration was about 20 g/L in each fermentation and the feeding solution used was concentrated nanofiltrated SSL, with approximately 400 g/L total sugar concentration. Initially, the immobilized cells were re-used after achieving a final succinic acid concentration of 45 g/L in the first fed-batch run, but the yield and productivity decreased significantly in subsequent fed-batch runs. For this reason, the cycle of five fed-batch fermentations was repeated with the first fed-batch run terminated when the final succinic acid concentration reached 39 g/L (9.8) in order to maintain the viability of the cells. Succinic acid yield was 0.51 g/g and the productivity was 0.65 g/L/h. In the second fed-batch run, the succinic acid production efficiency was reduced. After 50.5 h in the second fed-batch run, 23.3 g/L of succinic acid concentration with yield of 0.67 g/g and productivity of 0.46 g/L/h were achieved. The alginate beads were then filtrated and washed carefully with sterilized water and new medium was pumped into the reactor. The immobilized cells were re-used for a third fed-batch fermentation that led to the production of 16.9 g/L of succinic acid. Both yield (0.54 g/g) and productivity (0.37 g/L/h) decreased. Nevertheless, the immobilized microorganism produced a higher succinic acid concentration (20.1 g/L), but even lower yield (0.42 g/g) and productivity (0.29 g/L/h) in a fourth fed-batch fermentation. It should be stressed that 5.8 g/L sugars (mainly xylose and galactose) were not consumed. The same observation was also made by Corona-González et al. (2014), in repeated batch fermentations with *A. succinogenes* immobilized in agar.

The ratio of total by-products to succinic acid slightly increased from cycle to cycle. The ratio of lactic acid to succinic acid increased from 0.35 to 0.50, acetic acid to succinic acid from 0.18 to 0.24, whereas the ratio of formic acid to succinic acid decreased from 0.11 to 0.06. Regarding their final concentration in each fed-batch, 13.6 g/L of lactic acid, 4.1 g/L of formic acid and 6.9 g/L of acetic acid were produced during the first fed-batch run. During the second fed-batch, 9.4 g/L, 1.1 g/L and 4.9 g/L were produced of lactic, formic and acetic acids, respectively. In the third and fourth run, 6.4 g/L and 9.7 g/L of lactic acid were produced, 1.7 g/L and 1.2 g/L of formic acid and finally, 3.9 g/L and 4.6 g/L of acetic acid. Formic acid concentration decreased significantly from the first to the second run and remained constant to subsequent experiments. Acetic acid had a decreasing tendency until the

third fed-batch and increased again in the last one. Same tendency was also observed for lactic acid, the main by-product in each fermentation.

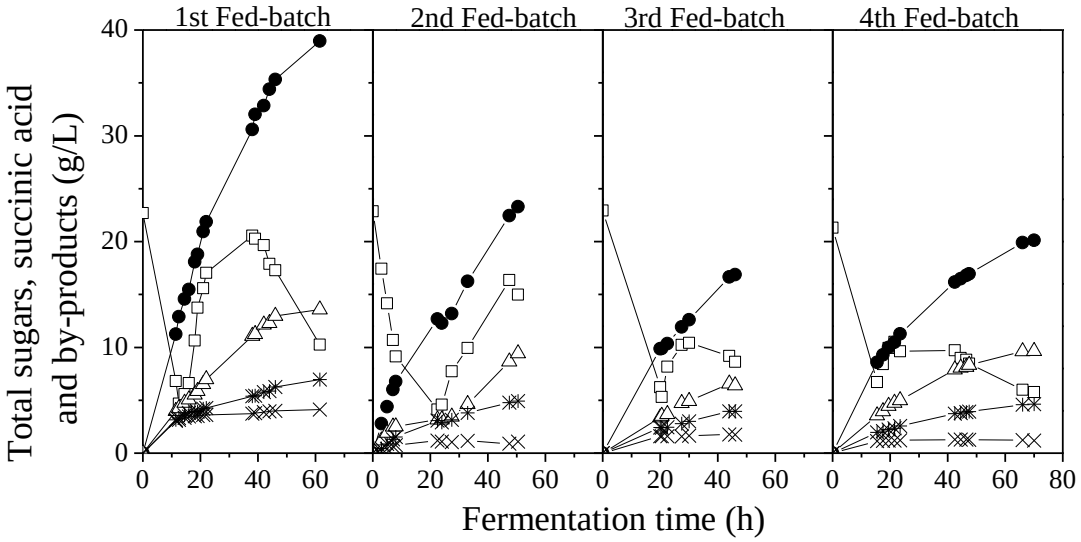



**Figure 9.8** Total sugar consumption (□) and production of succinic acid (●), lactic acid (△), formic acid (×) and acetic acid (∗) during four repeated fed-batch fermentations with entrapped *B. succiniciproducens* cells in alginate beads and nano-filtrated SSL as fermentation substrate

These results indicate that immobilization in alginate beads is an effective technique, as they were successfully re-used in four sequential fed-batch fermentations, leading to a total succinic acid production of 64.7 g out of 114.55 g consumed sugars. After the fourth cycle, the rupture of the beads was significant and this could explain the reduction on both yield and productivity. The initial size of the beads also affects the number of sequential fermentations that could be conducted. During each fermentation, bead size increases due to growth and after several cycles rupture is observed as a consequence of both cell growth and erosions caused by calcium discharge (Mishra et al., 2015). Mishra et al. (2015) studied the effect of bead size on ethanol production showing that medium and large size beads (diameter of 4-5 mm) could be re-used for more fermentation cycles than smaller beads (diameter of ~ 3 mm). Mishra et al. (2015) also claimed that rupture of smaller beads is faster because they tend to attach with each other due to the larger surface area. Moreover, calcium discharge occurs faster as a result of better mass transfer.

## 9.6. Conclusions

Immobilization of *B. succiniciproducens* led to the production of 45 g/L of succinic acid, which is the highest concentration achieved by this strain when cultivated on xylose-based hydrolysates. With the use of alginate beads, higher cell retention is achieved and the diffusion of nutrients is sufficient, yielding to high succinic acid concentrations in comparison to free cell cultures. The fact that the same biocatalyst can be used in repeated batch and fed-batch fermentations leads to the conclusion that this immobilization support could be used in large scale production of succinic acid as long as process optimization is carried out.

*The results presented in Chapter 9 have been published in Bioresource Technology:*

Alexandri M., Papapostolou H., Stragier L., Verstraete W., Papanikolaou S., Koutinas AA. (2017). Succinic acid production by immobilized cultures using spent sulphite liquor as fermentation medium. Bioresour Technol, 238: 214-222

# CHAPTER 10
# REFINING OF SBP FOR THE PRODUCTION OF VALUE-ADDED PRODUCTS AND SUGAR-RICH HYDROLYSATE

## 10.1 Introduction

Biomass refining involves the valorisation of a given raw material via its fractionation and bioconversion to value-added products such as fuels, chemicals, polymers or active ingredients (Koutinas et al., 2014). The corn wet milling industry as well as petroleum refineries constitute two profound examples of thorough exploitation of both technology flexibility and feedstock utilization efficiency (Kachrimanidou et al., 2015). The development of integrated biorefineries, based on the exploitation of wastes and by-products for the production of value-added products, would maximize profit, decreasing at the same time the dependence on petroleum-based commodities (Kachrimanidou et al., 2015). The sustainability of the process would be enhanced significantly if the production of chemicals from renewable resources is coupled with the extraction of value-added products (e.g. antioxidants, proteins, pectins) that would contribute to the overall economic viability of the process (Koutinas et al., 2014).

Sugar beet constitutes one of the most important crops, as it is number seven commodity worldwide, with annual production of about 270 million tonnes. Germany is one the most important producers of sugar beet in Europe, with annual production of 28 million tonnes, constituting 10% of worldwide production (Faostat, 2014). Sugar beet pulp (SBP) is the main by-product of the sugar production industry in Europe, resulting after sucrose extraction from the crop. It is estimated that its annual production accounts for approximately 5 million tonnes (on a dry basis) in Europe and about 1 million tonnes in the USA (Ziemínski et al., 2014). The current practice involves drying and pelletizing the pulp in a cost intensive process. The dried SBP is then sold as low-value animal feed. However, the further exploitation of SBP would reduce both cost and waste and at the same time would add value to the crop. SBP is rich in structural cell wall polymers such as cellulose (20-25%), hemicellulose (25-36%), pectins (20-25%), proteins (10-15%) and lignin (1-2%) (Bellido et al., 2015). Its high carbohydrate content, together with its low lignin concentration, renders SBP an appealing raw material for bioconversions. There are many studies focusing on the extraction of pectins from SBP, as it is already shown that they possess high emulsifying properties (Ma et al., 2013; Sun and Hughes, 1998; Olmos and Hansen, 2012). SBP has been used as feedstock mainly for bioethanol production (Berłowska et al. 2016; Salazar-Ordóñez

et al., 2013; Zheng et al., 2013), but also as raw material for the biotechnological production of acetone-butanol-ethanol (Bellido et al., 2015) and single cell protein (Labor et al., 1992), as well as for ferulic acid extraction (Aarabi et al., 2016) and anaerobic digestion (Koppar and Pullammanappallil, 2008). There are also many studies dealing with the hydrolysis of cellulose and hemicellulose, in order to obtain hydrolysates rich in monosaccharides that could be easily converted from the microorganisms to value-added fermentation products (Micard et al., 1996; Chamy et al., 1994; Kharina et al., 2016; Hamley-Bennet et al., 2016).

In this chapter, fractionation of SBP is initially carried out in order to produce value-added co-products. More specifically, phenolic compounds are extracted and analyzed in order to estimate their potential applications. Subsequently, pectins are precipitated from SBP, as they are also considered a potential co- product. The remaining solids are rich in cellulose and hemicellulose that could be hydrolysed by means of acid and enzymatic pretreatment, aiming to produce a sugar-rich hydrolysate that could be used as substrate for the microbial production of succinic acid.

## 10.2. Evaluation of the phenolic extract: TPC, antioxidant activity and determination of the major phenolic compounds

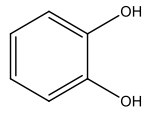
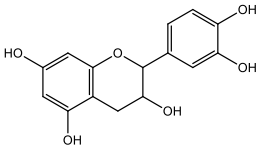
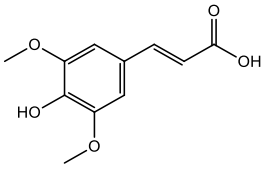
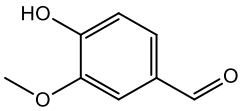
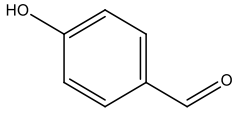
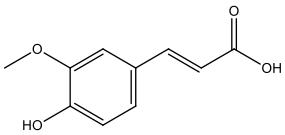
Two different sample-to-solvent ratios were studied for the extraction of the free phenolic compounds from the SBP. The extracting solvent was 60% aqueous ethanol solution. Ethanol is considered a “green solvent” and leads to satisfying results regarding phenolic extraction from plant matrices. When 1:25 ratio was tested, the TPC was 1.63 ± 0.09 mg GAE/g SBP, whereas when 1:12.5 ratio was evaluated, TPC was 0.88 ± 0.17 mg GAE/g SBP. The antioxidant activity of the extracts was also estimated using the DPPH radical. The radical scavenging activity (I%) of the extract using 1:25 and 1:12.5 ratios were 49% and 36%, respectively. The AAI of the extracts were 0.5 and 0.35, which categorises them as poor antioxidants. Since it is a crude plant extract, further purification using various methods such as chromatography, would improve its antioxidant activity.

The TPC results are in accordance with the study of Aarabi et al. (2015), where the methanolic extract of SBP had TPC values in the range of 1.28-2.55 mg GAE/ g SBP, depending on the reaction duration. Monhaly et al., (2013) also reported a TPC of 1.52 mg GAE/ g SBP when ethanol was the extracting solvent, whereas when methanol was employed the TPC was slightly higher (1.79 mg GAE/g). The authors also measured the TPC of two other agro-industrial by-products: potato peels and sesame cake. Among them, the highest

TPC was observed in potato peels ethanolic extract (2.74 mg GAE/g) followed by sugar beet pulp ethanolic extract, while sesame cake extract had the lowest value (0.55 mg GAE/g). Regarding the antioxidant activity of the extracts, when 200 ppm concentration was tested, the radical scavenging activity of the sugar beet pulp ethanolic extract was 42%, lower than the antioxidant activity of potato peels and sesame meal extracts (66% and 46%, respectively).

The individual phenolic compounds were also determined by means of HPLC coupled with a diode-array detector. There were 14 different peaks in the chromatogram, most of them having absorption maxima at 280 nm and six of them at about 320 nm. Among these compounds, it was possible to identify six of them (Table 10.1). The main compound was catechol, having a concentration of 640.5 ppm in the extract, followed by epicatechin with a concentration of 118.4 ppm. Sinapinic acid (51 ppm), vanillin (29.9 ppm), 4-hydroxybenzaldehyde (28.7 ppm) and ferulic acid (20.9 ppm) were also detected in the extract (Table 10.1). Catechol (1,2-dihydroxybenene) is a well-known phytochemical that is commonly found in plants, fruits and vegetables and it is synthesized through the shikimate pathway (Huang et al., 2014). Ferulic acid is a natural antioxidant that occurs in many cereals and it is also abundant in sugar beets (Ou and Sun, 2014; Aarabi et al., 2016). Ferulic acid is mainly bound to polysaccharides forming feruloylated oligosaccharides. Epicatechin constitutes the *cis*-diastereoisomer of catechin, belonging to the group of flavan-3-ols, which are part of the chemical group of flavonoids. This flavonoid naturally occurs in many vascular plants, but it is mainly found in green tea extracts, chocolate beans and wine (Tan et al., 2007). Its antioxidant effects have been studied intensively and it is shown to have numerous benefits in human health (Tan et al., 2007; Chen et al., 2015). Epicatechin was detected in sugar beet molasses in the study of Chen et al. (2015), together with gallic acid and cyaniding-3-*O*-glucoside.

**Table 10.1** Main phenolic compounds identified in the SBP ethanolic extract and their structures.

| Phenolic compound | Concentration (ppm) | Structure |
|---|---|---|
| Catechol | 640.5 |  |
| Epicatechin | 118.4 |  |
| Sinapinic acid | 51 |  |
| Vanillin | 29.9 |  |
| 4-hydroxybenzaldehyde | 28.7 |  |
| Ferulic acid | 20.9 |  |

## 10.3. Pretreatment of SBP

### 10.3.1. Acid pretreatment

The main components of SBP are cellulose (23.0 ± 3.3%), hemicellulose (19.5 ± 6.2%), pectins (30.3 ± 2.1%) and proteins (9.6 ± 0.6%). The very low lignin content (2.6 ± 0.4%) renders SBP an ideal substrate for microbial conversion, as no severe pretreatment techniques for the removal of lignin are required. In order to obtain a sugar-rich stream that could be further valorised for the biotechnological production of succinic acid, hemicellulose and cellulose should be degraded to their respective monosaccharides.

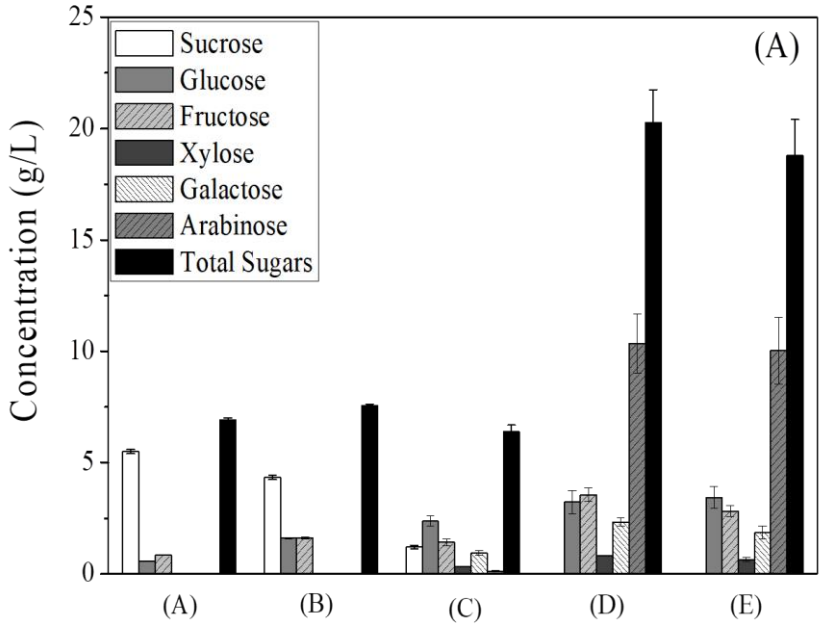
In the case of hemicellulose degradation, two kind of hydrolyses were evaluated: autohydrolysis and dilute acid treatment. Autohydrolysis is a green method, where only the raw material and water are present in the reaction. The advantages of this method over dilute acid pretreatment are mainly the low formation of inhibitory compounds (such as furfural, HMF and acetic acid), the low lignin solubilisation, the low cost as well as the low

environmental impact (Carvalheiro et al., 2005). The disadvantage of this method is that the derived liquors are not highly fermentable by microorganisms, since they are mainly composed by sugar oligomers rather than sugar monomers (Carvalheiro et al., 2005). Dilute acid pretreatment could also be performed at low cost, as it does not require specific equipment and it is effective in short reaction times (Taherzadeh and Karimi 2007). This pretreatment method is widely used in order to obtain hemicellulosic hydrolysates and increase the enzymatic digestion of cellulose (Chandel et al. 2011). Moreover, some studies have shown (Kumar et al., 2009; Pappas et al., 2014) that dilute acid pretreatment leads to structural alterations, such as hemicellulose and lignin degradation. It also increases cell wall porosity, thus assisting the enzymatic accessibility and digestion of cellulose. Furthermore, hemicellulose degradation is a key factor for the subsequent enzymatic digestion of cellulose, as the presence of hemicellulose blocks the access of the hydrolytic enzymes (Moxley et al., 2012).

Pappas et al. (2014) indicated that in most cases, the yield of enzymatic hydrolysis of cellulose is fast at the beginning of the reaction, following a logarithmic type that lasts for about 24 h. After that time, the glucose release rate decreases significantly. Mussato et al. (2008) suggested that, at the beginning of the reaction, the enzymes hydrolyse firstly the amorphous fraction, whereas the crystalline fraction is more difficult to be degraded.

Initially, hemicellulose degradation was evaluated using four different methods: autohydrolysis, acidification at pH 4 and dilute acid pretreatment with 0.5% $H_2SO_4$ or HCl. These methods were tested at 6% solid loading and at 121 $^oC$ for 15 min. Pretreatment was applied both on pellets and on ground SBP, in order to assess the need for mechanical pretreatment of the pulp. A suspension of 6% solids was also employed as control and the results are presented at Figure 10.1. A total sugar concentration of 6.92 g/L that was mainly consisted of sucrose (5.49 g/L), glucose (0.58 g/L) and fructose (0.85 g/L) was detected in the control sample. Autohydrolysis had no effect on hemicellulose degradation. In both cases (pellets and ground SBP), the sugars derived from hemicellulose breakdown (xylose, galactose or arabinose) were not detected. Acidification presented a low impact on hemicellulose hydrolysis, since low concentrations of xylose (0.33 g/L), galactose (0.93 g/L) and arabinose (0.12 g/L) were found in the liquor. When dilute acid treatment was applied either with $H_2SO_4$ or HCl, total sugar concentrations in the liquor reached values of 20.57 g/L and 18.79 g/L, respectively. The main monosaccharide derived from hemicellulose degradation was arabinose with a concentration of around 10 g/L when both acids were used. Besides arabinose, galactose was also present after pretreatment (about 2 g/L). Low

concentrations of xylose were also present in the hydrolysates, when either $H_2SO_4$ (0.82 g/L) or HCl (0.65 g/L) treatment was used. The acidic hydrolysates did not contain any sucrose, as it was completely hydrolysed to glucose and fructose (Figure 10.1A). Similar results were obtained when ground SBP was used for the hydrolysis, indicating that there is no need for further mechanical pretreatment (Figure 10.1B).



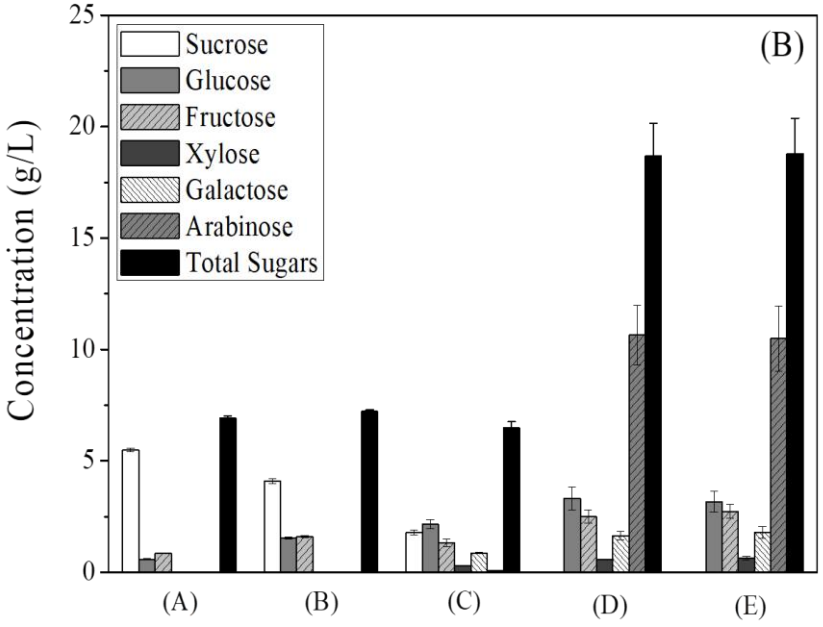



**Figure 10.1** Evaluation of hemicellulose hydrolysis expressed by the release of C5 and C6 sugars at different pretreatment conditions using (A) pellets and (B) ground SBP. On y axis: (A) control; (B) autohydrolysis; (C) autohydrolysis with acidification at pH 4; (D) pretreatment with 0.5% (v/v) $H_2SO_4$; (E) pretreatment with 0.5% (v/v) HCl

The highest conversion yield- together with the lowest by-product formation- was achieved when 0.5% $H_2SO_4$ was applied. More specifically, when HCl was used for the treatment, acetic acid, HMF and furfural were detected in the produced liquors at concentrations of 2.3 g/L, 295.9 mg/L and 20.3 mg/L, respectively. On the other hand, when $H_2SO_4$ was employed, all inhibitory by-products mentioned above were identified at lower concentrations (1.6 g/L, 152.4 mg/L and 11.7 mg/L, respectively). The same profile was observed when ground SBP was used for the hydrolysis. HMF and furfural were found at concentrations of 274.6 mg/L and 19.7 mg/L, in the case of HCl treatment, and 167.5 mg/L and 14 mg/L, in the case of dilute acid hydrolysis with $H_2SO_4$. Acetic acid concentrations were 2.6 g/L and 2 g/L when HCl and $H_2SO_4$ treatment was employed, respectively.

In order to identify the optimum conditions that will lead to the highest possible sugar release from hemicellulose, three factors were evaluated, namely: solid loading, hydrolysis time and initial acid concentration. Table 10.2 presents the results, when four different initial solid loadings (6, 7.5, 10 and 15%) were applied at two different hydrolysis times (15 min and 30 min). Increasing the amount of the raw material during hydrolysis (15 min), led to higher sugar concentrations in the hydrolysate. In all cases, the main sugar monomer was arabinose, with concentrations varying from 10.58- 23.23 g/L, for solid loadings from 6%- 15%. The concentrations of galactose and xylose did not differ much among the different samples, with an average concentration of 2.52 g/L and 0.77 g/L, respectively. Glucose and fructose were also present as a result of sucrose hydrolysis during the process. The conversion yield, accounting only for the sugars derived from hemicellulose degradation, shows that the highest value is obtained when 7.5% solids are used (80.5%), followed by 6% solids (79.6%). More specifically, when 7.5% solids were treated, 23.5 g of total sugars/ 100 g dry SBP were produced, whereas the lowest amount of sugars (11.3 g/ 100 g) was observed when 15% solids were used. The by-products formation was also monitored in all cases and the results are presented in Figure 10.2. The main by-product was acetic acid, with a concentration proportionate to solid loading, followed by HMF and furfural. The hydrolysate from the hydrolysis of 15% SBP contained the highest concentration of acetic acid (3.06 g/L). On the other hand, the highest concentration of HMF and furfural was found in the 10% solids hydrolysate, with values of 437.95 mg/L and 25.75 mg/L, respectively (Figure 10.2A).

When the hydrolysis time increased to 30 min, the highest total sugar concentration was accomplished when 10% solids were used and was equal to 37.02 g/L or 21.2 g sugars/ 100 g SBP. Arabinose was again the main monosaccharide, with a concentration of 24.36 g/L (13.95 g/100 g SBP), followed by galactose (1.63 g/100g SBP) and xylose (0.40g/ 100 g SBP). However, the highest biomass conversion was again achieved at 7.5% solids (26.38 g sugars/100 g SBP), followed by 6% solids (25.81 g sugars/ 100 g SBP). The lowest sugar release was observed when 15% solids were hydrolysed (approximately 9.04 g total sugars/100 g SBP). According to these results, it is obvious that among the different solid loadings the less effective one is 15%. Analysis of the inhibitory compounds indicates a similar profile as in case of 15 min reaction time. Acetic acid was the main by-product formed in all the hydrolysates and its highest concentration was observed at 15% solid loading. HMF had the same concentration (~ 437 mg/L) in both 10% and 15% solids, whereas the highest concentration of furfural was monitored at 10% solids and was equal to 44.22 mg/L (Figure 10.2B).

**Table 10.2** Concentrations (g/L) of the individual sugars obtained after acid pretreatment

| Sample | Sucrose | Glucose | Xylose | Galactose | Arabinose | Fructose | Total sugars |
|---|---|---|---|---|---|---|---|
| ***Blank samples*** | | | | | | | |
| 6 % | 5.49 ± 0.09 | 0.58 ± 0.02 | - | - | - | 0.85 ± 0.01 | 6.92 ± 0.09 |
| 7.5 % | 7.18 ± 0.02 | 0.79 ± 0.05 | - | - | - | 1.15 ± 0.02 | 9.12 ± 0.06 |
| 10 % | 7.21 ± 0.12 | 0.78 ± 0.03 | - | - | - | 1.13 ± 0.02 | 9.12 ± 0.13 |
| 15 % | 11.69 ± 0.06 | 1.53 ± 0.03 | - | - | - | 1.94 ± 0.05 | 15.16 ± 0.09 |
| ***15 min acid pretreatment*** | | | | | | | |
| 6 % | - | 3.28 ± 0.34 | 0.82 ± 0.05 | 2.33 ± 0.08 | 10.58 ± 0.49 | 3.56 ± 0.36 | 20.57 ±0.70 |
| 7.5 % | - | 4.39 ± 0.52 | 0.68 ± 0.02 | 2.91 ± 0.18 | 14.09 ± 1.33 | 4.75 ± 0.30 | 26.82 ±1.47 |
| 10 % | - | 4.38 ± 0.73 | 0.78 ± 0.09 | 2.64 ± 0.06 | 18.21 ± 2.16 | 4.73 ± 0.01 | 30.74 ± 2.28 |
| 15 % | - | 7.35 ± 0.88 | 0.78 ± 0.02 | 2.21 ± 0.10 | 23.23 ± 1.11 | 7.75 ± 1.69 | 41.32 ± 2.21 |
| ***30 min acid pretreatment*** | | | | | | | |
| 6 % | - | 3.85 ± 0.02 | 0.72 ± 0.02 | 3.16 ± 0.50 | 11.36 ± 0.21 | 3.04 ± 0.07 | 22.13 ± 0.55 |
| 7.5 % | - | 5.12 ± 0.52 | 0.75 ± 0.04 | 3.48 ± 0.67 | 15.13 ± 1.80 | 4.33 ± 0.30 | 28.81 ± 2.01 |
| 10 % | - | 4.38 ± 2.57 | 0.71 ± 0.05 | 2.84 ± 0.28 | 24.36 ± 1.83 | 4.73 ± 2.67 | 37.02 ± 4.14 |
| 15 % | - | 6.11 ± 0.87 | 0.74 ± 0.06 | 3.32 ± 0.10 | 17.61 ± 3.72 | 5.36 ± 1.69 | 33.14 ± 4.18 |

These results are in accordance to the study of Bellido et al. (2015). Taking into account that the raw material used had higher hemicellulose content than the one used in this study (33.7%), the authors obtained 5.1 g of glucose, 5.4 g of fructose and 15.8 g or arabinose per gram of SBP. Gunan-Yucel et al. (2015) also employed acid hydrolysis with $H_2SO_4$ on SBP resulting in 45.3 g/L sugars, 3.8 g/L total furan compounds and 2.9 g/L of phenolic compounds.

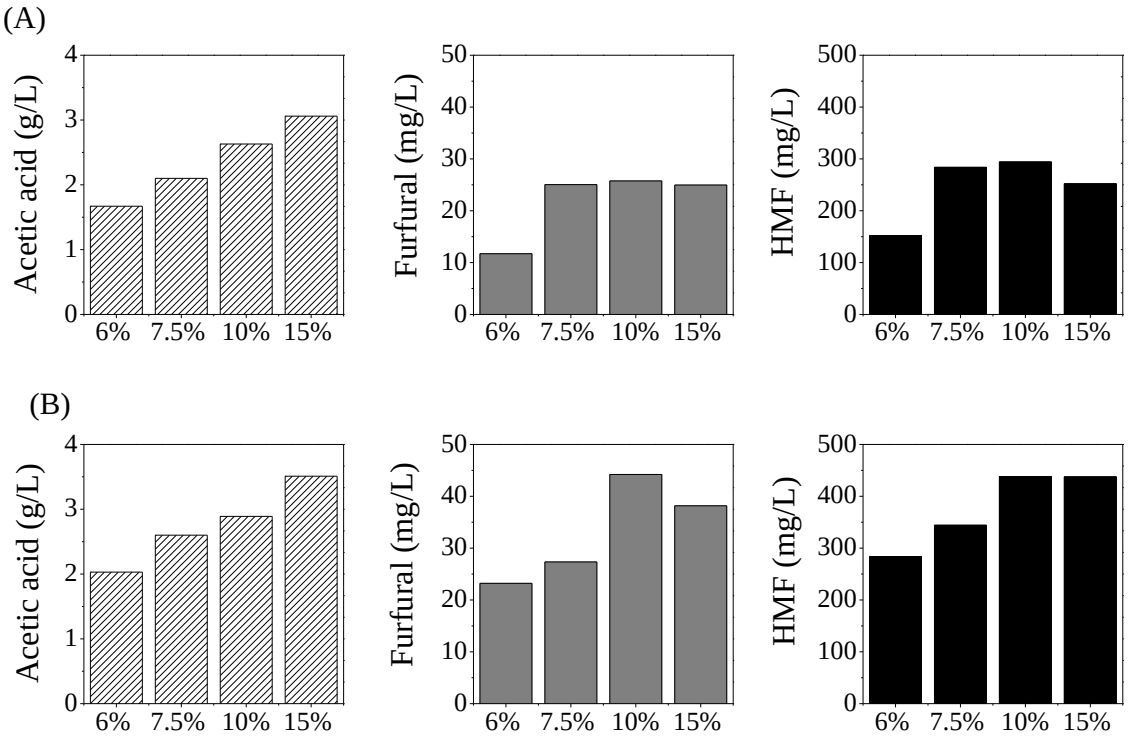



**Figure 10.2** By-product formation during acid pretreatment with 0.5% $H_2SO_4$ at 121 $^oC$, for (A) 15 min and (B) 30 min

Finally, 1% $H_2SO_4$ was tested at 30 min reaction time and at solid loadings of 6, 7.5 and 10%. Fifteen per cent solids were excluded since they resulted to the poorest degree of hydrolysis in the previous experiments. Figure 10.3 presents the concentration of monosaccharides as well as the concentration of total sugars acquired, in 1% $H_2SO_4$. For 6%, 7.5% and 10% solid loadings, final sugar concentration was 18.67 g/L (21.78 g/100g), 24.34 g/L (22.29 g/100 g) and 27.87 g/L (15.96 g/100 g) respectively, values that are lower in comparison to the treatment with 0.5% $H_2SO_4$.

Based on these results, the optimum conditions for the hemicellulose degradation in SBP are the dilute acid pretreatment with 0.5% $H_2SO_4$, at 121 $^oC$ for 30 min. This result could be explained by the fact that when the acid pretreatment method is more severe, sugar degradation could also occur, leading to poor hydrolysis yield (Bösch et al., 2010).

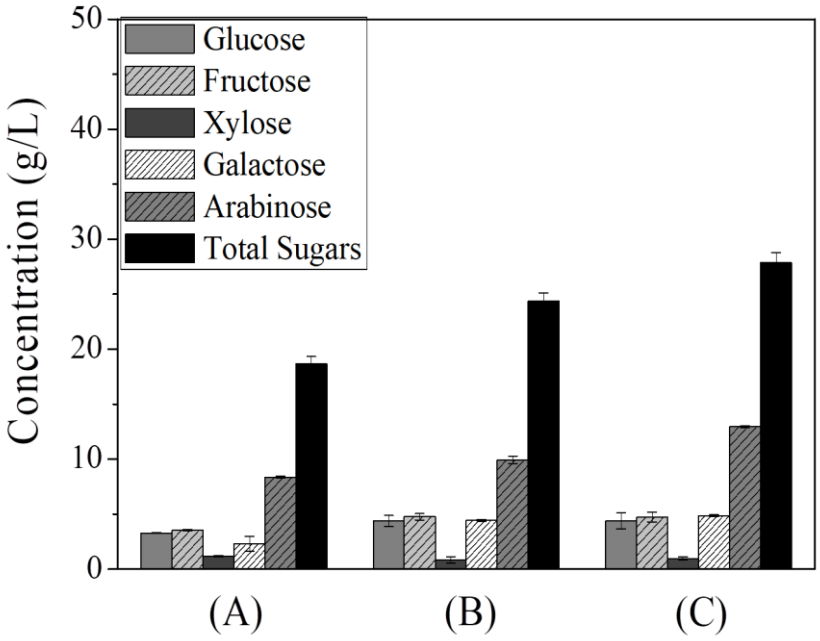



**Figure 10.3** Evaluation of hemicellulose hydrolysis using 1% $H_2SO_4$ at 121 $^oC$ for 30 min, with solid loadings of (A) 6%; (B) 7.5% and (C) 10%

### 10.3.2. Enzymatic hydrolysis

As it is already stated, the enzymatic degradation of cellulose is directly related to the pretreatment methods as both lignin and hemicellulose affect the digestibility. The commercial enzyme preparation Accellerase 1500 was tested for the cellulose degradation in SBP. Firstly, the lowest dosage (0.1 mL per gram of cellulose) was studied, using different solid-to-liquid ratios, after the acid hydrolysis (Figure 10.4). For both 15 and 30 min of acid hydrolysis, the highest final glucose concentration was achieved when 10% solids were utilised, with values of 8.9 g/L and 9.6 g/L, respectively. The poor hydrolysis of hemicellulose, when 15% solids were used led also to poor cellulose degradation. After 15 min acid hydrolysis, the subsequent enzymatic hydrolysis resulted to the production of very low concentration of glucose (under the detection limit of the HPLC) or no production at all. Employing 30 min hydrolysis, some glucose was detected, but it was the lowest value observed from all the different cases (1.76 g/L).

Cellulose hydrolysis yield reached 31.1% when 6% solids were pretreated with $H_2SO_4$ for 15 min. The second highest value was 25.8% from 7.5% solids, under the same acid hydrolysis conditions. When 10% solids were applied, a slightly highest hydrolysis yield (23.7%) was achieved when the acid hydrolysis step duration was 30 min. Similar results

were reported by Bellido et al. (2015), who obtained a higher glucose concentration in 10% solids but cellulose degradation was higher when 5% and 7.5% solids were hydrolysed.

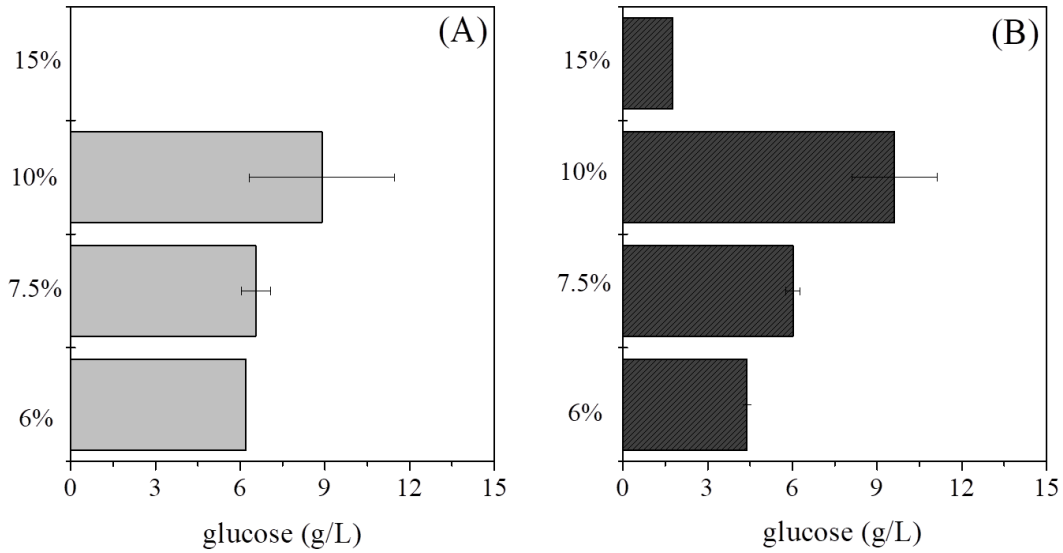



**Figure 10.4** Glucose release from cellulose hydrolysis using 0.1 mL/g cellulose of Accellerase 1500 in acid pretreated SBP for (A) 15 min and (B) 30 min using different solid ratios

Since the produced hydrolysate will subsequently be used as fermentation substrate, the combination of hydrolysis that leads to the highest final sugar concentration would be selected for further experiments. Acid pretreatment of 10% solids with 0.5% $H_2SO_4$ for 30 min with subsequent enzymatic hydrolysis produces 46.6 g/L of total reducing sugars (or 26.7 g of total sugars per 100 g of SBP), with a total hydrolysis yield of 62.3%.

The highest total hydrolysis yield (74.4%) was achieved when 7.5% solids were treated for 30 min with 0.5% $H_2SO_4$, followed by enzymatic degradation of cellulose. The lowest value was observed when 15% solids were treated either for 15 or 30 min, with a total hydrolysis yield equal to 26.1% and 22.2%, respectively.

### 10.3.3. Bioreactor hydrolysis

Hydrolyses in 10 L bioreactors were subsequently carried out, aiming to produce enough hydrolysate for microbial fermentation. Solid loading of 10% treated for 30 min with 0.5% $H_2SO_4$ at 121$^{o}$C was selected as it led to a final sugar concentration of more than 40 g/L. Since 0.1 mL/g of Accellerase 1500 was already tested, a higher enzyme loading was selected

in order to improve the cellulose hydrolysis yield of the substrate. The enzyme dosage was 0.5 mL/g of cellulose and the results are presented in Table 10.3 The total sugar concentration reached a value of 43.6 g/L. The main sugars were glucose (19.2 g/L) and arabinose (15.8 g/L). Increasing the dosage loading of Accellerase 1500, the yield of cellulose conversion to glucose increased from 23.7% to 53.9%. ADF, NDF and ADL analysis of the residual solids showed that after both acidic and enzymatic treatment, the cellulose content was 5.5%, hemicellulose content was 7.9% and lignin was 1.7%. According to this analysis, cellulose degradation occurred at a yield of 95% however the cellulose to glucose yield was only 36.6%. This result is attributed to the fact that during cellulose hydrolysis disaccharides like cellobiose are also produced. The residue was also analyzed for its protein content and results showed a quantity of 20% indicating that the hydrolysis residue could be an alternative nitrogen source.

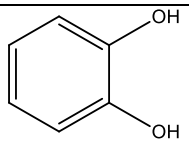
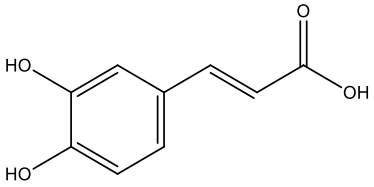
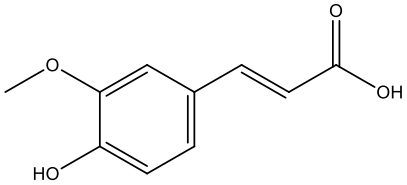
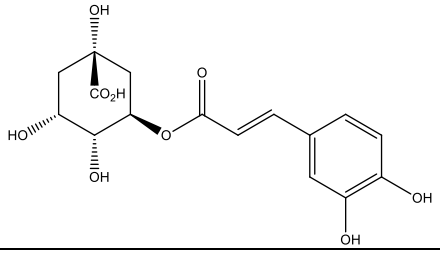
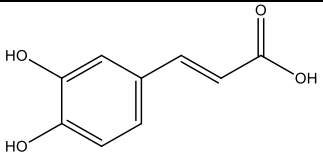
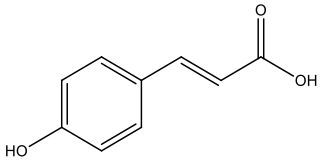
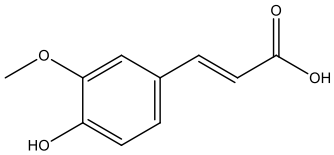
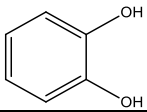
Inhibitor formation was similar to the results obtained from shake flask experiments. More specifically, the final concentrations of HMF, furfural and acetic acid in the hydrolysate were 442.5 mg/L, 55.5 mg/L and 4.1 g/L respectively. Analysis of the individual phenolic compounds was also carried out as phenolics can act as antioxidants and can highly inhibit microbial growth (Alexandri et al., 2016a). During hydrolysis, more phenolic compounds (bound or esterified) are released, even if solvent extraction was applied before. The spectrum obtained from the hydrolysate after acidic hydrolysis was quite different than the extract (Table 10.4). Caffeic and chlorogenic acid were identified in the hydrolysate at concentrations of 211.7 ppm and 215.9 ppm. Ferulic acid and catechol were also present, having concentrations of 178.1 ppm and 511.4 ppm. After the enzymatic hydrolysis, the concentrations of both caffeic and ferulic acid were slightly increased to 221.5 ppm and 207.3 ppm, respectively, whilst p-coumaric was also identified (237.5 ppm). Ferulic acid is reported to be present in high amounts in SBP and it is also a component of some hemicelluloses (Wyman et al. 2004). As already mentioned, ferulic acid is mainly present in form of feruloylated oligosaccharides. The mild acid hydrolysis of plant cell walls or the enzymatic treatment could release ferulic acid in the hydrolysate (Nacz and Shahidi, 2004). In sugar beet, this phenolic compound has been found to be linked via ester bonds at C-2 and C-6 positions to arabinofuranose and galactopyranose respectively. More specifically, according to the studies of de O. Buanafina (2009), almost 45-50% of ferulic acid is esterified to galactose, while 50-55% is bound to arabinose. The presence of *p*-coumaric acid is mainly related to lignin, but it can be also connected to polysaccharides (Scheller and Ulvskov, 2010). The most common monosaccharides linked to ferulic and *p*-coumaric acids are xylose

and arabinose, even though they can be also bound to galactose and glucose but in lesser extent (Ou and Sun, 2014). Levigne et al., (2004) found that in sugar beet cell walls, ferulic acid is esterified with two arabinose moieties at positions *O*-5 and *O*-2. Both compounds are well-known antioxidants, with various applications in food industry, cosmetics and pharmaceuticals (Ou and Sun, 2014).

**Table 10.3** Compositional analysis of the SBP hydrolysate obtained after acid and enzymatic hydrolysis using 10% solids, 0.5% $H_2SO_4$ at 121 $^oC$ for 30 min and 0.5 mL Accellerase 1500/g cellulose. Hemicellulose and cellulose hydrolysis yields are also presented

| **Component** | **Concentration (g/L)** |
|---|---|
| Glucose | 19.2 |
| Xylose | 0.9 |
| Galactose | 3.7 |
| Arabinose | 15.8 |
| Fructose | 4.0 |
| Total sugars | 43.6 |
| Acetic acid | 4.1 |
| Furfural (mg/L) | 55.2 |
| HMF (mg/L) | 442.5 |
| *Compositional analysis after both acid and enzymatic hydrolysis (%)* | |
| Cellulose | 5.5 |
| Hemicellulose | 7.9 |
| Lignin | 1.7 |
| Protein | 20 |
| *Hydrolysis yield (%)* | |
| Cellulose | 36.6 |
| Hemicellulose | 59.4 |

**Table 10.4** Concentration and structure of phenolic compounds present in the hydrolysate after acid and enzymatic pretreatment

| **Phenolic compounds after acid pretreatment** | | |
|---|---|---|
| **Compound** | **C (ppm)** | **Structure** |
| Catechol | 511.3 |  |
| Caffeic acid | 211.7 |  |
| Ferulic acid | 178.1 |  |
| Chlorogenic acid | 215.9 |  |
| **Sum** | **1117.1** | |
| **Phenolic compounds after enzymatic hydrolysis** | | |
| Caffeic acid | 221.5 |  |
| p-coumaric acid | 237.5 |  |
| Ferulic acid | 207.3 |  |
| catechol | 1083.6 |  |
| **Sum** | **1749.8** | |

## 10.4. Conclusions

In this chapter, SBP was fractionated into three different streams: a phenolic-rich extract, a pectin-rich fraction and a sugar-rich hydrolysate. The HPLC analysis showed that the main phenolic compounds were catechol and epicatechin, whilst also sinapinic and ferulic acids, vanillin and 4-hydroxybenzaldehyde were also present in lower quantities. In its crude form, the extract does not express high antioxidant activity, but the application of the adequate purification method would possible enhance its antioxidant properties. The pectin fraction should be also analyzed and tested for its emulsifing properties. The sugar-rich hydrolysate containing mainly glucose and arabinose will be used as fermentation substrate for the biotechnological production of succinic acid.

# CHAPTER 11
# BIO-PROCESS DEVELOPMENT FOR THE PRODUCTION AND PURIFICATION OF SUCCINIC ACID FROM SBP DERIVED HYDROLYSATES

## 11.1. Introduction

In the previous chapter, a sugar-rich hydrolysate was successfully produced via acid and enzymatic pretreatment of SBP. The aim of this chapter is the further valorization of the hydrolysate via its bioconversion to succinic acid. As in the case of SSL, the bacterial strains *A. succinogenes* and *B. succiniciproducens* were tested for their ability to grow and produce succinic acid using the sugars present in the SBP hydrolysate. Firstly, bioreactor fermentations were carried out in synthetic media and then in SBP hydrolysate. The most efficient fermentation conditions were selected for scale-up to 50 L. The biotechnologically produced succinic acid was separated from the fermentation broth and purified by means of monopolar and bipolar electrodialysis.

## 11.2. Bioreactor Fermentations using the strain *A. succinogenes*

### 11.2.1. Bioreactor fermentations in synthetic medium

*A.succinogenes* is a well-studied succinic acid producer having the ability to grow and produce succinic acid in various substrates (Pateraki et al., 2016a). As the produced hydrolysate from SBP mainly contains glucose and arabinose, firstly - for comparison reasons - two bioreactor batch fermentations were carried out using only glucose or only arabinose as carbon source.

In the case of pure glucose, the initial sugar concentration was almost 45 g/L and its consumption started rapidly (Figure 11.1). Almost all glucose was consumed at about 43 h and 29.1 g/L of succinic acid were produced with a yield of 0.74 g SA pre g glucose and a productivity of 0.68 g/L/h. Formic and acetic acid were also produced with final concentrations of 1.8 g/L and 6.4 g/L, respectively. When arabinose was used as carbon source (approximately 29.4 g/L initial concentration), a longer lag phase of almost 8 h was observed. The succinic acid concentration was 21.4 g/L after 48 h (Figure 11.2). The succinic acid yield on total arabinose was 0.81 g/g, a higher value than the one observed when glucose was used the sole carbon source. However, the productivity was lower (0.45 g/L/h). By-

product formation was also observed (6.5 g/L), with acetic acid being the main by-product, followed by formic acid (2.1 g/L). Arabinose fermentation resulted to a higher production of by-products, which became more evident when calculating the ratio of each by-product to succinic acid. More specifically, when arabinose was used, the ratio was 1:0.10:0.30:0.40 (SA:FA:AA:total by-products on a mass basis), whereas in the case of glucose the respective ratio was 1:0.07:0.22:0. 29.

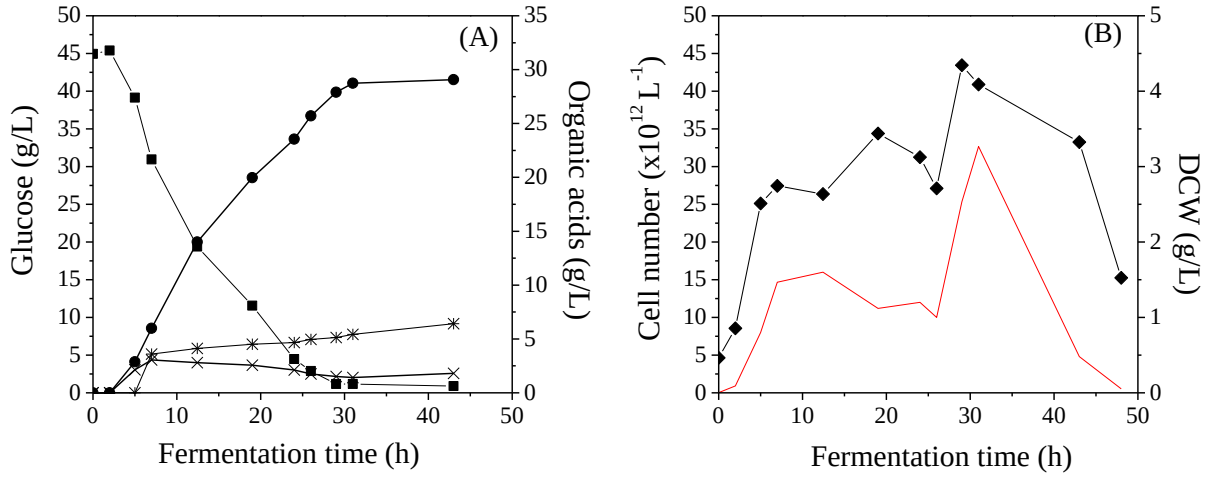



**Figure 11.1** Batch fermentation of *A. succinogenes* in synthetic media using glucose as single carbon source, where (A) shows glucose consumption (■), succinic acid production (●), formic acid production (×) and acetic acid production (⁕), and (B) presents the total number of living cells per L broth (red line) as well as the dry cell weight DCW (♦)

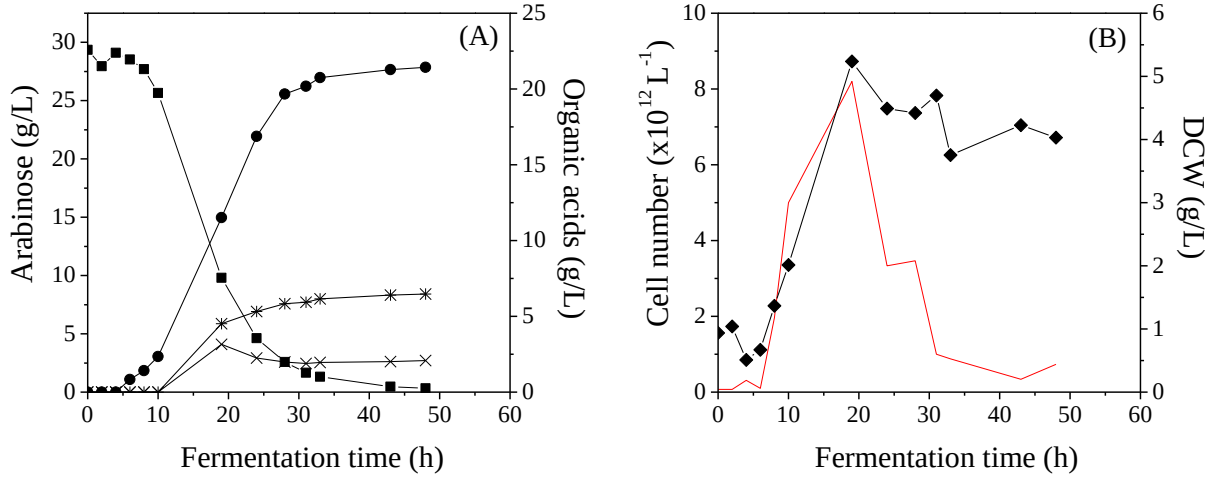



**Figure 11.2** Batch fermentation of *A. succinogenes* in synthetic media using arabinose as single carbon source, where (A) shows arabinose consumption (■), succinic acid production (●), formic acid production (×) and acetic acid production (⁕), and (B) presents the total number of living cells per L broth (red line) as well as the DCW (♦)

The reported yields on succinic acid production from glucose as single carbon source are in the range of 0.50-0.94 g/g (Beauprez et al., 2010). The range is quite wide mainly depending on the complexity of the medium used. Almqvist et al. (2016) reported a yield of 0.56 g/g from glucose (initial concentration 10 g/L) in cultures carried out in Duran bottles using yeast extract as nitrogen source. Salvachua et al. (2016), conducted bioreactor batch fermentations with glucose concentrations in the range of 40-100 g/L. The highest yields (0.72 g/g and 0.69 g/g) were achieved at 60 g/L and 40 g/L initial glucose concentration, respectively, values similar to the ones obtained in this study, with the difference that corn steep liquor was used as additional nitrogen source. Arabinose uptake as single carbon source was investigated by Almqvist et al. (2016), reporting a yield of 0.44 g/g which is lower than the one (0.81 g/g) obtained in this study.

### 11.2.2. Bioreactor fermentations using SBP hydrolysate and yeast extract

The next step involved the investigation of the use of SBP hydrolysate as fermentation substrate supplemented with 5 g/L of yeast extract as nitrogen source. For the first run, a hydrolysate obtained from the hydrolysis of 6% solids was selected in order to assess the ability of the microorganism to grow and produce succinic acid in this substrate. The initial total sugar concentration at the beginning of the fermentation was 19.5 g/L, of which glucose was 8.5 g/L, arabinose was 7.7 g/L and the total concentration of fructose, galactose and xylose were 3.3 g/L (Figure 11.3). The strain started consuming the sugars during the first three hours of fermentation, having a distinct preference to glucose. When glucose and probably also fructose were depleted the strain started consuming the pentoses. The same observation was also made by other researchers when *A. succinogenes* was cultivated in sugar mixtures containing glucose and a pentose, mainly xylose (Pateraki et al., 2016a; Salvachua et al., 2016a). Sugars were depleted after 30 h of fermentation and the final succinic concentration achieved was 13.5 g/L with a yield of 0.74 g/g and a productivity of 0.45 g/L/h. The results were considered as quite promising taking into account that the yield achieved was similar to the one achieved (0.74 g/g) using pure glucose as substrate. The production of formic acid was higher (4.2 g/L) in comparison to the one obtained in synthetic media, whereas acetic acid (5.4 g/L) was slightly lower than the one observed in defined medium with glucose or arabinose. The ratio of each by-product to succinic acid significantly increased in comparison to the defined substrate, having a ratio of 1:0.31:0.54:0.85 for SA:FA:AA:total by- products.

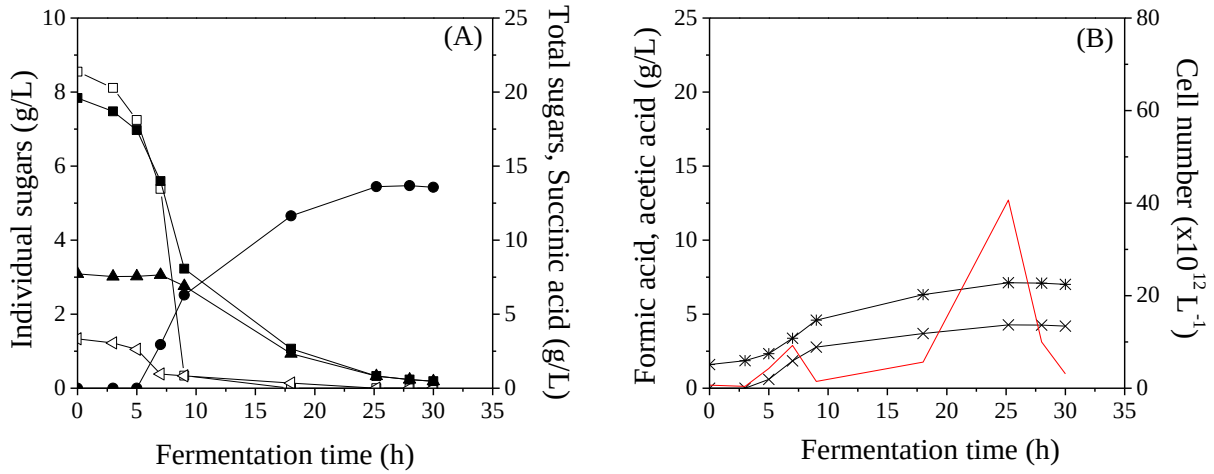



**Figure 11.3** Preliminary batch fermentation of *A. succinogenes* using SBP hydrolysate (hydrolysis using 6 % solid loading), where (A) presents glucose (□), xylose+fructose+galactose (▲), arabinose (◁), total sugars (■), succinic acid (●) and (B) presents formic acid (×), acetic acid (∗) and the total number of living cells per L broth (red line)

Since the microorganism was able to utilize the sugars obtained from hemicellulose and cellulose hydrolysis of SBP, a hydrolysate with higher sugar content was then evaluated. The hydrolysate was obtained by hydrolysing 10% solids with 0.5% $H_2SO_4$ for 30 min followed by the addition of 0.5 mL Accellerase 1500 per g of cellulose. The composition of the hydrolysate has been described previously (Table 10.3). Due to the dilution that occurs after the addition of the mineral solution, yeast extract and inoculum, the initial sugar concentration was approximately 31 g/L. Glucose (15.5 g/L) was the main sugar present in the hydrolysate, followed by arabinose (10.7 g/L), fructose (2.5 g/L), galactose (1.7 g/L) and xylose (0.6 g/L) (Figure 11.4). Glucose was depleted in the first 10 h. When glucose was exhausted (and probably fructose as well), then the strain started consuming the other sugars. As it is has been reported *A. succinogenes* shows lower preference to galactose (Salvachua et al., 2016a) or does not consume it at all (Pateraki et al., 2016b; Almqvist et al., 2016). So we could assume that from the peak representing three sugars (xylose, galactose and fructose), galactose was the last to be consumed. Nevertheless, at the end of the fermentation (29 h) only a small amount of arabinose (about 0.4 g/L) was unconsumed. Succinic acid production was initiated after 4 h and reached a final concentration of 19.9 g/L, with a yield on total sugars equal to 0.71 g/g and productivity of 0.69 g/L/h. In this fermentation the main by-product was acetic acid (7 g/L), followed by formic acid (4.5 g/L). Surprisingly, the ratio of

each by-product to succinic acid was lower than the corresponding ratio observed in the fermentation where lower sugar concentration was tested. More specifically, the ratio of total-by products to succinic acid was 0.58 instead of 0.85 and the ratio SA:FA:AA was 1:0.23:0.35 in comparison to 1:0.31:0.54 observed in the fermentation carried out with low sugar concentration.

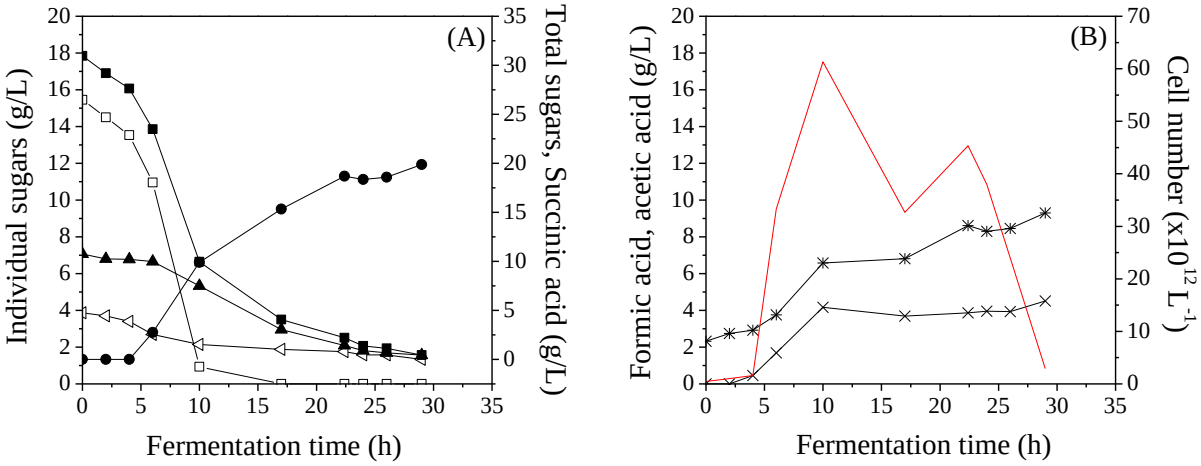



**Figure 11.4** Batch fermentation of *A. succinogenes* using SBP hydrolysate (10 % solid loading), where (A) presents glucose (□), xylose+fructose+galactose (▲), arabinose (◁), total sugars (■)and succinic acid (●) and (B) presents formic acid (×), acetic acid (∗) and the total number of living cells per L broth (red line)

### 11.2.3. Bioreactor fermentations using SBP hydrolysate as both carbon and nitrogen source

In order to create an efficient biorefinery based on SBP valorisation, the possibility to use the hydrolysate as both carbon and nitrogen source should be investigated. As it is already mentioned, the initial raw material contains around 8% protein that after hemicellulose and cellulose degradation this value increases to about 20%. Hydrolysis of proteins leads to the production of amino acids and peptides, expressed by the increased concentration of free amino nitrogen (FAN) that can be directly assimilated by the microorganisms eliminating the need for yeast extract addition in the medium.

Two fermentations were carried out using the same hydrolysate that was obtained using 10% solid loading. In the first fermentation, the hydrolysate was not supplemented with yeast extract due to the addition of Fermgen enzyme preparation in the hydrolysate, while in the second fermentation, the hydrolysate was supplemented with yeast extract. The initial sugar concentration was around 32 g/L in both fermentations. The same inoculum was used in

both cases. When yeast extract was added into the medium, sugar consumption started rapidly (Figure 11.5A) and the fermentation finished faster than the fermentation where the hydrolysate was treated with Fermgen enzyme preparation (Figure 11.5B). Nevertheless, when the enzyme preparation was employed, the succinic acid concentration reached 19.6 g/L and the yield of succinic acid produced to total sugars was 0.78 g/g, a value slightly higher than the one observed in the fermentation supplemented with yeast extract. The increased succinic acid yield could be easily explained by the fact that by-product formation was really low when no yeast extract was used. More specifically, the final formic acid and acetic acid concentrations were only 2 g/L and 2.7 g/L, respectively. The succinic acid to by-product ratio decreased significantly (1:0.11:0.16:0.26) in comparison to the fermentation carried out with yeast extract.

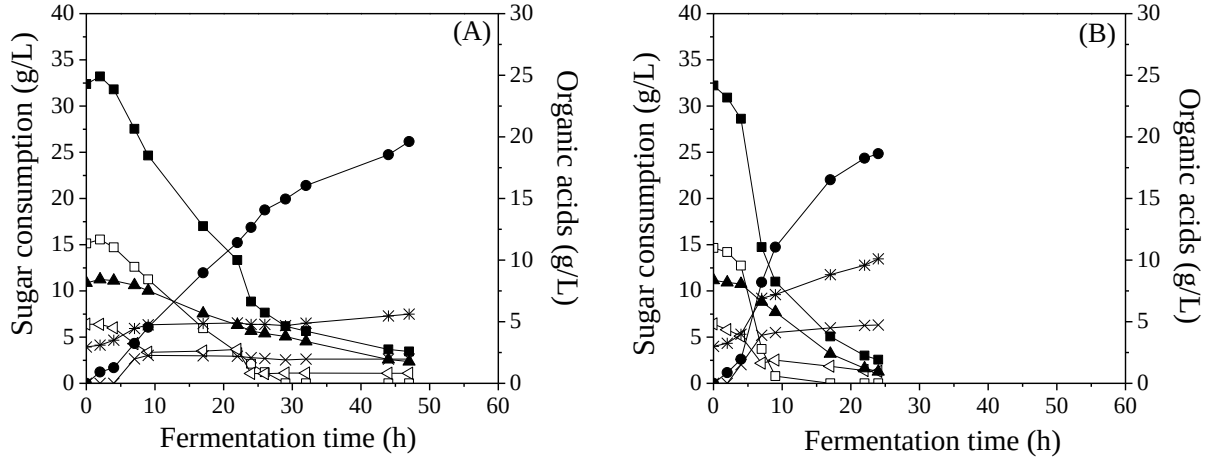



**Figure 11.5** Batch fermentation of *A. succinogenes* using (A) SBP hydrolysate treated with Fermgen enzyme preparation and without addition of yeast extract and (B) SBP hydrolysate supplement with yeast extract. Glucose (□), xylose+fructose+galactose (▲), arabinose (◁) and total sugars (■), succinic acid (●), formic acid (×) and acetic acid (∗)

To further assess the efficiency of the enzymes present in Fermgen, two fermentations were carried out using the same inoculum, one using a hydrolysate produced with SBP pre-treated with Fermgen enzyme preparation (Figure 11.6A) and the other without addition of yeast extract or treatment with Fermgen (Figure 11.6B). The initial total sugar concentration was nearly the same (around 40 g/L), since the two fermentations had an initial sugar concentration difference of 3.6 g/L. Glucose was a bit higher when the Fermgen treated SBP hydrolysate (20.6 g/L in comparison to 18.8 g/L) was used. In both cases, sugar consumption started almost at the same time (Figure 11.6) followed by succinic acid production. The preference on glucose was evident, but there was also a parallel - even though slower -

consumption of the other sugars, before glucose depletion. Both fermentations finished almost at the same time, resulting in the production of 23.8 g/L (Figure 11.6A) and 25.3 g/L (Figure 11.6B) of succinic acid concentration. Total sugar to succinic acid conversion yields were also similar (0.67 g/g and 0.65 g/g). The productivity was slightly higher in the case that the Fermgen treated SBP hydrolysate (0.49 g/L/h) was used, in contrast to the fermentation carried out with yeast extract supplementation or treatment with Fermgen enzyme preparation (0.44 g/L/h).

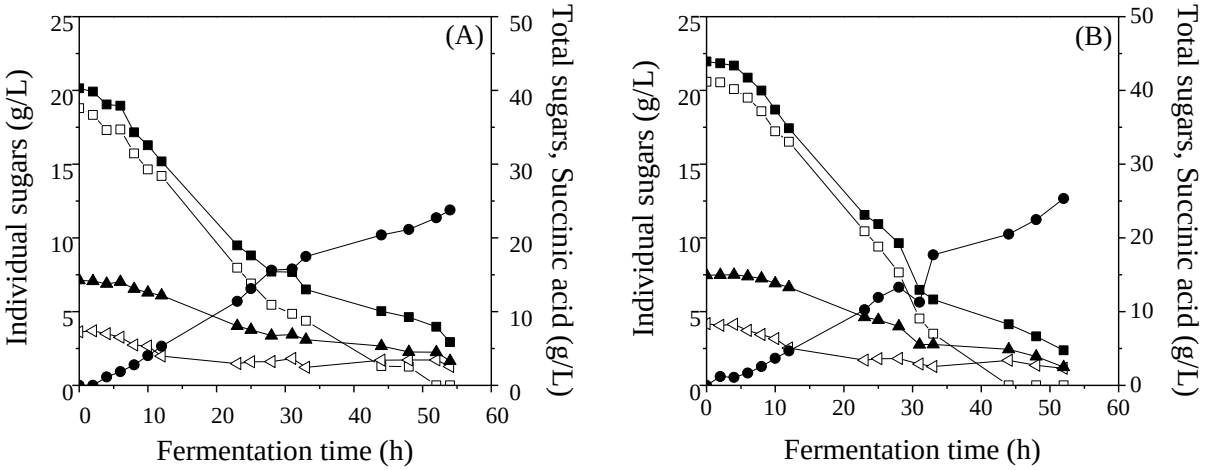



**Figure 11.6** Batch fermentation of *A. succinogenes* using (A) SBP hydrolysate produced via treatment with Fermgen enzyme preparation without addition of yeast extract and (B) SBP hydrolysate without yeast extract addition or treatment with Fermgen enzyme preparation. Glucose (□), xylose+fructose+galactose (▲), arabinose (◁) total sugars (■), succinic acid (●)

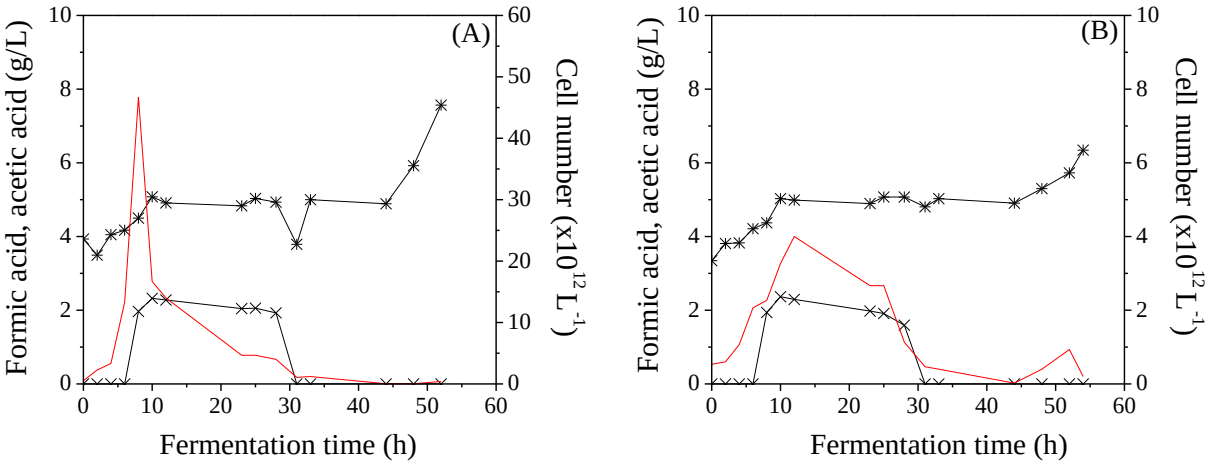



**Figure 11.7** Batch fermentation of *A. succinogenes* using (A) Fermgen treated SBP hydrolysate without addition of yeast extract and (B) SBP hydrolysate without yeast extract

supplementation. Formic acid (×), acetic acid (∗) and total number of living cells per L broth (red line)

Formic acid production started at about 8 h, reaching its highest value after 2 h (2.4 g/L) followed by a slowly decreased trend to complete consumption after 31 h. The exact same trend was observed in both fermentations (Figure 11.7). It has been reported that *A. succinogenes* may metabolise formate to $CO_2$ and $H_2O$ (Brink and Nicol, 2014). The acetic acid concentrations in both cases were around 5 g/L. The succinic acid to by-product ratio was low (0.16 and 0.17) at the end of both fermentations as the only by-product present in the broth was acetic acid.

The experiments presented in Figures 11.5 – 11.7 indicate that SBP hydrolysate could be used as a complete nutrient source, if more work is carried out on protein hydrolysis in order to improve the supply of nitrogen sources during fermentation. Considering the downstream separation process, it is really important to achieve such a low by-product formation as the one achieved in the fermentations carried out without addition of yeast extract exploiting the protein hydrolysis achieved after supplementation with Fermgen enzyme preparation. It should be stressed that the lowest fermentation duration was achieved with yeast extract supplementation (Figure 11.5B) indicating that protein hydrolysis needs optimisation. Further experiments should be carried out in order to optimize this process and to combine it with the separation and purification of succinic acid from the fermentation broth.

### 11.2.4. Bioreactor fed-batch fermentations

In order to achieve higher succinic acid production efficiency, fed-batch experiments were subsequently carried out. During the batch phase, yeast extract was added in order to reduce the fermentation duration as was observed in the fermentations presented in Figure 11.5 and Figure 11.6. Two strategies were followed during feeding where in the first fermentation the feeding solution used did not contain any yeast extract (Figure 11.8), while in the second fermentation the feeding solution contained yeast extract (Figure 11.9).

Figure 11.8 presents the consumption of sugars and the production of succinic acid and by-products in the fermentation where the feeding solution did not contain any yeast extract (FBW fermentation). The initial sugar concentration was approximately 29 g/L. Feeding started when total sugar concentration reached 5.5 g/L (13 h) and glucose was

completely consumed. The by-product concentration did not change significantly when feeding started. The fermentation lasted 50 h and the final succinic acid concentration was 31.1 g/L with a yield of 0.8 g/g and total volumetric productivity of 0.62 g/L/h. Interestingly, a slightly higher formic acid (7.5 g/L) concentration was produced in comparison to acetic acid (6.2 g/L), which is the main by-product in most fermentations carried out with *A. succinogenes*. The ratios of by-products to succinic acid were 0.27 (FA:SA), 0.23 (AA:SA) and 0.50 (total by-products to SA). In the end of fermentation, 1.6 g/L of sugars, mainly galactose, were left unconsumed. The carbon balance also reveals that the metabolism was mainly oriented towards succinic acid production, as the C-moles were 0.67 for succinic acid, 0.13 for formic acid and 0.17 for acetic acid.

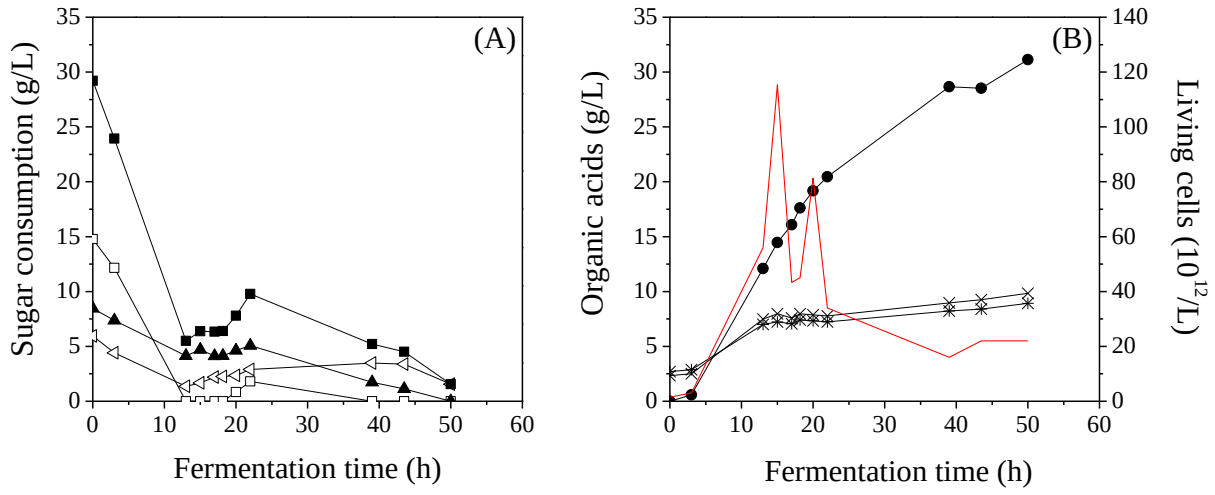



**Figure 11.8** Fed-batch fermentation using SBP hydrolysate without addition of yeast extract in the feeding solution. (A) sugar consumption and (B) organic acids production and living cells. Glucose (□), xylose+fructose+galactose (▲), arabinose (◁), total sugars (■), succinic acid (●), formic acid (×), acetic acid (∗) and total number of living cells per L broth(red line)

Figure 11.9 presents the consumption of sugars and the production of succinic acid and by-products in the fermentation where the feeding solution contained 20 g/L of yeast extract (FBYE fermentation). The FBYE fermentation started with a total sugar concentration of approximately 27 g/L (Figure 11.9). Feeding started after 12 h when glucose was almost completely consumed. The preference towards glucose was again evident. The fermentation was terminated after 35 h, when the final succinic acid concentration was 29 g/L, with a yield on total sugars of 0.94 g/g and a productivity of 0.83 g/L/h. In the FBYE fermentation, acetic acid was the main by-product with a final concentration of 6.3 g/L, while formic acid concentration was 3 g/L. It is clear that when yeast extract was added to the feeding solution,

the fermentation was faster and both yield and productivity were higher than the FBW fermentation. Interestingly, by-product formation was much lower than the FBW experiment. The ratio of SA:FA:AA:total by-products in the FBYE was 1:0.12:0.25:0.37, in comparison to 1:0.27:0.23:0.50 observed in the FBW fermentation.

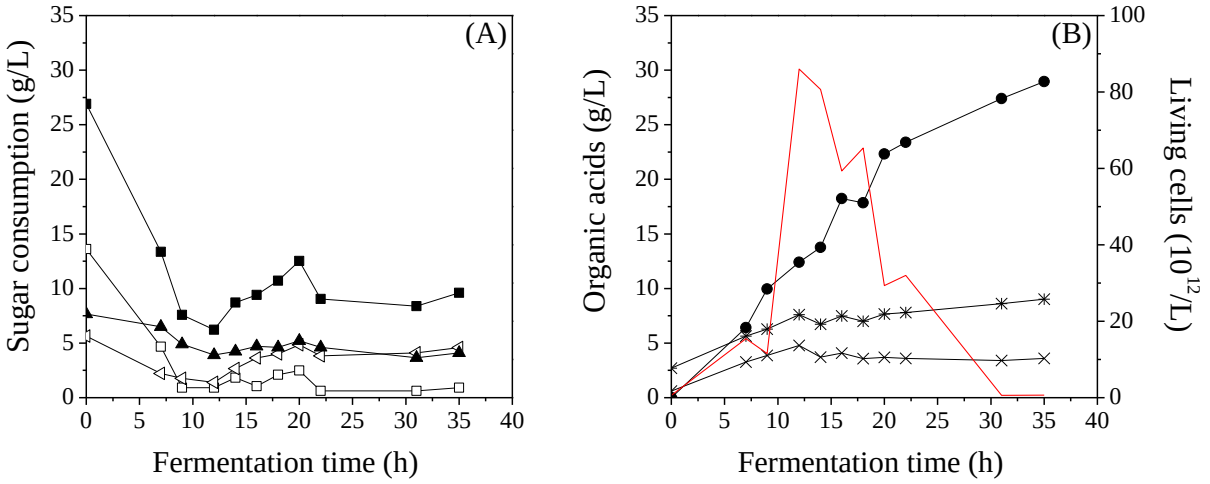



**Figure 11.9** Fed-batch fermentation using SBP hydrolysate and a feeding solution supplemented with yeast extract. (A) sugars consumption and (B) organic acids production and living cells. Glucose (□), xylose+fructose+galactose (▲), arabinose (◁), total sugars(■), succinic acid (●), formic acid (×), acetic acid (✳) and the total number og living cells per L broth (red line)

### 11.2.5. Bioreactor fermentations with filtrated SBP hydrolysate

Even though the final yields and productivities of succinic acid obtained by using the raw SBP hydrolysate were quite satisfactory, a pre-treatment via filtration was also tested in order to evaluate its effect on the fermentation. Firstly, filtration with 0.45 μm membrane was tested and a batch fermentation was carried out with the permeate stream. No specific sugar rejection was observed after filtration and fermentation started with 37.9 g/L initial sugar concentration (Figure 11.10). Sugar consumption started rapidly, but succinic acid production was initiated after 4 h. The succinic acid production rate was slower than in all the experiments carried out using raw SPB hydrolysate. The succinic acid concentration was 20.2 g/L after 26 h. The final concentration of by-products was similar to the ones obtained in the batch fermentations presented in Figure 11.3 and Figure 11.4. The sugar to succinic acid conversion yield was 0.6 g/g, which is quite lower than all fermentations presented in Figure 11.3 and Figure 11.4. The productivity achieved was 0.78 g/L/h.

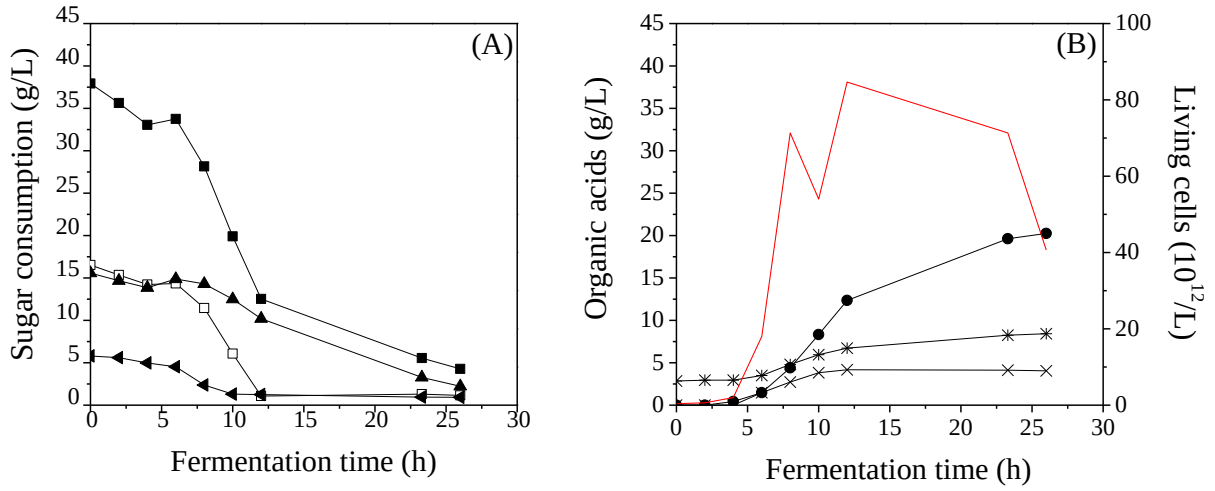



**Figure 11.10** Batch fermentation using microfiltrated (0.4 μm) SBP hydrolysate. (A) sugar consumption and (B) organic acids production and living cells. Glucose (□), xylose+fructose+galactose (▲), arabinose (◀), total sugars (■), succinic acid production (●), formic acid (×), acetic acid (✳) and the total number of living cells per L broth (red line)

Subsequently, microfiltration with 0.2 μm membrane pore size was carried out and the obtained permeate was subjected to microbial fermentation. Again, no specific sugar rejection was observed and the initial total sugar concentration at the beginning of the fermentation was 30.7 g/L. The microorganism started consuming the sugars present in the hydrolysate without any inhibition with subsequent succinic acid production (Figure 11.11). After 24 h, succinic acid concentration was 16.9 g/L, while 2 g/L of total sugars (mainly galactose and arabinose) were left unconsumed. The final yield (0.60 g/g) was the same as in case that filtration with 0.4 μm membrane pore size was used. The productivity was slightly lower (0.70 g/L/h) than the one observed with the SBP hydrolysate filtrated with 0.4 μm membrane. Interestingly, by-product formation was much higher than the untreated hydrolysate, especially their ratio to succinic acid. The ratio of total by-products to SA was 0.70 and the individual ratios of FA:SA and AA:SA were 0.29 and 0.40, respectively. As a conclusion, since the overall succinic acid production efficiency is not significantly enhanced, there is no need for applying filtration to the SBP hydrolysate.

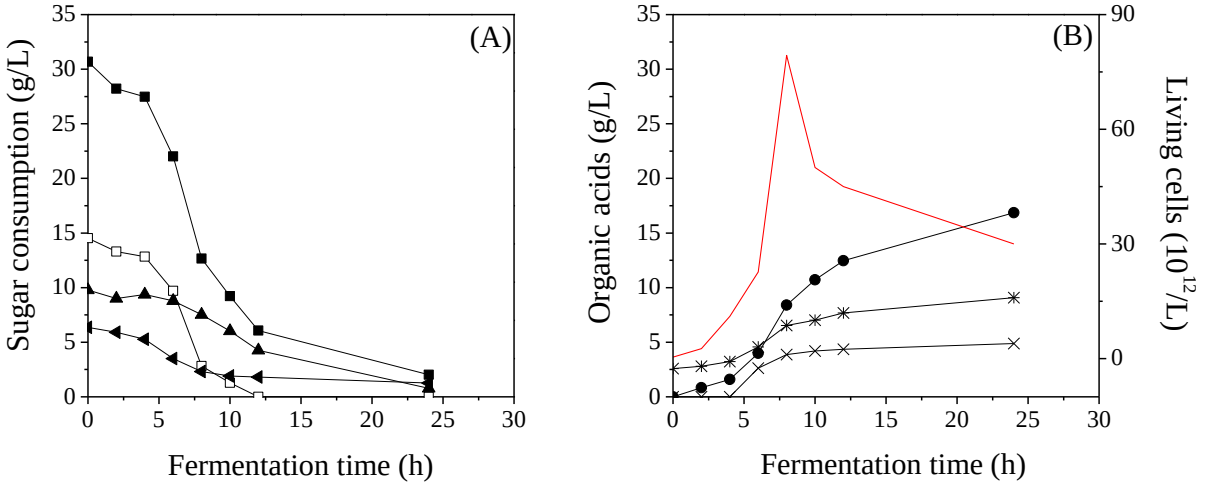



**Figure 11.11** Batch fermentation using microfiltrated SBP hydrolysate. (A) sugar consumption and (B) organic acids production and living cells. Glucose (□), xylose+fructose+galactose (▲),arabinose (◀), total sugars (■), succinic acid (●), formic acid (×), acetic acid (✳) and the total number of living cells (red line)

A fed-batch fermentation (Figure 11.12) with micro-filtrated SBP hydrolysate was also carried. As observed in the previous fed-batch fermentations, glucose consumption was also rapid (Figure 11.12). The feeding solution used was micro-filtrated-concentrated SBP hydrolysate. After 22 h, accumulation of sugars was observed and for this reason the addition of feeding medium was stopped. However, even after 62 h, there was no sugar consumption. The final succinic acid concentration was 24.1 g/L with a yield of 0.66 g/g. It is obvious that both yield and final succinic acid concentration were much lower than the values obtained using the untreated SBP hydrolysate (Figure 11.9). Productivity was high (1.07 g/L/h), but the low final succinic acid concentration together with the high amount of sugars that were left unconsumed lead to the conclusion that the strain performs better when no filtration is carried out prior to fermentation.

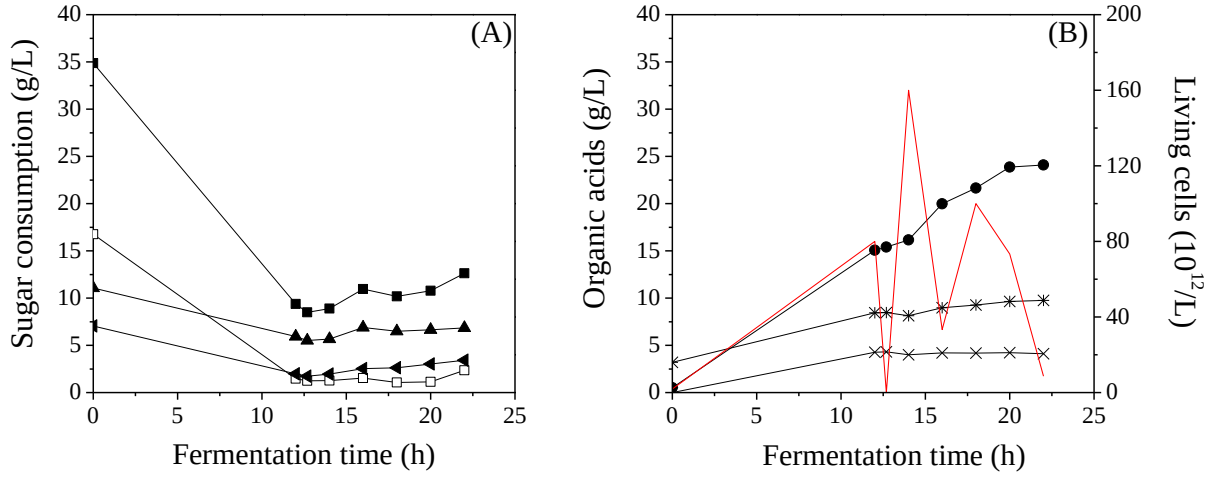



**Figure 11.12** Fed-batch fermentation using microfiltrated SBP hydrolysate with a 0.2 μm membrane pore size. (A) sugar consumption and (B) organic acids production and living cells. Glucose (□), xylose+fructose+galactose (▲), arabinose (◀), total sugars (■), succinic acid (●), formic acid (×), acetic acid (✳) and the total number of living cells (red line)

### 11.2.6. Fed-batch fermentation in pilot scale at 72 L bioreactor

The production of succinic acid by *A. succinogenes* using SBP hydrolysate was subsequently evaluated at pilot scale using a 72 L bioreactor (Figure 11.13). Since there was no significant improvement via microfiltration, no pretreatment of the hydrolysate was applied. The initial sugar concentration was 23 g/L. After 6 h, glucose was depleted and it was not detected again during fermentation. The final succinic acid concentration was 30.1 g/L after 40 h. The formic acid was also consumed towards the end of the fermentation. More specifically, its production ceased after 9 h (before the onset of feeding), and after that time it was gradually consumed completely. The final acetic acid concentration was 7.1 g/L. Succinic acid yield and final volumetric productivity were 0.90 g/g and 0.75 g/L/h, respectively. Since acetic acid was the only by-product present at the end of the fermentation, its ratio to succinic acid was 0.25, which is one of the lowest ratios observed in this study. This ratio is lower than the fed-batch fermentations carried out in lab scale (Figure 11.8 and Figure 11.9). To conclude, the process was successfully implemented to a larger scale, indicating that SBP could be efficiently employed for the biotechnological production of succinic acid using the strain *A. succinogenes*.

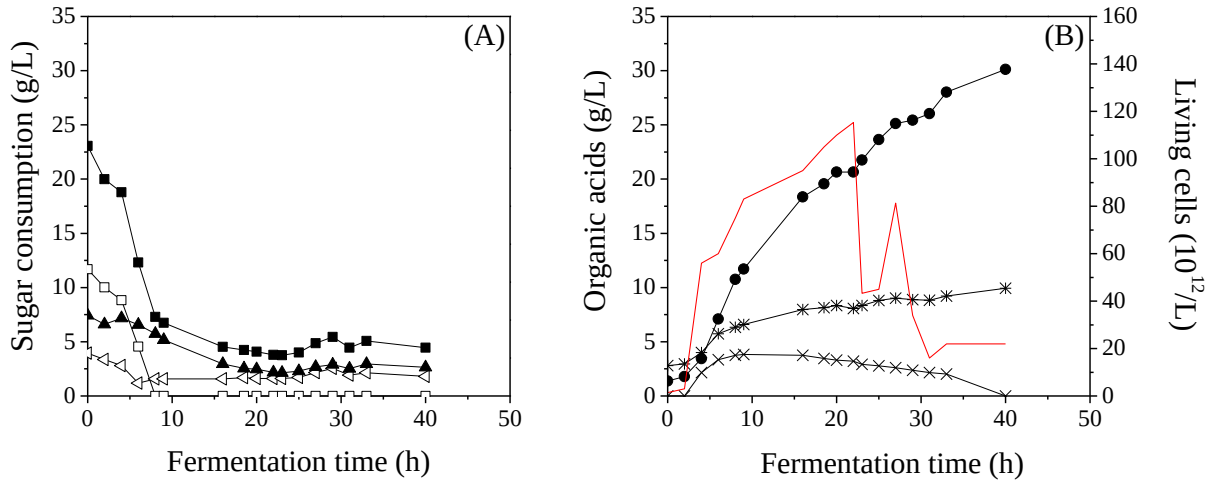



**Figure 11.13** Fed-batch fermentation using SBP hydrolysate and yeast extract in the feeding solution. (A) sugar consumption and (B) organic acids production and living cells. Glucose (□), xylose+fructose+galactose (▲), arabinose (◁), total sugars (■), succinic acid (●), formic acid (×), acetic acid (∗) and the total number of living cells per L broth (red line)

### 11.3. Bioreactor fermentations carried out with the strain *B. succiniciproducens*

*B. succiniciproducens* is a newly isolated strain and it is not widely tested regarding its ability to produce succinic acid from renewable resources. Salvachúa et al. (2016) used *B. succiniciproducens* in fermentations with lignocellulosic hydrolysate as substrate leading to 30 g/L of succinic acid concentration. Initially, microbial growth and succinic acid production were evaluated in a synthetic medium containing arabinose with initial concentration of about 31.6 g/L. In Figure 11.14, it is evident that the strain *B. succiniciproducens* started to consume the sugar without any lag phase. Succinic acid production started almost immediately, whereas formic and acetic acid production was detected after 6 h. Lactic acid production was also observed but only at the end of the fermentation (18 h). Arabinose was consumed after 26 h, resulting in the production of 18.4 g/L succinic acid, 2.3 g/L lactic acid, 4.6 g/L formic acid and 5.1 g/L acetic acid. The yield of arabinose bioconversion to succinic acid was 0.64 g/g with a total volumetric productivity of 0.71 g/L/h. Even though the final product yield was lower in comparison to *A. succinogenes*, productivity was much higher with this strain. Besides lactic acid production, which is not observed in *A. succinogenes* fermentations, *B. succiniciproducens* produced double quantity of formic acid. The ratios of each by-product and total by-products to succinic acid was 1:0.12:0.25:0.28:0. 53 (SA: LA: FA: AA: total by-products).

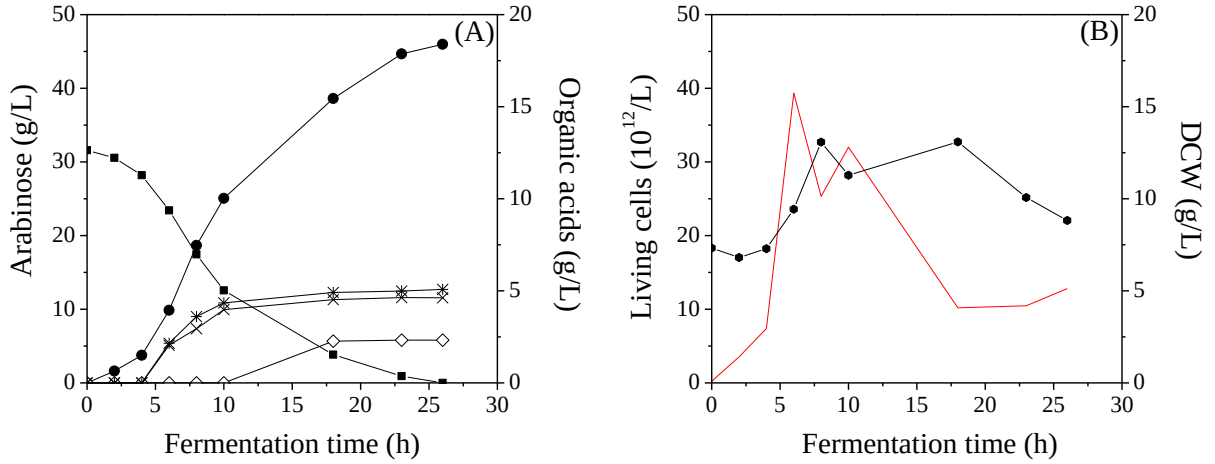



**Figure 11.14** Batch fermentation of *B. succiniciproducens* carried out in synthetic medium using arabinose as single carbon source. (A) shows arabinose consumption (■), succinic acid production (●), lactic acid production (◊), formic acid production (×), acetic acid production (∗), and (B) presents the total number of living cells per L broth (red line) as well as the DCW (♦)

A SPB hydrolysate with low sugar concentration was subsequently tested, in order to evaluate the ability of the strain *B. succiniciproducens* to produce succinic acid and to compare its production efficiency with the one achieved with *A. succinogenes*. The results are presented in Figure 11.15. The microorganism was able to consume almost all the sugars present in the hydrolysate, leading to the production of 14.3 g/L of succinic acid. The product yield was 0.64 g/g and the final volumetric productivity was 0.34 g/L/h. As in the case of *A. succinogenes*, glucose was consumed rapidly after 7 h and then the strain started to consume the mixture of fructose, galactose and xylose. Besides formic and acetic acids, *B. succiniciproducens* also produces lactic acid as a by-product. Acetic acid had the highest concentration with a value of approximately 5 g/L, followed by formic and lactic acids with concentrations of 4.4 g/L and 4.2 g/L, respectively. As a consequence, the ratio of total by-products to SA was much higher (1.13) than the corresponding fermentation (0.85) carried out with *A. succinogenes* (Figure 11.3).

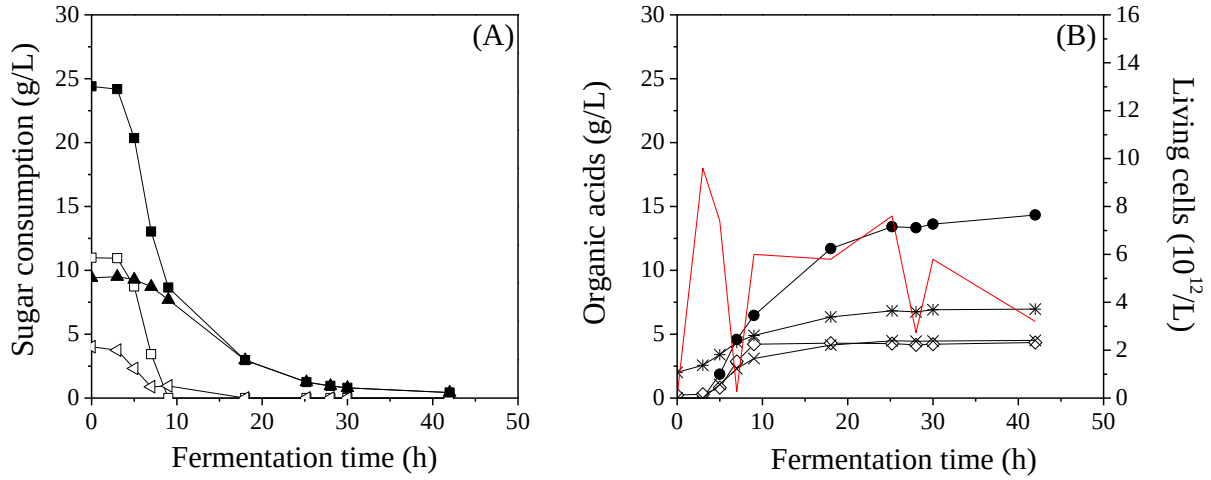



**Figure 11.15** Batch fermentation using sterilized SBP hydrolysate. (A) sugar consumption and (B) organic acids production and living cells. Glucose (□), xylose+fructose+galactose (▲), arabinose (◁), total sugars (■) consumption, succinic acid (●), lactic acid (◊), formic acid (×), acetic acid (✳) and the total number of living cells per L broth (red line)

Figure 11.16 presents the fermentation results using pasteurized SBP hydrolysate. The initial sugar concentration was 26.8 g/L, a slightly higher value than the one used in the sterilized medium (Figure 11.15). The initial concentration of sugars was 12.8 g/L glucose, 9.7 g/L arabinose and 4.3 g/L total concentration of fructose, galactose and xylose. Sugar consumption started rapidly, with the usual preference to glucose. When glucose and fructose were depleted, arabinose consumption was initiated. All sugars were totally consumed after 45 h, leading to 15.5 g/L of succinic acid, with a yield of 0.63 g/g and productivity of 0.46 g/L/h. Acetic acid (5.4 g/L) was the main by-product followed by lactic and formic acids with similar concentrations of 4.5 g/L and 4.9 g/L, respectively. The ratio of each by-product to SA was quite high, especially in comparison to *A. succinogenes*, as SA:LA:FA:AA was 1:0.32:0:30:0.37 and the ratio of total by-products to SA was 0.99.

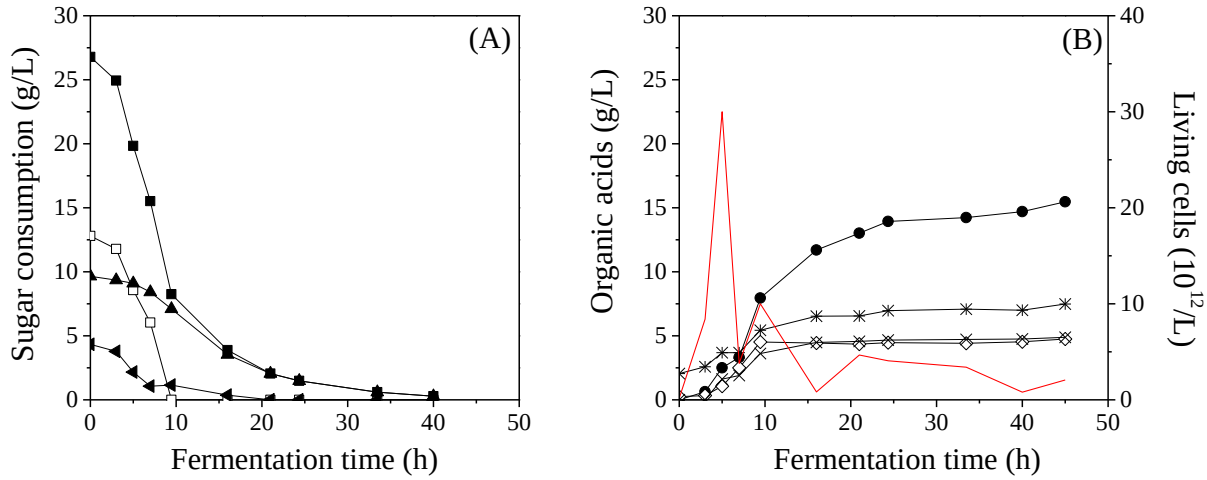



**Figure 11.16** Batch fermentation using pasteurized SBP hydrolysate. (A) sugar consumption and (B) organic acids production and living cells. Glucose (□), xylose+fructose+galactose (▲), arabinose (◀), total sugars (■), succinic acid production (●), lactic acid (◊), formic acid (×), acetic acid (∗) and the total number of living cells per broth (red line)

In order to reduce the by-product formation, microfiltration was tested. Firstly, filtration of 0.45 µm membranes was evaluated and the results are presented at Figure 11.17. From 30.5 g/L initial sugar concentration, 15.2 g/L of succinic acid were produced, resulting in a yield of 0.55 g/g on total sugars and final volumetric productivity of 0.51 g/L/h. All by-products had similar final concentrations (around 5 g/L), with a ratio of total by-products to succinic acid of 0.95.

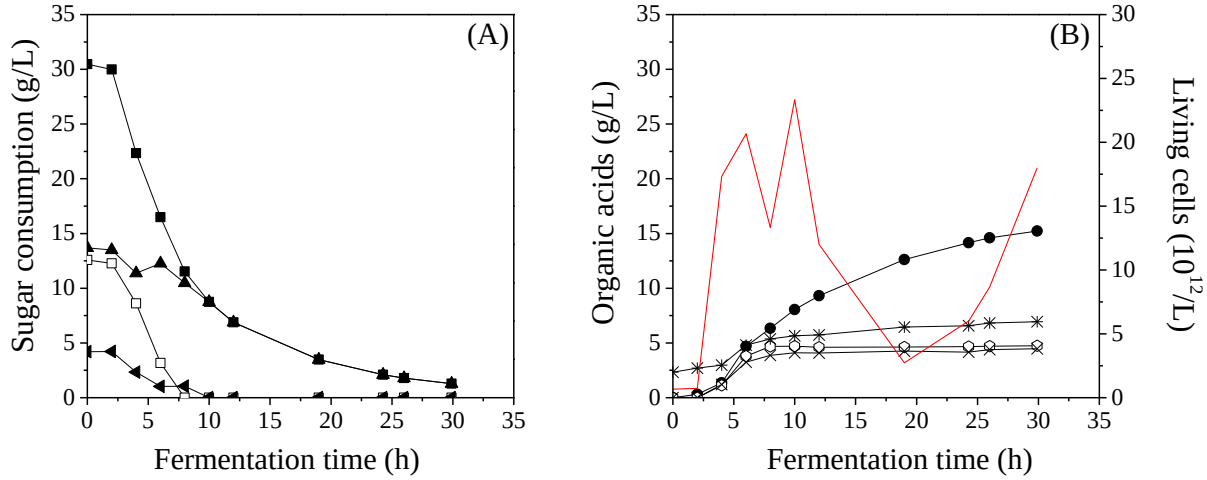



**Figure 11.17** Batch fermentation using microfiltrated SBP hydrolysate with 0.45 µm membrane pore size. (A) sugar consumption and (B) organic acids production and living cells. Glucose (□), xylose+fructose+galactose (▲), arabinose (◀), total sugars (■), succinic acid

(●), lactic acid (◊), formic acid (×), acetic acid (∗) and the total number of living cells per L broth (red line)

Since there was a slight improvement regarding by-product formation, the hydrolysate was then microfiltrated, using a 0.2 μm membrane (Figure 11.18). The initial sugar concentration (36.2 g/L) was higher than in the previous batch experiment presented in Figure 11.17. As previously observed, glucose was the first sugar to be depleted and arabinose consumption initiated when glucose was less than 2 g/L. Succinic acid reached 15.6 g/L resulting in final volumetric productivity of 0.60 g/L/h. Product yield was 0.48 g/g, lower than in previous fermentations. Lactic acid concentration was also much higher (7.4 g/L) than formic acid (4.4 g/L) and acetic acid (4.5 g/L). Salvachua et al. (2016b), also observed an increase in lactic acid concentration in fermentations of *B. succiniciproducens*, concluding that this strain produces more lactic acid when does not starve for energy. The ratio of total by-products increased to 1.27 which is higher than the fermentation carried out with filtrated SBP hydrolysate using a 0.45 μm membrane.

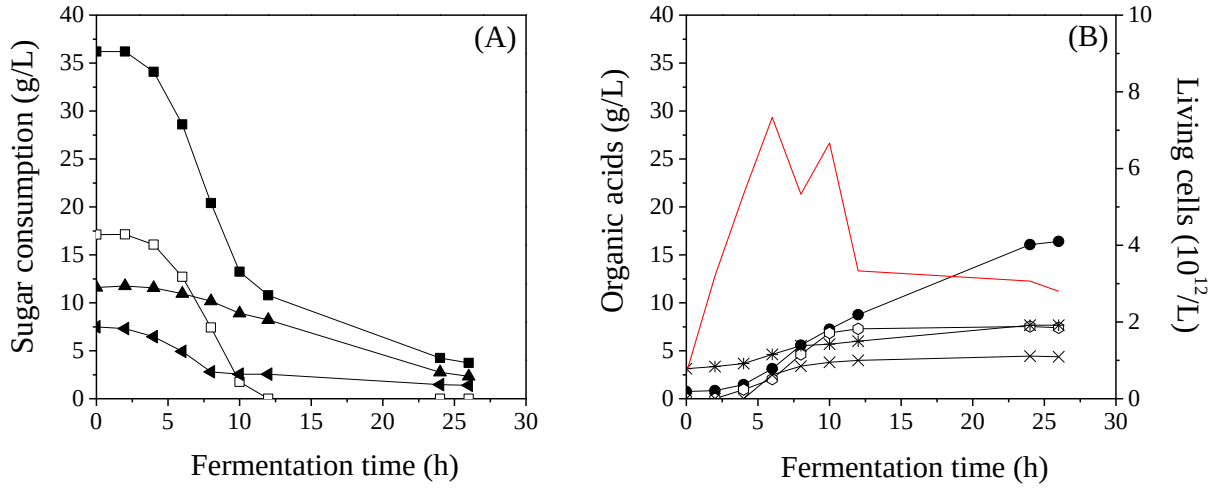



**Figure 11.18** Batch fermentation using microfiltrated SBP hydrolysate. (A) sugar consumption and (B) organic acids production and living cells. Glucose (□), xylose+fructose+galactose (▲), arabinose (◀), total sugars (■), succinic acid production (●), lactic acid (◊), formic acid (×), acetic acid (∗) and the total number of living cells per L broth (red line)

## 11.5. Conclusions

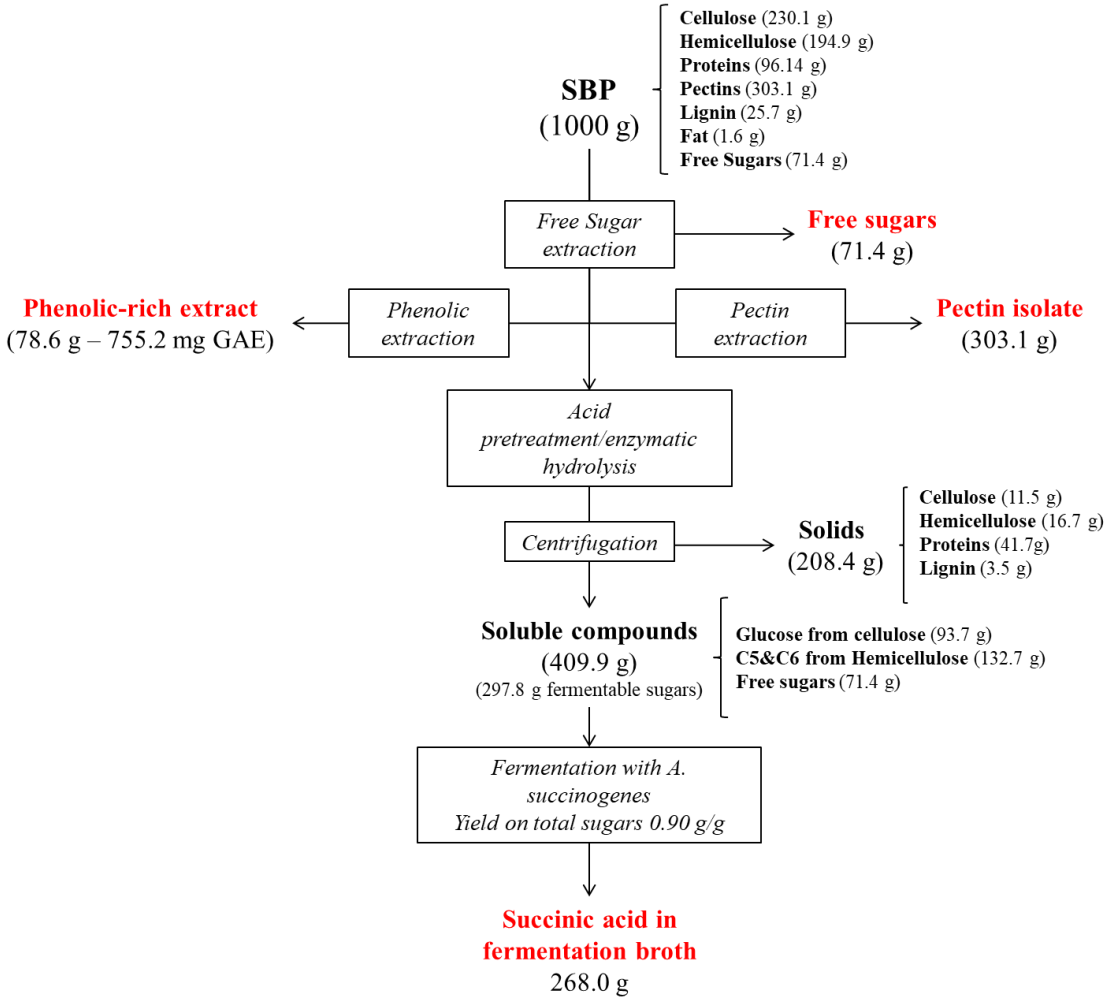
SBP hydrolysate was efficiently bio-converted to succinic acid using the strain *A. succinogenes*. The microbial strain produced approximately 30 g/L in fed-batch fermentation with a yield of 0.80 g/g. The process was also successfully scaled-up in a 50 L bioreactor resulting in the same final succinic acid concentration but higher yield (0.90 g/g) and productivity (0.75 g/L/h). Fermentation results without the addition of yeast extract showed that the strain can also use the proteins and amino acids in the hydrolysate, but succinic acid productivity was reduced. The use of the commercial protease preparation Fermgen could be employed in order to overcome this problem. Even though more work should be carried out, the preliminary results show that SBP hydrolysate could be used as both carbon and nitrogen source, resulting in significant decrease of the overall process cost.

The strain *B. succiniciproducens* was also tested but its performance was not as satisfactory as in the case of *A. succinogenes*. The strain could grow and produce succinic acid but the by-product formation was higher than in the case of *A. succinogenes*. By-product concentration was also higher in comparison to the fermentations where SSL was used as substrate. Filtration was employed in order to remove possible inhibitory compounds, but the fermentation performance was not significantly improved. Table 11.1 presents an overview of all the fermentations carried out using SBP-derived hydrolysates.

**Table 11.1** Overview of the fermentations carried out using SBP-derived hydrolysates

| Strain | Fermentation mode | Initial sugars (g/L) | Consumed sugars (g/L) | SA produced (g/L) | SA yield (g/g) | SA productivity (g/L/h) | Formic acid (g/L) | Lactic acid (g/L) | Acetic acid (g/L) |
|---|---|---|---|---|---|---|---|---|---|
| *A.succinogenes* | Batch, YE | 19.5 | 19.5 | 13.5 | 0.74 | 0.45 | 4.2 | - | 5.4 |
| | Batch,YE | 31.0 | 31.0 | 19.9 | 0.71 | 0.69 | 4.5 | - | 7.0 |
| | Batch, Addition of Fermgen | 32.4 | 28.9 | 19.6 | 0.78 | 0.42 | 2 | - | 2.7 |
| | Batch, no YE | 40.3 | 34.4 | 23.8 | 0.67 | 0.44 | 0.0 | - | 3.6 |
| | FB, no YE in feeding | 29.2 | 154.7* | 31.1 | 0.80 | 0.62 | 7.5 | - | 6.2 |
| | FB, yeast extract in feeding | 26.9 | 127.6* | 29.0 | 0.94 | 0.83 | 3.0 | - | 6.3 |
| | Batch, microfiltration (0.45 μm) | 37.9 | 33.6 | 20.2 | 0.60 | 0.78 | 4.0 | - | 5.6 |
| | Batch, microfiltration (0.2 μm) | 30.7 | 28.7 | 16.9 | 0.60 | 0.70 | 4.9 | - | 6.5 |
| | Fed-Batch, microfiltration (0.2 μm) | 34.9 | 149.5* | 24.1 | 0.66 | 1.07 | 4.1 | - | 6.6 |
| | Fed-Batch pilot-scale | 23.1 | 1260.8 | 30.1 | 0.90 | 0.75 | 0.0 | - | 7.1 |
| *B.succiniciproducens* | Batch | 24.4 | 24.0 | 14.3 | 0.64 | 0.34 | 4.5 | 4.1 | 5.0 |
| | Batch, pasteurized medium | 26.8 | 26.8 | 15.5 | 0.63 | 0.46 | 4.9 | 4.5 | 5.4 |
| | Batch, microfiltration (0.45 μm) | 30.5 | 29.2 | 15.2 | 0.55 | 0.51 | 4.5 | 4.7 | 4.6 |
| | Batch, microfiltration (0.45 μm) | 36.2 | 32.5 | 15.6 | 0.48 | 0.60 | 4.4 | 7.4 | 4.5 |

*grams of consumed sugars




**Scheme 11.1** Mass balances based on 1 Kg of SBP

The work presented in chapters 10 and 11 is promising and proposes a biorefinery scheme that could be employed by the sugar industry, giving value to a by-product that is produced in high amounts. Scheme 11.1 presents the overall process that is proposed in this work, showing that from 1 kg of SBP it is possible to produce 78.6 mg expressed in GAE of a phenolic-rich extract, 303.1 g pectins and 268 g of succinic acid.

# CHAPTER 12
# SEPARATION AND PURIFICATION OF SUCCINIC ACID FROM THE FERMENTATION BROTH

## 12.1. Introduction

The development of a cost-effective upstream process for biotechnologically produced succinic acid can be achieved through the utilization of renewable feedstocks and the production of value-added co-products. In the previous chapters, SSL – a low value industrial side stream - was employed as substrate for the production of succinic acid. The use of solvent extraction with ethyl acetate coupled with nanofiltration led to the production of two value-added co-product streams: antioxidants and lignosulphonates. Similar to SSL, SBP was fractionated to pectins, antioxidants and succinic acid. In this way both SSL and SBP can be fully exploited and the cost of the upstream process could be reduced. The subsequent development of an efficient separation scheme in order to obtain succinic acid crystals of high purity is also of major importance for the development of an economically viable bio-based succinic acid production process. It is already highlighted that for the production of bio-polymers (such as polybutylene-succinate), succinic acid crystals should have a purity of more than 99% (Pateraki et al., 2016a). As the downstream process can account for around 80% of total succinic acid production cost (Luque et al., 2009), it is crucial to establish an efficient and cost-competitive separation and purification protocol.

In this chapter, five different methods were evaluated regarding succinic acid separation and purification from SSL and SBP-derived fermentation media. The methods under consideration include calcium precipitation, salting-out, reactive extraction, direct crystallization and bipolar electrodialysis membranes (BEM). The first four methods were applied on SSL-derived media, while BEM was tested on SBP-based media. BEM was carried out at Leibniz Institute of Agricultural Engineering and Bioeconomy (ATB) in Potsdam, Germany.

Regarding SSL-derived media, since the fermentation medium has a distinct dark color mainly due to the presence of remaining lignosulphonates, different concentrations of activated carbon were also tested. Treatment with activated carbon is a preliminary separation step that in most cases is necessary in order to remove the compounds responsible for the dark color of the fermentation media. Crystallization was also the final step in every separation

scheme tested. Evaluating the time needed for this step, was another objective of this chapter as it highly affects both yield and purity of the succinic acid crystals.

The separation protocol followed in case of SBP-derived media was based on the well-established method followed by ATB for lactic acid separation and purification. The objective was to assess whether or not the same process could be applicable for succinic acid as well. For this separation scheme, cells were removed via microfiltration and color impurities via resins. Finally, instead of crystallization, succinic acid recovery was carried out using a spray dryer. The aim of this chapter is to provide an overview of the possible separation schemes that could be applied for succinic acid purification from fermentation broths, in terms of final succinic acid yield and productivity.

## 12.2. Treatment with activated carbon

The use of activated carbon is necessary for every DSP using simulated or actual fermentation broths for the removal of impurities (e.g. proteins, pigments, LS) that contribute to the dark coloration of the broth. Depending on the origin of the solution used to evaluate the different DSP, varying quantities of activated carbon were required. In pure-commercial organic acid solutions (medium 1), the use of activated carbon was not necessary. When medium 3 (actual fermentation broth obtained via *A. succinogenes* cultivation in synthetic medium) was used, 3% (w/v) of activated carbon was sufficient for complete discoloration of the broth. However, in real SSL-based fermentation broths produced by *B. succiniciproducens* (medium 4) higher quantities of activated carbon were required for effective color removal. Five different quantities of activated carbon (0%, 3%, 7.5%, 12.5% and 20%, w/v) were tested in medium 4 in order to evaluate the efficiency of color removal with respect to the added amount of activated carbon. The color removal was evaluated by measuring the optical density at 400 nm of the five solutions and the results are shown in Figure 12.1. It is obvious that in order to achieve almost complete color removal in medium 4, around 12.5% (w/v) of activated carbon should be applied.

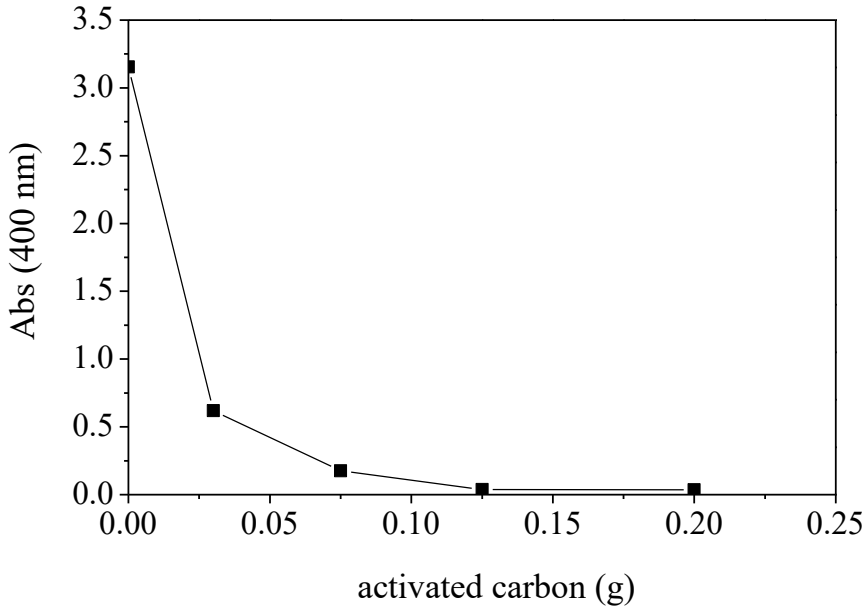



**Figure 12.1** Reduction of color of medium 4 with different concentrations of activated carbon

It is obvious that in order to achieve almost complete color removal in medium 4, 12.5% of activated carbon should be applied. This higher percentage of activated carbon compared to synthetic or pure solutions is attributed mainly to the concentration of lignosulphonates which is around 10 g/L and is the main compound responsible for the dark color of the medium. According to the obtained results, when SSL was used as fermentation substrate, even a small amount of activated carbon (e.g. 3%) could substantially reduce the dark color of the solution. However, 12.5% was the minimum quantity required in order to achieve acceptable color removal. It is worth noting that fermentation broths containing LS concentration up to 100 g/L were not decolorized. In Image 12.1 the effect of different concentrations of activated carbon is also presented.

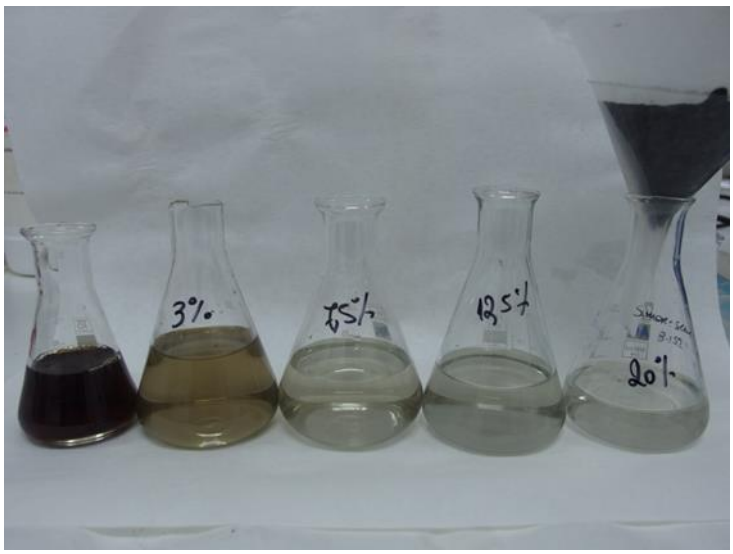



**Image 12.1** Effect of the concentration of activated carbon on the discoloration. First from the left: medium before the addition of activated carbon

The addition of activated carbon leads to a loss of succinic acid depending on the quantity used, probably due to the fact that succinic acid is more easily absorbed to the activated carbon. Figure 12.2 presents the percentage of succinic acid removal when the different studied concentrations were applied in a pure succinic acid solution (PS). It is obvious that increasing quantities of activated carbon lead to higher succinic acid losses which can reach approximately 52% when 20% (w/v) of activated carbon is used. These losses could be eliminated if the activated carbon is hydrated either with water or even better with fermentation broth. For this reason, the losses of succinic acid via treatment with activated carbon are not taken into account when the final process yield is calculated.

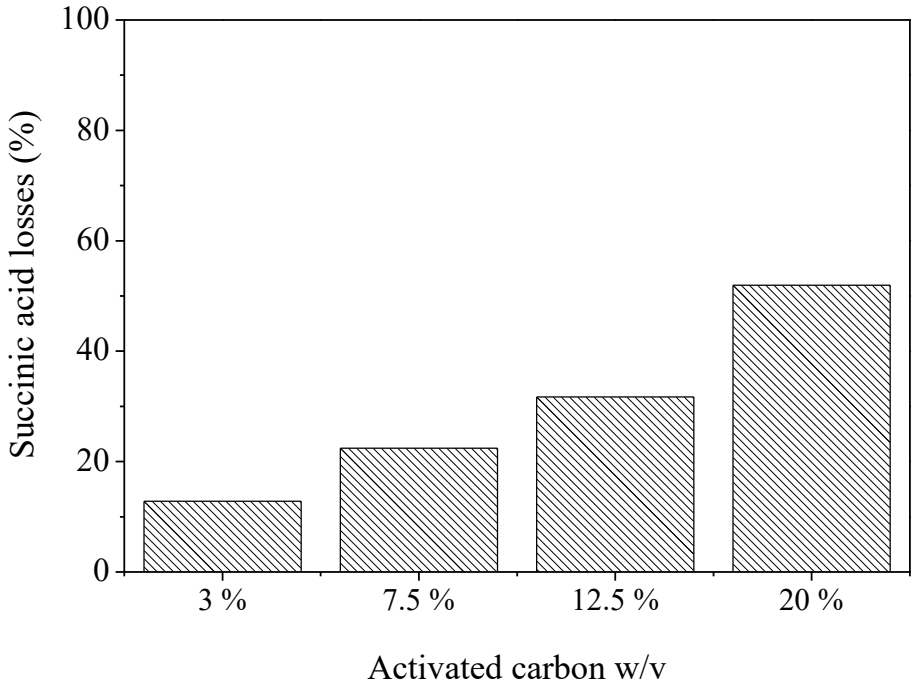



**Figure 12.2** Succinic acid losses observed at different concentrations of activated carbon

### 12.3. Effect of crystallization time

The crystallization step is necessary in order to produce succinic acid crystals of high purity. In the chemical industries, batch crystallization is a common unit operation. The crystallization process should be optimized in order to obtain the appropriate crystal size distribution as well as a good repeatability from batch to batch (Feng and Berglund, 2002). The cooling rate is very important as it affects directly not only supersaturation but also the crystal size distribution. In industrial applications, there is a lack of kinetic data regarding crystallization profile. Aiming to optimize this process, the crystallization rate of succinic acid was monitored by calculating the concentration of diluted succinic acid in a synthetic solution containing an initial succinic acid concentration of approximately 190 g/L. Figure 12.3 presents the concentration of soluble succinic acid as a function of time at different temperatures, starting from 80 °C to 4 °C. Crystallization was carried out under continuous stirring. Within 8 h of crystallization, the soluble succinic acid concentration was around 36 g/L at 5°C which is close to its theoretical solubility at 4°C (30 g/L). This result indicates that when temperature reaches the desirable value, the crystallization of succinic acid reaches a plateau. This occurred in less than 100 minutes in our experiment. Agitation is also important, as it is proven that affects both mass transfer and crystal growth (Feng and Berglund, 2002).

Crystallization is also affected by the impurities that are present in the solution. Feng and Berglund (2002) studied the effect of the addition of acetic acid on the cooling profile of succinic acid showing that 2 mol% of acetic acid lead to reduced crystal growth rate. For this reason, the application of an evaporation step prior to crystallization is a crucial in order to produce succinic acid crystals of high purity.

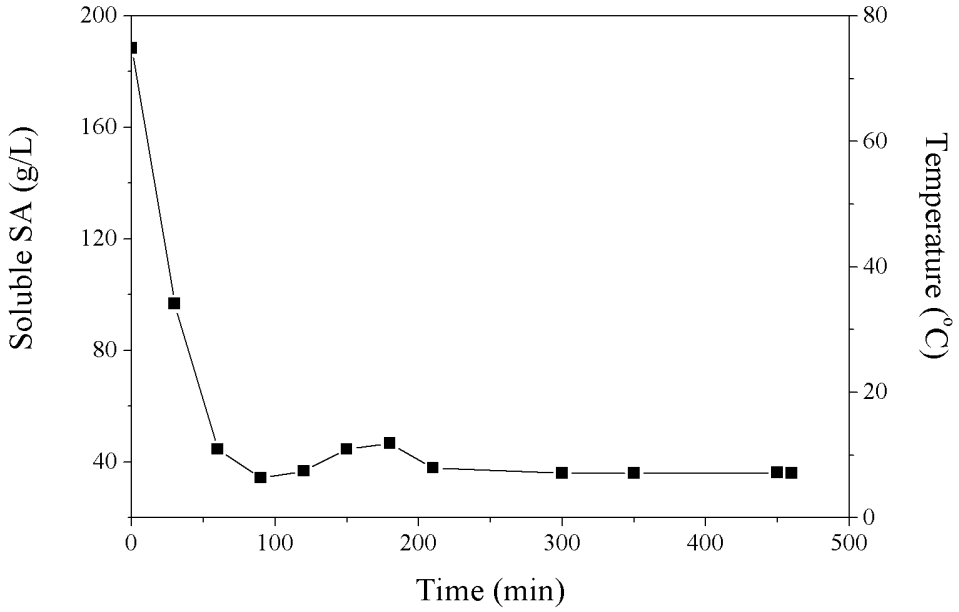



**Figure 12.3** Soluble succinic acid concentration as a function of time at different temperatures following a temperature ramp starting at 80 ºC to 4 ºC

## 12.4. Calcium precipitation method

The use of the calcium precipitation method is traditionally applied in the industry mainly for the recovery of citric and lactic acids (Cheng et al., 2012). The first studies regarding the recovery of succinic acid using calcium hydroxide in laboratory scale were described by Berglund et al. (1991) and Datta et al. (1992). In the study of Datta et al. (1992), the final purity of succinic acid was 94.2%. This method is generally considered quite effective for the removal of the residual sugars, proteins and other by-products. Nevertheless, this process is very slow and requires high energy consumption (Luque et al. 2009) and in this study it was mainly tested for comparison reasons.

The calcium precipitation method was applied in two different fermentation broths, one derived from a fermentation using *A. succinogenes* (SNFB) and the other using *B. succiniciproducens* (RFB). The initial composition of the two broths is shown in Table 12.1. Both samples were treated in the same way with exception of the activated carbon step. For the SNFB, 3% (w/v) of activated carbon was applied, whilst for the RFB, a concentration of

12.5% (w/v) of activated carbon was necessary in order to achieve complete color removal. The steps of the process are described in detail in *Chapter 6* (Materials and Methods). After crystallization, succinic acid yield of the overall process was 7.8% in case of SNFB and 13.1% in the case of RFB, whilst purity was 87.2% and 81%, respectively. In the SNFB medium, the main impurities detected by HPLC were formic and acetic acids with final concentrations of 0.3 g/L and 0.2 g/L respectively. In the RFB medium, the main impurity was lactic acid (ca. 4 g/L) in the aqueous solution (45 mL) created after the dilution of separated succinic acid crystals. Lactic acid has similar properties to succinic acid, making their separation quite difficult. Residual sugars or LS were not detected in the succinic acid crystals.

The final succinic acid yield achieved was similar to the values reported in the literature. Luque et al. (2009) applied the calcium precipitation method for succinic acid recovery from a semi-defined medium containing 35.3 g/L of succinic acid, 8.1 g/L acetic acid, 6.3 g/L formic acid and 1 g/L of pyruvic acid. Succinic acid yield was 13%, whilst crystal purity was only 30%. Datta et al. (1992) reported a purity of 89.6% using calcium precipitation followed by filtration and crystallization. It is obvious that the final succinic acid yield and purity is directly related to its initial concentration in the fermentation broth and also to the nature of the substrate used as in the fermentation stage (e.g. defined medium or renewable feedstocks).

**Table 12.1** Initial composition (g/L) of the two fermentation broths tested for succinic acid separation and the final recovery efficiency achieved using the calcium precipitation method.

| Broth | Solution | Xylose (g/L) | Succinic acid (g/L) | Lactic acid (g/L) | Formic acid (g/L) | Acetic acid (g/L) | LS (g/L) | $Yield_{SA}$ (%) | $Purity_{SA}$ (%) |
|---|---|---|---|---|---|---|---|---|---|
| ***SNFB*** | Initial [1] | 2.8 | 20.3 | 0 | 4.6 | 7.6 | 0 | 7.8 | 87.2 [3] |
| | End [2] | 0 | 6.5 | 0 | 0.3 | 0.2 | 0 | | |
| ***RFB*** | Initial [4] | 9.3 | 41.2 | 12.3 | 2.0 | 9.9 | 10.0 | 13.1 | 81.0 [3] |
| | End [5] | 0 | 18.0 | 3.9 | 0 | 0 | 0 | | |

[1]starting volume of 550 mL
[2]dilution of 1 g crystals in 133 mL
[3]based on total crystal weight
[4]starting volume of 150 mL
[5]dilution of 1 g crystals in 45 mL

## 12.5. Salting-out

Salting-out extraction (SOE) is a separation method that is applied in order to extract a hydrophilic molecule from an aqueous solution by using an organic solvent as the extractant and a salt as the *salting-out* reagent. The advantages of this technique include the low-cost, the low interfacial tension, the good resolution, the high yield and capacity and the simplicity of scaling up the system (Jiang et al., 2009; Li et al., 2009). In addition, cells and proteins are simultaneously removed from the fermentation broth, avoiding centrifugation and/or filtration steps. Total yields of the downstream process were higher than 60% and purities higher than 91% were achieved when salting out was applied in synthetic fermentation media as a separation method for the recovery of succinic acid (Li et al., 2009; Li et al., 2011; Sun et al., 2014).

SOE was applied at the fermentation broths SNFB and RFB (Table 12.1), following the method reported by Sun et al. (2014). After adjusting the pH to 3, acetone (the extractant) and $(NH_4)_2SO_4$ (the salting-out reagent) were added to the fermentation broths. No biomass removal or activated carbon treatment was employed as primary separation steps. After 8 h, three very distinct phases were observed:

- the upper phase contained the organic solvent that is rich in succinic acid
- the middle phase contained the biomass
- the lower phase was the aqueous one containing residual sugars, proteins and other impurities.

Phase separation was carried out via decanting. The succinic acid rich phase was subsequently treated with activated carbon, with the adequate concentrations previously descripted in Section 10.4 in the case of SNFB and RFB. After color removal, acetone was recovered via vacuum evaporation. Interestingly, in both cases succinic acid yield was 50% and purity 86%, as about 25% of xylose was also extracted in the organic phase.

## 12.6. Direct crystallization method

The direct crystallization method was initially reported by Luque et al. (2009). This method is based on the low solubility of succinic acid at 4 $^{o}$C. Most fermentations are carried out at a pH range of 6-7, above the $pK_a$ value of the acid. Acidification of the broth is necessary in order to convert succinate salts into succinic acid. Acidification of the broth could be carried out either using an acid or cation-exchange resins. In both cases, the pH of the solution was around 2.0 as in this value succinic acid is present in its undissociated form

and can be crystallized in higher yields than mono- or di-succinate (Lin et al., 2010). In both cases, the next step involved vacuum evaporation that should be carried out in order to eliminate the volatile organic acids, acetic and formic acids, that are produced as by-products during succinic acid production via fermentation and to increase approximately five times the final concentration of succinic acid. This method was employed in pure organic acid solutions (PR), in a simulated fermentation broth (SFB) and finally in a real fermentation broth (RFB) produced by the bacterial strain *B. succiniciproducens* (RFB) (Table 12.2).

**Table 12.2** Composition of simulated and actual fermentation broths used for the direct crystallization method

| **Component (g/L)** | **Pure solution (PS)** | **Simulated (SFB) & Real (RFB) fermentation broths from *B. succiniciproducens*** |
|---|---|---|
| Xylose | 10.2 | 9.3 |
| Succinic acid | 39.3 | 41.2 |
| Lactic acid | 11.1 | 12.3 |
| Formic acid | 2.0 | 2.0 |
| Acetic acid | 9.6 | 9.9 |
| LS | 0 | 10.0 |

In the case of pure organic acid solution (PS), the steps involving the addition of activated carbon and acidification were omitted. In the case of SFB, the solution was treated with activated carbon but not with resins or an acid as the pH value was already around 2. Results are presented in Figure 12.4. When PS was tested, the succinic acid yield and purity were 72.7% and 87.1%, respectively. In the case of SFB, the succinic acid yield (60.1%) was lower than PS, but higher purity was observed (89.8%). In the RFB, the use of resins resulted in enhanced yield and purity of succinic acid crystals. More specifically, when acidification was employed, the succinic acid yield was 57%, whilst in the case that resins were applied the succinic acid yield reached a value of 79%. Purity was also higher in the case of resin utilization (96%), whereas direct crystallization with resins resulted in a final purity of 90%. These results show the significance of the primary separation steps on succinic acid recovery. Activated carbon absorbs various impurities leading to higher purity of the final product. With the use of resins, better conversion of succinate salts to succinic acid is achieved resulting in higher product recovery after crystallization. To conclude, the highest succinic acid yield

(79%) and purity (96%) were obtained by using ion exchange resins. The rest 4% which is attributed to impurities was not detectable by HPLC.

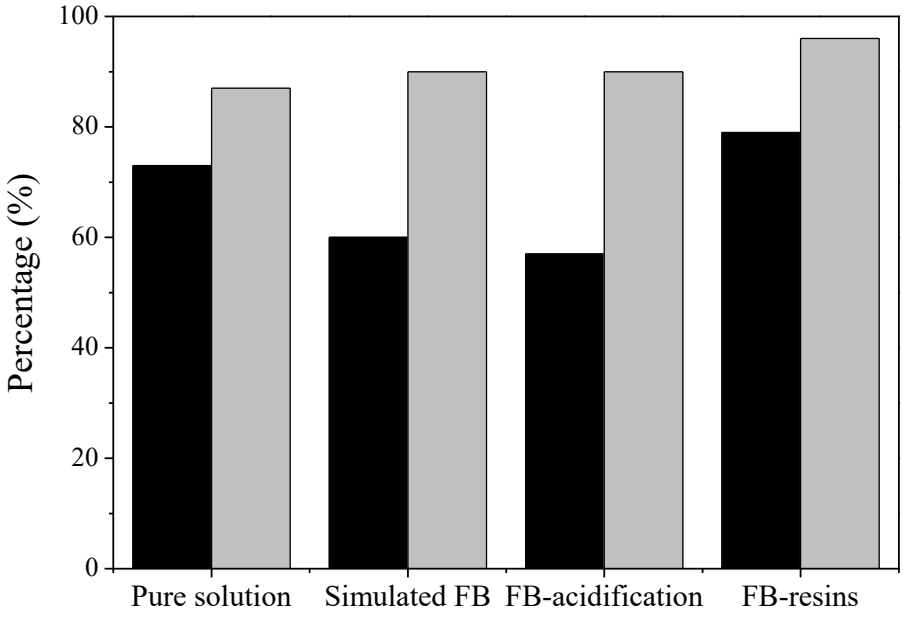



**Figure 12.4** Succinic acid yield (black bars) and purity (grey bars) obtained using the direct crystallization method

Lin et al. (2010) were the first to report the direct crystallization method using ion-exchange resins. In their study, using real fermentation broth using the strain *A. succinogenes*, succinic acid yield and purity reached 89.5% and 99%, respectively. The lower succinic acid yield and purity obtained in this study could be attributed to the complexity of the initial substrate used in the fermentation. An NMR analysis of the crystals obtained could elucidate the nature of the contaminants, so the process could be adequately adapted to achieve crystal purities as high as 99%.

### 12.7. Reactive extraction

Reactive extraction using high molecular weight amines is a well-known method for the extraction of organic acids (Kurzrock and Weuster-Botz, 2011). Regarding succinic acid recovery, long-chain aliphatic primary, secondary and tertiary amines are proposed for its reactive extraction from aqueous solutions (Hong & Hong, 2004; Hong et al., 2002; Hong et

al., 2004; Song et al., 2007). Primary amines show high solubility in the aqueous phase. Secondary amines present the highest distribution coefficients but tend to form amides during regeneration by distillation. Primary, secondary and tertiary amines can only extract the undissociated form of the acid. In contrast, quaternary ammonium salts can extract both dissociated and undissociated forms of the acid, but they are difficult to be regenerated by back-extraction (Kurzrock and Weuster-Botz, 2011). Consequently, tertiary amines are the most promising extractants for reactive extraction of carboxylic acids (Lee et al., 2008). It must be stressed the importance of the $pK_a$ of the acid as well as the pH of the aqueous solution. When tertiary amines are used, the pH of the aqueous solution must be under the $pK_a$ value, because at pH values above $pK_a$ the dissociated form of the acid is increased, leading to low extraction yields. Furthermore, as far as dicarboxylic acids are concerned, the distribution coefficient ($k_d$) value decreases significantly at pH values between $pK_{a1}$ and $pK_{a2}$ (Huh et al., 2006). The selection of the various reactive extraction systems was decided after a thorough literature review on reactive extraction systems for the recovery of succinic acid (Kurzrock et al., 2011).

Secondary, tertiary amines and one primary amine in various solvents were selected for succinic acid separation from fermentation broths. Initially, five amines, four dissolved in 1-octanol and one in 2-octanol were tested in a pure succinic acid solution. The pH of the solution was adjusted to 2.0 in order to ensure that all the succinic acid is present in its undissociated form. The results are presented in Figure 12.5. The vast majority (13 out of 15) of the reactive extraction systems were able to remove more than 90% of succinic acid, with trioctylamine (TOA) in 1-octanol reaching a removal yield of approximately 94%.

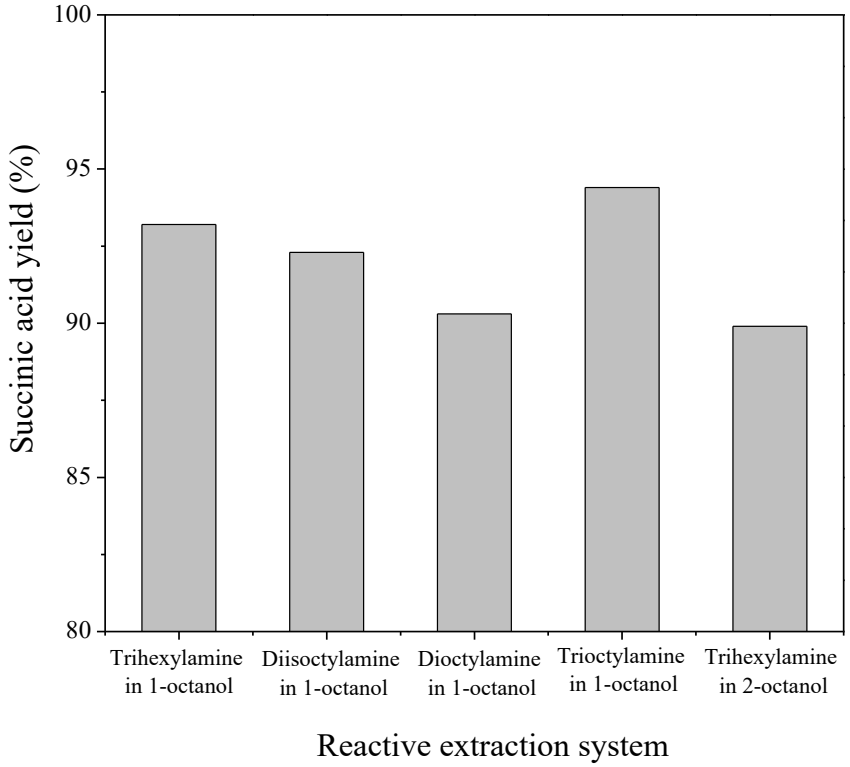



**Figure 12.5** Succinic acid extraction yield using reactive extraction in pure solutions

The efficiency of reactive extraction and the selectivity of the amines selected were subsequently tested using a real fermentation broth where other carboxylic acids were also present. Two different pH values were studied (pH 2 and 5) and the results are shown in Table 12.3 and Table 12.4, respectively. At pH value of 2, all organic acids are co-extracted in high removal yields. Dioctylamine in 1-octanol, dioctylamine in 1-hexanol, TOA in 1-hexanol and diisoctylamine in 1-octanol are the systems that almost all the organic acids were extracted and only the residual sugars remained in the aqueous phase. In this way, the produced organic acids can be separated from the residual sugars and possibly from various impurities.

**Table 12.3** Succinic, lactic and acetic acid extraction yields using different reactive extraction systems at pH 2.0.

| System | Succinic acid yield | Lactic acid yield | Acetic acid yield |
|---|---|---|---|
| TOA + 1-octanol | 94.0 | 93.2 | 71.8 |
| Trihexylamine + 1-octanol | 93.4 | 90.2 | 70.8 |
| Dioctylamine + 1-octanol | 100.0 | 93.8 | 100.0 |
| Trihexylamine + 1-hexanol | 93.2 | 82.5 | 69.2 |
| Trixexylamine + 1-butanol | 93.8 | 91.4 | 75.4 |
| Trihexylamine + 2-octanol | 79.7 | 67.4 | 53.0 |
| Diisoctylamine + 1-butanol | 97.3 | 100.0 | 86.7 |
| octylamine + 1-hexanol | 90.6 | 100.0 | 74.0 |
| Dihexylamine + 1-hexanol | 93.4 | 100.0 | 84.1 |
| Dioctylamine + 1-hexanol | 97.4 | 100.0 | 94.2 |
| Tripentylamine + 1-butanol | 88.5 | 100.0 | 78.1 |
| TOA + 1-hexanol | 98.2 | 100.0 | 93.7 |
| Methyldioctylamine + MIBK | 95.8 | 100.0 | 80.2 |
| Diisoctylamine + dixehylamine + 1-octanol + 1-hexanol | 97.0 | 100.0 | 86.4 |
| Diisoctylamine + 1-octanol | 99.0 | 100.0 | 90.7 |

An alternative use of reactive extraction is to use this method as a primary separation step in order to remove the by-products and especially lactic acid (when *B. succininiciproducens* is employed). In order to achieve this, the same reactive extraction systems were applied at pH value of 5. Interestingly, some systems were able to extract more by-products leaving the majority of succinic acid into the aqueous phase. Dihexylamine in 1-hexanol and tripentylamine in 1-butanol could extract the whole amount of formic acid and almost 50% of lactic acid that is less easily removed via vacuum evaporation and crystallization. The use of a pH value of 5 is a useful alternative in the case of *B. succiniciproducens* and not so much in the case of *A. succinogenes* as the latter does not produce lactic acid as by-product.

**Table 12.4** Succinic, lactic and acetic acid extraction yields using different reactive extraction systems at pH 5.

| System | Succinic acid yield | Lactic acid yield | Formic acid yield | Acetic acid yield |
|---|---|---|---|---|
| Dioctylamine + 1-octanol | 34.2 | 25.6 | 34.5 | 12.1 |
| Trihexylamine + 2-octanol | 10.7 | 36.0 | 38.9 | 36.4 |
| Trixexylamine + 1-butanol | 11.9 | 46.6 | 46.9 | 43.0 |
| Trihexylamine + 1-hexanol | 12.4 | 36.8 | 41.6 | 39.8 |
| TOA + 1-octanol | 10.6 | 34.1 | 38.1 | 36.1 |
| Diisoctylamine + 1-octanol | 13.2 | 29.1 | 32.7 | 31.7 |
| Diisoctylamine + 1-butanol | 17.4 | 52.5 | 100.0 | 54.9 |
| octylamine + 1-hexanol | 14.2 | 39.6 | 100.0 | 37.2 |
| Dihexylamine + 1-hexanol | 14.3 | 67.1 | 100.0 | 76.4 |
| Dioctylamine + 1-hexanol | 22.4 | 34.7 | 100.0 | 40.1 |
| Tripentylamine + 1-butanol | 8.9 | 56.4 | 100.0 | 42.1 |
| TOA + 1-hexanol | 16.6 | 51.7 | 100.0 | 51.0 |
| Methyldioctylamine + MIBK | 18.7 | 44.9 | 100.0 | 35.7 |
| Diisoctylamine + dixehylamine + 1-octanol + 1-hexanol | 3.5 | 7.0 | 100.0 | 47.8 |
| Diisoctylamine + 1-octanol | 14.8 | 19.8 | 100.0 | 53.0 |

In order to recover the succinic acid from the acid-amine complex as well as the amine employed (and subsequently recycle it back to the reactive extraction system), it is necessary to establish an efficient and yet economic back-extraction process. Two different back extraction techniques were tested: temperature swing and pH-swing. Two systems were selected for the back extraction tests which were distinguished from the previous experiments: TOA in 1-hexanol and dioctylamine in 1-octanol. TOA in 1-hexanol was tested in various temperature-swing trials than the other reactive extraction system. The back-extraction with pH-swing is based on the dependence of the undissociated acid to the pH value of the aqueous

solution. A higher pH value than the $pK_a$ of succinic acid inhibits the formation of the amine-acid complex making possible to back-extract the acid into the aqueous phase. An increased temperature is another way to destroy the complex, because the reactions at the interface of the aqueous and the organic phase involve proton transfer or hydrogen-bond formation and are expected to be exothermic.

In Figure 12.6, the results of back-extraction of the two selected systems using temperature and pH-swing are presented. With pH-swing, all succinic acid was recovered when TOA in 1-hexanol was used for reactive extraction, whereas almost 65% of succinic acid was back-extracted from dioctylamine in 1-octanol. As TOA is a tertiary amine the steric hindrance facilitates the back-extraction via pH-swing, while the bonding between succinic acid and dioctylamine, which is a secondary amine, is stronger (Hong and Hong, 2003). The disadvantage of this method is that the acid is recovered in a salt form and acidification is required once again. Temperature-swing was less efficient than pH-swing, as no more than 30% of succinic acid was recovered. In the case that TOA was used, the recovery yields achieved were again higher than the ones obtained with dioctylamine. Regarding by-products, all were back-extracted in the aqueous phase. Nevertheless, the processes that follow this step (i.e. evaporation and crystallization) eliminate the residual by-products leading to highly pure succinic acid crystals.

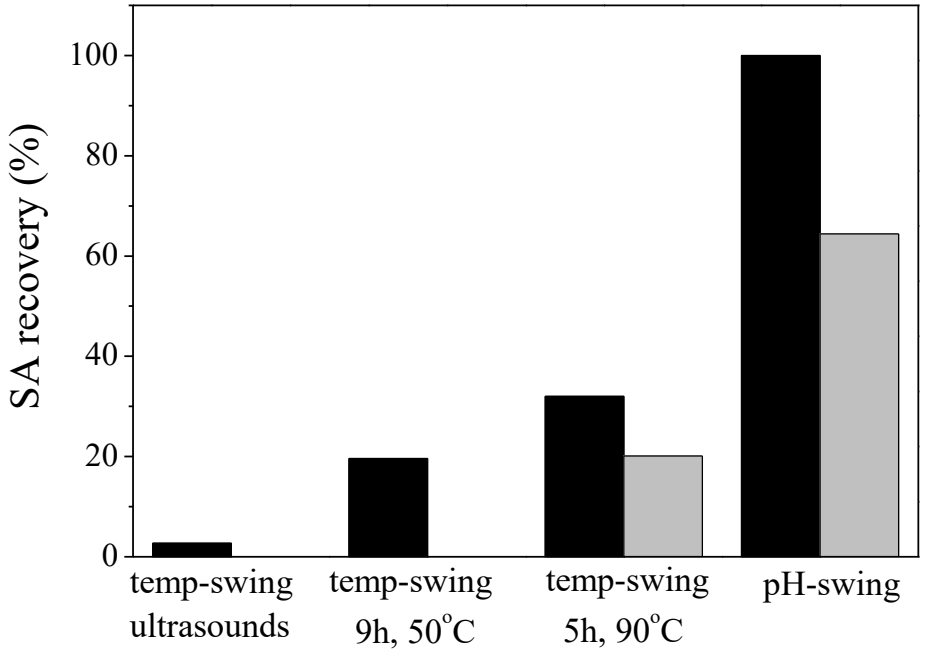



**Figure 12.6** Back-extraction of succinic acid from TOA/1-hexanol (black bars) and from dioctylamine/1-octanol (grey bars).

## 12.8. Bipolar electrodialysis membranes

- *Downstream process using BEM in fermentation broths using synthetic media*

A preliminary experiment was first carried out using the fermentation broth from *A. succinogenes* obtained using synthetic media (glucose and arabinose). After mixing the two fermentation broths the total volume was 5.3 L, having a succinic acid concentration equal to 25.8 g/L. The broth was subsequently microfiltrated in order to remove the biomass, leading to 4.7 L of a permeate stream having 25.6 g/L SA and a retentate stream (0.6 L) that contained 24.8 g/L succinic acid (Table 12.5).

From this step, product recovery accounted approximately 88%. Moreover, almost 40% of proteins (expressed as $N_{kjel}$) were removed during this step. The next step involved softening in order to remove the cations, mainly $Mg^{2+}$ and $Ca^{2+}$ that could damage the membranes. Bipolar electrodialysis was subsequently employed, generating three different streams, namely acid-rich stream, base and salt. Succinic acid was mainly found in the acid-rich stream (15.4 g/L), whereas a minor quantity was passed to the salt stream (0.30 g/L).

The final purification steps involved decolorisation, anion and cation exchange chromatography and the results obtained from each step are presented in Table 12.5. Anion exchange resins were the step that involved the highest succinic acid losses, as product recovery from the decolorisation step to chromatography was 67.6%. Nevertheless, this step reduced significantly the ions present in the succinic acid-rich stream. The final step of the downstream process involved the use of spray dryer in order to obtain succinic acid crystals. At the end of the process 47.3 g of product were recovered. ICP-MS analysis, shown in Table, reveals that the main impurity present in the final product is $SO_4^{2-}$ (6077 mg/L). Total process yield was 34.6% and product purity was 96%

**Table 12.5** Overview of downstream processing of fermentation broth from synthetic medium using glucose and arabinose.

| Purification step | Residual sugars (g/L) | Succinic acid (g/L) | Formic acid (g/L) | Acetic acid (g/L) | Total $N_{Kjel}$ (mg/L) | $P_{total}$ (mg/L) | $SO_4^{2-}$ (mg/L) | $Na^+$ (mg/L) | $Mg^{2+}$ (mg/L) | Other anions (mg/L) | Other cations (mg/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Fermentation Broth | n.d | 25.8 | 1.83 | 6.97 | 684.0 | 341.0 | 4490.0 | 13960.0 | 2166.0 | 1105.0 | 313.2 |
| Microfiltration Permeate | n.d | 25.6 | 1.82 | 7.01 | 478.0 | 1661.0 | 4100.0 | 13428.0 | 2125.0 | 1042.0 | 276.7 |
| Microfiltration Retentate | n.d | 24.8 | 1.04 | 6.47 | 2170.0 | 181.0 | 5182.0 | 14093.0 | 2021.0 | 1091.0 | 303.7 |
| Softening | n.d | 13.5 | n.d | 3.67 | 232.0 | 58.1 | 1787.0 | 9513.0 | 0.15 | 572.0 | 98.2 |
| Bipolar ED acid | n.d | 15.4 | 1.15 | 4.66 | 132.0 | 61.4 | 1951.0 | 617.3 | 0.17 | 809.0 | 8.1 |
| Bipolar ED base | n.d | n.d | n.d | n.d | n.d | 3.1 | 26.5 | 16927.0 | n.d | 22.0 | 112.9 |
| Bipolar ED salt | n.d | 0.30 | n.d | n.d | 120.0 | 11.3 | 56.5 | 292.0 | 0.12 | 1.0 | 2.4 |
| Decolorization | n.d | n.d | n.d | 3.2 | 10.1 | 42.2 | 1511.0 | 18.5 | 0.06 | 549.0 | 0.74 |
| Anion exchange resins | n.d | 6.60 | n.d | 5.3 | 3.89 | 22.1 | 1054.0 | 13.9 | 0.07 | 0.9 | 1.6 |
| Cation exchange resins | n.d | 5.9 | n.d | 4.7 | 0.15 | <4.0 | 977.0 | 0.5 | 0.03 | 1.3 | 0.7 |
| Succinic acid solution after spray-dryer | n.d | n.d | n.d | 48.0 | 35.7 | 24.8 | 6077.0 | 14.9 | 0.56 | n.d | 13.3 |

- *Downstream process based on BEM using fermentation broth using SBP-based madia*

At the end of the pilot scale fermentation, 43.4 L of fermentation media containing approximately 30 g/L of sodium and magnesium succinate were collected. Besides succinate, there was also an amount of acetate (about 10 g/L) and residual sugars (4.4 g/L, mainly arabinose and galactose). There was a considerable amount of $SO4^{2-}$ (Table 12.6), due to the acid pretreament of SBP. As in the case of the synthetic medium the first step involved microfiltration in order to separate the biomass and other solids. Recovery of succinate in the permeate stream was approximately 86%, whilst there were some losses in the retentate. These losses could be eliminated if the retentate is recycled, aiming also to recover the remaining nutrients that are still present (and they are also concentrated) in this stream.

Before the succinate- rich stream was introduced in the monopolar electrodialysis configuration, softening was carried out in order to remove magnesium and calcium salts. This step resulted in a concentrate stream that contained 56 g/L of succinate, as well as a diluate, having less than 2 g/L of succinic acid. Before introducing the concentrate stream in the bipolar electrodialysis configuration, a second softening step was again required since the concentration of magnesium and calcium cations was high (Table 12.6).

Bipolar electrodialysis resulted in three different streams, the acid, the base and the salt stream. Succinic acid was mainly present in the acid stream (34.9 g/L), whereas losses of about 18% were observed in the salt stream. The acid stream was further treated with decolorising resins as well as anion- and cation- exchange chromatography in order to remove more impurities that are still present in the succinic acid rich stream. As it was observed also in the synthetic fermentation medium, the highest overall process losses occurred after the anion exchange chromatography, since only 40% of succinic acid was recovered.

Succinic acid-rich stream generated from anion exchange chromatography was finally introduced into the spray dryer. In the end of the process, 270 grams of succinic acid were recovered. The overall yield of the process was 20.7% and crystal purity was 79%. The main impurities were the residual sugars (about 6 g/L in a succinic acid solution of 50 g/L), as well as $SO4^{2-}$ with a concentration of 5.4 g/L. Both yield and purity are very low in comparison to other studies that present recovery rates of about 80% and purity of more than 99%. Optimization of the overall process could also lead to higher product recovery yields as well as higher purity

**Table 12.6** Overview of downstream process of SBP fermentation broth

| Purification step | Residual sugars (g/L) | Succinic acid (g/L) | Formic acid (g/L) | Acetic acid (g/L) | Total $N_{Kjel}$ (mg/L) | $P_{total}$ (mg/L) | $SO_4^{2-}$ (mg/L) | $Na^+$ (mg/L) | $Mg^{2+}$ (mg/L) | Other anions (mg/L) | Other cations (mg/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Fermentation Broth | 4.4 | 30.0 | n.d | 10.0 | 1705.0 | 296.0 | 12468.0 | 22970.0 | 1812.0 | 723.0 | 1025.0 |
| Microfiltration Permeate | 4.3 | 29.5 | n.d | 9.5 | 927.0 | 114.0 | 7761.0 | 17159.0 | 273.0 | 476.0 | 455.0 |
| Microfiltration Retentate | 4.1 | 24.5 | n.d | 9.9 | 5957.0 | 1320.0 | 11954.0 | 22826.0 | 1791.0 | 686.0 | 990.0 |
| Softening | 1.9 | 19.6 | n.d | 11.8 | 641.0 | 79.1 | 7558.0 | 16999.0 | 270.0 | 462.0 | 451.0 |
| Monopolar ED concentrate | n.d | 56.0 | n.d | 34.8 | 299.0 | 100.0 | 22503.0 | 45709.0 | 747.0 | 1635.0 | 1303.0 |
| Monopolar ED diluate | 0.94 | 1.94 | n.d | n.d | 747.0 | 52.1 | 445.0 | 2469.0 | 7.99 | 3.1 | 26.0 |
| Softening | n.d | 31.4 | n.d | 13.5 | 173.0 | 55.5 | 13430.0 | 26133.0 | 0.13 | 927.0 | 618.0 |
| Bipolar ED acid | n.d | 34.9 | n.d | 13.2 | 119.0 | 73.5 | 19713.0 | 1821.0 | n.d | 1430.0 | 52.0 |
| Bipolar ED base | n.d | n.d | n.d | n.d | 33.6 | <4.0 | 55.4 | 24051.0 | n.d | 23.0 | 482.0 |
| Bipolar ED salt | n.d | 7.23 | n.d | 5.0 | 102.0 | <4.0 | 986.0 | 555.0 | 0.10 | 5.8 | 6.3 |
| Decolorization | n.d | 24.0 | n.d | 10.1 | 43.3 | 52.1 | 13282.0 | 1539.0 | 0.13 | 987.7 | 29.3 |
| Anion exchange resins | n.d | 6.3 | n.d | 6.5 | 25.5 | - | 789.0 | 914.4 | 0.44 | 3.7 | 45.4 |
| Cation exchange resins | n.d | 6.0 | n.d | 6.3 | 10.0 | - | 746.0 | 0.41 | 0.03 | 4.2 | 0.8 |
| Condensate vacuum distillation | n.d | 11.47 | n.d | 10.10 | n.d | - | 1447.0 | 3.53 | 0.14 | 17.3 | 8.9 |
| Succinic acid solution after spray-dryer | 5.78 | 39.5 | n.d | n.d | 9.61 | - | 5412.0 | 12.9 | 0.18 | 7.8 | 7.6 |

## 12.9. Conclusions

Succinic acid recovery and purification is a demanding process involving many steps in order to achieve the purity required for biopolymer production. The purity of the succinic acid crystals has to be around 99.9%, while the recovery yield should be as high as possible in order to maintain the succinic acid production cost at low levels. An overview of the results obtained from the evaluation of four different downstream separation schemes are presented in Table 12.7. In terms of succinic acid yield and purity, direct crystallization using ion-exchange resins is the most promising downstream separation scheme as it leads to the highest succinic acid recovery yield and more than 95% purity of succinic acid crystals. However, reactive extraction can also be considered as an alternative method as final succinic acid purity reached 97.2%.

**Table 12.7** Summary of the downstream separation processes tested for succinic acid purification in terms of succinic acid yield and purity achieved

| Method | Yield (%) | Purity (%) |
|---|---|---|
| Calcium precipitation | 13 | 81.0 |
| Direct crystallisation / acidification | 57 | 90.0 |
| Direct crystallisation / Resins | 79 | 96.0 |
| Salting-out extraction | 50 | 86.0 |
| Reactive Extraction | 73 | 97.2 |
| BEM | 20.7 | 79.0 |

# CHAPTER 13
# CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Biotechnological production of succinic acid is of major importance as this di-carboxylic acid is nowadays considered as a key platform chemical for the bio-economy era. Succinic acid finds already numerous industrial applications, from the food industry to the production of bio-polymers such as polybutylene succinate. In the bio-economy era, the applications of succinic acid will expand to the production of different bulk/ intermediate chemicals as for example the production of 1,4-butanediol. However, upstream and downstream separation costs still hinder its large scale production using microbial fermentations.

Lignocellulosic biomass is characterized by its abundance as well as its compositional diversity. As it contains more than 70% of sugars, it is an excellent substrate for the biotechnological production of chemicals, such as succinic acid. As it was highlighted in Chapter 1, one of the main impediments for biorefinery development is the transportation of the feedstock to centralised facilities. The incorporation of the biotechnological conversion of waste and by-product streams on site, in already existing industrial sectors would provide a smoother transition to the bio-economy era. Two industrial sectors that are favorable for biorefinery development is the pulp and paper industry and the sugar industry.

SSL is a by-product stream generated in vast amounts during the acidic sulphite pulping process in the pulp and paper mills. As it contains high concentrations of fermentable sugars, it can be employed for the production of fermentation products. However, more than 70% of its carbohydrate content is consisted of C5 sugars that many microbial strains are not able to utilize. Moreover, its high content of LS (lignin fragments produced during sulphonation, Chapter 2), together with the presence of phenolic compounds, acetic acid and furans may inhibit fermentation efficiency. The valorization of SSL for the production of chemicals has not yet been studied in detail. In this thesis, SSL was evaluated as fermentation substrate for the biotechnological production of succinic acid using two wild-type strains, namely: *A. succinogenes* and *B. succiniciproducens* and at the same it was fractionated to value-added products.

In Chapter 7, the extraction of phenolic compounds from SSL using ethyl acetate was optimized. Using design of experiments of the main parameters affecting extraction efficiency (initial pH of the diluted SSL and solvent-to-liquid ratio) the highest TPC value equal to 7.5 g

GAE/L SSL was achieved at the optimum conditions (pH of the ten times diluted SSL equal to 2.22 and solvent-to-liquid ratio of 3.67:1). The extract also exhibited very strong antioxidant activity (AAI=3.64), rendering a potential marketable product for utilization as active ingredient in various applications. The main phenolic compounds present in the extract were also identified and ellagic and gallic acids were found to be the predominant ones. The aqueous stream produced after solvent extraction of phenolic compounds was then subjected to microbial fermentation for succinic acid production. Both studied strains performed better when SSL treated with solvent extraction was employed as fermentation media. Especially *B. succiniciproducens*, whose growth was highly inhibited in seven times diluted and untreated SSL, was able to grow and produce succinic acid in the detoxified stream.

The next step was to enhance succinic acid production and fractionate SSL to more value-added products (Chapter 8). Solvent extraction using acetone and isopropanol was studied for LS separation from SSL. Extraction with isopropanol resulted in higher LS removal than acetone (80%). The pretreated SSL with isopropanol was then used as fermentation media for fed-batch fermentations using *A. succinogenes* and *B. succiniciproducens*, resulting in 19 g/L of succinic acid concentration in both cases. When solvent extraction with ethyl acetate (for phenolic compounds removal) was coupled with nanofiltration (for LS separation), the produced detoxified sugar-rich stream led to the production of 39 g/L of succinic acid when the strain *B. succiniciproducens* was employed. This fractionation scheme resulted to the production of 1.15 g of an antioxidant-rich extract, 28.1 g LS and succinic acid and 4.28 g succinic acid from 100 g SSL. The results of Chapter 8 indicate that conventional pulp and paper mills could be converted into integrated biorefineries.

In Chapter 9, cell immobilization was applied in order to increase the final succinic acid concentration as well as succinic acid yield and productivity from SSL. Two different supports were evaluated; delignified cellulosic material (DCM) and alginate beads. Both techniques were firstly tested in seven times untreated SSL in repeated batch fermentations using the strain *A. succinogenes*. When the strain was immobilized on DCM, succinic acid production was increased to 64.5%. The same biocatalyst was also used in three sequential batch fermentations, using again untreated seven times diluted SSL, but succinic acid production was declining in each cycle. More stable results were obtained when cells were immobilized in alginate beads, with higher yields and productivities in comparison to the repeated batch fermentations using DCM as support. The alginate beads were re-used in 5 sequential repeated batch fermentations using untreated SSL, and succinic acid yield was

from 0.58-0.71 (g/g) in comparison to 0.51-0.57 (g/g) obtained from DCM fermentations. Since LS were the main inhibitory compound, even when immobilization was employed, ultrafiltration was subsequently tested. Fed-batch fermentations with *A. succinogenes* immobilized in DCM resulted in the production of 27.3 g/L succinic acid, with a yield of 0.65 g/g, whilst immobilization in alginate beads led to the production of 36.8 g/L succinic acid with a much higher yield (0.81 g/g). Since nanofiltration was proved to be an effective method when free cells were utilized, SSL pretreated via nanofiltration was employed as fermentation substrate with immobilized cells in alginate beads. This technique was tested also on *B. succiniciproducens*. The succinic acid production from both strains was significantly enhanced and the highest values in this thesis were obtained. More specifically, *A. succinogenes* produced 35.4 g/L of succinic acid (40.5% increment in comparison to free cells), whereas succinic acid production by *B. succiniciproducens* reached a value of 45 g/L, with a yield of 0.66 g/g. The value obtained by *B. succiniciproducens* is the highest reported in the literature so far, from the conversion of an industrial waste containing mainly C5 sugars and from a wild-type strain. Finally, since the best results were obtained with *B. succiniciproducens*, this strain was evaluated on repeated fed-batch fermentations. It was possible to preform four sequential fed-batch fermentations, leading to the production of 64.7 g succinic acid out of 114.55 g of consumed sugars.

Besides SSL, another industrial by-product, sugar beet pulp (SBP) was fractionated to value-added co-products. The free phenolic compounds were extracted using solid-to-liquid extraction with aqueous ethanol. The total phenolic content of the extract was 1.63 mg GAE/g SBP, with a radical scavenging activity of 49%. The main phenolic compounds identified in the extract were catechol at a concentration of 640.5 ppm and epicatechin at a concentration of 118.4 ppm. The next fraction with added-value was pectins that were separated from SBP by using HCl. The remaining solids were subjected to acid pretreatment and enzymatic hydrolysis in order to produce a sugar-rich substrate for succinic acid fermentations. In the best hydrolysis conditions (10% solid loading, 0.5% $H_2SO_4$ at 121 $^oC$ for 30 min and addition of 0.5 mL Accellerase /g cellulose), the hydrolysate presented a total sugar concentration of 40 g/L. Microbial fermentations with the aforementioned bacterial strains showed that SBP-derived hydrolysate is a suitable substrate for bio-based succinic acid. In contrast to SSL-based media, *A. succinogenes* performed better in this hydrolysate. In fed-batch fermentation, the strain was able to produce about 30 g/L of succinic acid, with a yield of 0.94 g/g, without any pretreatment. The process was successfully scaled-up in pilot scale, giving comparable results to the lab-scale fermentation.

Five different downstream separation methods were evaluated considering the final yield and purity of succinic acid crystals. Two methods were the most promising; direct crystallisation using cation-exchange resins and reactive extraction. Direct crystallisation method yielded in 79% succinic acid crystals, with a purity of 96%, while the yield of succinic acid separated through reactive extraction was 73%, with a crystal purity of 97.2%.

The results obtained in this study regarding the development of advanced biorefineries based on industrial waste streams indicate a promising proposal on the integration of bio-processes. Nevertheless, more research should be carried out aiming to further improve the proposed biorefinery concept. The main subjects that should be optimized include: 1) the improvement of the bioconversion of both by-product streams in terms of yield and productivity, 2) especially in case of SBP-derived hydrolysates an efficient pretreatment method should be developed in order to increase succinic acid production, 3) fractionation of phenolic compounds from both streams and study on their potential applications (*e.g.* for wood protection), 4) techno-economic evaluation of the proposed biorefinery schemes in order to assess the economic viability of the process and 5) Life cycle analysis.

Regarding the bacterial bioconversion process, even though the concentration achieved from the immobilized cells of *B. succiniciproducens* is among the highest in the literature obtained from xylose, yield and productivity should be further optimized. A possible approach is the utilization of corn steep liquor that is been currently suggested by some researchers. The bioprocess based on SBP, should be improved both in terms of final succinic acid and productivity. In this thesis, it has been shown that after the pretreatment more phenolic compounds are released. In Chapter 7, it has been also reported that both microbial strains are affected by the presence of phenolic compounds in their environment. The effective removal of the phenolic compounds from the SBP hydrolysates (*e.g.* via absorption with resins) would possibly increase fermentation efficiency and at the same one more stream rich in antioxidants could be co-produced.

The phenolic-rich extracts from both by-product streams contain well-known antioxidants and the DPPH assay revealed their antioxidant activity. Additional fractionation could result in different phenolic streams, with different antioxidant activities (possibly stronger) and hence different functionalities. Separation of different phenolic fractions would give more value to the process, thus lowering the upstream cost even more. These fractions could be also tested in order to completely assess their potential applications, as for example their toxicity towards fungi that attack on wood.

Techno-economic evaluation is crucial for assessing the economic viability of the overall proposed biorefineries. The cost of every process should be calculated together with the potential profit that could occur from the fractionation of either SSL or SBP. Finally, the application of Life Cycle Analysis (LCA) would complete the sustainability assessment of SSL- and SBP-based biorefineries. LCA development would help to identify and revise accordingly the processes that consume more energy in order to establish a complete sustainable procedure.

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***Hyperlinks***

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