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

PhD Thesis

Bioprocess development and transcriptomic analysis for succinic acid production via fermentation using waste streams from the pulp and paper industry

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Athens, 2018

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Ανάπτυξη βιοδιεργασιών και μεταγραφομική ανάλυση για την παραγωγή ηλεκτρικού οξέος μέσω μικροβιακών ζυμώσεων με χρήση αποβλήτων από βιομηχανικές διεργασίες παραγωγής χαρτιού

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Bioprocess development and transcriptomic analysis for succinic acid production via fermentation using waste streams from the pulp and paper industry

Department of Food Science & Human Nutrition
Laboratory of Food Engineering, Processing & Preservation of Agricultural Products

Abstract

The scope of this thesis is the evaluation of spent sulphite liquor (SSL) derived as by-product stream from the sulphite pulping process of *Eucalyptus globulus* wood for the production of succinic acid via microbial fermentations. The study was initiated with the analysis of the composition of SSL and the evaluation of the inhibitory effect of SSL components and metabolic products on succinic acid production and bacterial growth. The evaluation of acetic acid, furfural and methanol showed that concentrations of 12, 1 and 4 g/L, respectively, are inhibitory for *Actinobacillus succinogenes* growth, while concentrations of 12, 3 and 8 g/L, respectively, are inhibitory for *Basfia succiniciproducens* growth. Lignosulphonates also posed major inhibitory effect on succinic acid production at concentrations higher than 50 g/L. Based on the high lignosulphonate concentration of the thick SSL used in this study, it was concluded that pretreatment of SSL should be carried out before fermentation. The critical concentrations of succinic acid, lactic acid, formic acid and acetic acid that inhibit bacterial growth and succinic acid production were 55, 60, 18 and 38 g/L, respectively.

Ultrafiltration and nanofiltration of SSL was employed in order to evaluate the sequential extraction of lignosulphonates and the production of bio-based succinic acid using the bacterial strains *A. succinogenes* and *B. succiniciproducens*. Furthermore, this pretreatment step produced a permeate liquid stream with low lignosulphonate content that led to efficient succinic acid production. Ultrafiltration with membranes of 10, 5 and 3 kDa molecular weight cut-off result in significant losses of lignosulphonates (26 - 50%) in the permeate stream, while nanofiltration using membrane with 500 Da molecular weight cut-off results in high retention yields of lignosulphonates (95.6%) in the retentate stream and 66% of total sugars in the permeate stream. Fed-batch bioreactor cultures using permeates from ultrafiltrated SSL resulted in similar succinic acid concentration (27.5 g/L) and productivity (0.4 g/L/h) by both strains. When permeates from nanofiltrated SSL were used, the strain *B. succiniciproducens* showed the highest succinic acid concentration (33.8 g/L), yield (0.58 g per g of consumed sugars) and productivity (0.48 g/L/h). Ultrafiltration of SSL resulted in higher succinic acid production per t of SSL used, whereas nanofiltration resulted in higher LS recovery per t of SSL used. The nanofiltration of 1 t of thick SSL could lead to the production of 306.3 kg of lignosulphonates and 52.7 kg of succinic acid when *B.*

succiniciproducens is used or 51.8 kg of succinic acid when *A. succinogenes* is used. The ultrafiltration of 1 t of thick SSL using a 3 kDa membrane could result in the production of 237 kg of lignosulphonates and 71.8 kg of succinic acid when *B. succiniciproducens* is used.

The metabolic potential of *A. succinogenes* was evaluated through RNA expression of the genes that encode the enzymes involved in succinic acid production when the bacterial strain was cultivated on glucose, xylose and SSL. Ultrafiltrated SSL was selected as the substrate to analyse RNA expression levels, which were compared with respective expression levels observed in glucose and xylose bioreactor cultures. Xylose and glucose were selected because they constitute 73% and 10% of the total sugars contained in SSL. A transcriptomic approach of the key enzymes of glucose and xylose catabolism, carboxylic acid production as well as oxidative phosphorylation led to an improved understanding on the energy consuming metabolic pathways. The transcriptomic analysis was carried out in batch cultures. A cDNA library was constructed at different phases of the fermentation where major metabolic changes in extracellular metabolites or biomass production were observed. RT-qPCR was used to determine the expression levels of the genes of interest throughout the fermentation. The bottlenecks of the fermentative production of succinic acid by *A. succinogenes* were addressed with particular focus on the effect of glucose and xylose catabolism on pathways that involve ATP consumption and NADH oxidation. All subunits of ATP synthase were highly expressed in SSL. In particular, ATP synthase F0 was higher expressed in SSL. Phosphoenol-pyruvate carboxykinase (*PEPCK*) expression was delayed when xylose was present in the medium. Despite the fact that extracellular lactic acid was not detected low expression levels of lactic acid dehydrogenase (*LDH*) were observed in all substrates.

Finally, a breakthrough technology was applied in fed-batch *B. succiniciproducens* cultures that integrates succinic acid production via fermentation and *in situ* separation via electrochemical membrane extraction. The current drives the charged carboxylic acids across an anion exchange membrane by electromigration from the high-volume bioreactor into a low-volume extract that contains the succinic acid in higher concentrations. These membranes are permeable to many carboxylic acid anions (e.g. acetic acid, succinic acid), but impermeable to cells and solids, resulting in a combined extraction, clarification, acidification and concentration step in a single unit.

Scientific area: Industrial Biotechnology

Keywords: Bioprocessing, Fermentation, Renewable resources, Succinic acid, Transcriptomics

Ανάπτυξη βιοδιεργασιών και μεταγραφομική ανάλυση για την παραγωγή ηλεκτρικού οξέος μέσω μικροβιακών ζυμώσεων με χρήση αποβλήτων από βιομηχανικές διεργασίες παραγωγής χαρτιού

Τμήμα Επιστήμης Τροφίμων & Διατροφής του Ανθρώπου
Εργαστηριο Μηχανικής & Επεξεργασίας Τροφίμων

Περίληψη

Ο σκοπός της διατριβής αυτής έγκειται στην αξιολόγηση του παράπλευρου ρεύματος που παράγεται από βιομηχανίες χαρτοπολτού (SSL) για την παραγωγή ηλεκτρικού οξέος μέσω μικροβιακών ζυμώσεων. Η μελέτη επικεντρώθηκε αρχικά στην ποσοτικοποίηση των κυριοτέρων συστατικών. Ακολούθως μελετήθηκε η παρεμπόδιση συγκεκριμένων συστατικών του SSL στην παραγωγή ηλεκτρικού οξέος και στην μικροβιακή ανάπτυξη. Οι συγκεντρώσεις του οξικού οξέος, της φουρφουράλης και της μεθανόλης που παρεμποδίζουν την ανάπτυξη του *Actinobacillus succinogenes* είναι 12, 1 and 4 g/L αντίστοιχα, ενώ οι αντίστοιχες συγκεντρώσεις για τον *Basfia succiniciproducens* είναι 12, 3 and 8 g/L, αντίστοιχα. Τα λιγνοσουλφονικά άλατα αποτελούν παρεμποδιστικά συστατικά σε συγκεντρώσεις μεγαλύτερες από 50 g/L. Το γεγονός ότι το SSL που χρησιμοποιήθηκε σε αυτή τη μελέτη περιέχει υψηλή συγκέντρωση λιγνοσουλφονικών αλάτων οδήγησε στην απόφαση να προεπεξεργαστούμε το αρχικό SSL μέσω υπερδιήθησης ή νανοδιήθησης. Οι συγκεντρώσεις του ηλεκτρικού οξέος, του γαλακτικού οξέος, του μυρμηγκικού οξέος και του οξικού οξέος που παρεμποδίζουν την παραγωγή ηλεκτρικού οξέος είναι 55, 60, 18 και 38 g/L τόσο για τον *A. succinogenes* όσο και για τον *B. succiniciproducens*.

Η επεξεργασία του SSL μέσω υπερδιήθησης ή νανοδιήθησης αξιολογήθηκε προκειμένου να εκτιμήσουμε τη δυνατότητα διαχωρισμού λιγνοσουλφονικών αλάτων και σακχάρων τα οποία χρησιμοποιήθηκαν ακολούθως για την παραγωγή ηλεκτρικού οξέος με χρήση των στελεχών *A. succinogenes* και *B. succiniciproducens*. Η χρήση υπερδιήθησης με μεμβράνες με μοριακό βάρος αποκοπής της τάξης των 10, 5 και 3 kDa είχε ως αποτέλεσμα την απώλεια σημαντικών ποσοτήτων λιγνοσουλφονικών αλάτων (26 - 50%) στο διήθημα. Η χρήση νανοδιήθησης με μεμβράνες με μοριακό βάρος αποκοπής της τάξης των 500 Da οδήγησε σε υψηλό βαθμό διαχωρισμού λιγνοσουλφονικών αλάτων (95.6%). Η πραγματοποίηση ζυμώσεων ημι-διαλείποντος έργου με χρήση διηθήματος από επεξεργασμένο SSL μέσω υπερδιήθησης οδήγησε στην παραγωγή 27.5 g/L ηλεκτρικού οξέος με παραγωγικότητα 0.4 g/L/h όταν χρησιμοποιήθηκαν και οι δύο μικροοργανισμοί. Η υψηλότερη συγκέντρωση ηλεκτρικού οξέος (33.8 g/L), παραγωγικότητα (0.48 g/L/h) και βαθμός μετατροπής σακχάρων σε ηλεκτρικό οξύ (0.58 g/g) επετεύχθησαν με το στέλεχος *B.*

succiniciproducens όταν αναπτύχθηκε σε διήθημα που παρήχθη μέσω νανοδιήθησης του SSL. Η νανοδιήθηση 1 τόνου SSL δύναται να οδηγήσει στην παραγωγή 306.3 kg λιγνοσουλφονικών αλάτων και 52.7 kg ηλεκτρικού οξέος όταν χρησιμοποιηθεί ο μικροοργανισμός *B. succiniciproducens*. Η υπερδιήθηση 1 τόνου SSL με χρήση μεμβράνης με μοριακό βάρος αποκοπής της τάξης των 3 kDa δύναται να οδηγήσει στην παραγωγή 237 kg λιγνοσουλφονικών αλάτων και 71.8 kg ηλεκτρικού οξέος όταν χρησιμοποιηθεί ο μικροοργανισμός *B. succiniproducens*.

Η δυναμική του μεταβολισμού του *A. succinogenes* αξιολογήθηκε μέσω της έφρασης του RNA των γονιδίων που είναι υπεύθυνα για τα ένζυμα που συμμετέχουν στην παραγωγή ηλεκτρικού οξέος, σε υποστρώματα που περιέχουν γλυκόζη, ξυλόζη και στο παραπροϊόν της βιομηχανίας χαρτοπολτού. Επιλέχθηκε η μέθοδος της υπερδιήθησης για την προεπεξεργασία του SSL που χρησιμοποιήθηκε σαν υπόστρωμα για την μελέτη της έκφρασης του RNA. Πραγματοποιήθηκε σύγκριση των αποτελεσμάτων από τη ζύμωση σε SSL με ζυμώσεις που πραγματοποιήθηκαν σε γλυκόζη ή ξυλόζη. Η ξυλόζη και η γλυκόζη επιλέχθηκαν διότι αποτελούν τα κύρια σάκχαρα που περιέχονται στο SSL με περιεκτικότητα 73% και 10%, αντίστοιχα. Η μεταγραφομική ανάλυση των σημαντικότερων ενζύμων που συμμετέχουν στον καταβολισμό της γλυκόζης και της ξυλόζης, στον κύκλο των τρικαρβοξυλικών οξέων και της οξειδωτικής φωσφορυλίωσης, οδήγησαν στην καλύτερη κατανόηση των μεταβολικών διαδικασιών. Η μεταγραφομική ανάλυση πραγματοποιήθηκε σε ζυμώσεις διαλείποντος έργου σε εργαστηριακό βιοαντιδραστήρα. Σε διαφορετικές φάσεις της ζύμωσης, όπου παρατηρήθηκαν αλλαγές στους εξωκυτταρικούς μεταβολίτες, κατασκευάστηκαν cDNA βιβλιοθήκες. Τα επίπεδα έκφρασης των γονιδίων «στόχων», σε διαφορετικές χρονικές στιγμές κατά τη διάρκεια της ζύμωσης, αναλύθηκαν με τη χρήση της RT-qPCR. Τα καθοριστικά σημεία του μεταβολισμού του *A. succinogenes* αναγνωρίστηκαν. Ιδιαίτερο ενδιαφέρον παρουσίασαν τα μεταβολικά μονοπάτια που συμμετέχουν στην κατανάλωση ATP και στην οξείδωση του NADH σε συνδυασμό με τον καταβολισμό της γλυκόζης και της ξυλόζης. Όλες οι υπομονάδες της ATP συνθάσης υπερεκφράστηκαν στο SSL. Συγκεκριμένα, η F0 περιοχή της ATP συνθάσης υπερεκφράστηκε στο SSL. Η έκφραση της φωσφοενολοπυροσταφυλικής καρβοξυκινάσης (*PEPCK*) ήταν χαμηλότερη όταν το θρεπτικό μέσο περιείχε ξυλόζη. Παρά το γεγονός ότι δεν ανιχνεύθηκε εξωκυτταρικό γαλακτικό οξύ, ανιχνεύθηκε η έκφραση της γαλακτικής δεϋδρογονάσης (*LDH*) σε όλα τα υποστρώματα.

Τέλος, μία καινοτόμος τεχνολογία εφαρμόσθηκε σε καλλιέργειες ημι-διαλείποντος έργου με τον *B. succiniciproducens*, η οποία συνδυάζει την παραγωγή ηλεκτρικού οξέος μέσω ζύμωσης με ταυτόχρονο διαχωρισμό του ηλεκτρικού οξέος μέσω μίας ηλεκτροχημικής κυψέλης στην οποία χρησιμοποιείται μία ανιονική μεμβράνη. Το ηλεκτρικό ρεύμα οδηγεί τα

καρβοξυλικά οξέα από το υγρό της ζύμωσης, διαμέσου της ανιονικής μεμβράνης, σε ένα μικρότερου όγκου διάλυμα το οποίο περιέχει υψηλή συγκέντρωση ηλεκτρικού οξέος. Οι μεμβράνες αυτές είναι διαπερατές σε πολλά καρβοξυλικά οξέα (π.χ. οξικό οξύ, ηλεκτρικό οξύ), αλλά δεν είναι διαπερατές από μικροβιακά κύτταρα και στερεά συστατικά. Αυτό έχει ως αποτέλεσμα να επιτυγχάνεται διαχωρισμός του ηλεκτρικού οξέος από το υγρό της ζύμωσης σε συνδυασμένο με την ταυτόχρονη μετατροπή του άλατος των καρβοξυλικών οξέων στην όξινη μορφή γεγονός το οποίο οδηγεί στη μείωση των σταδίων καθαρισμού του ηλεκτρικού οξέος (π.χ. απομάκρυνση μικροβιακών κυττάρων και προσμίξεων που περιέχονται στο υγρό της ζύμωσης).

Επιστημονική περιοχή: Βιομηχανική βιοτεχνολογία

Λέξεις-κλειδιά: Βιοδιεργασίες, Ζύμωση, Ανανεώσιμες πρώτες ύλες, Ηλεκτρικό οξύ, Μεταγραφομική

Dissemination activities and research visits

List of publications - Related to this thesis

Henrik Almqvist, **Chrysanthi Pateraki**, Maria Alexandri, Apostolis Koutinas, Gunnar Lidén, Succinic acid production by *Actinobacillus succinogenes* from batch fermentation of mixed sugars. Journal of Industrial Microbiology & Biotechnology 43 (2016) 1117-1130. doi:10.1007/s10295-016-1787-x

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Chrysanthi Pateraki, Henrik Almqvist, Dimitris Ladakis, Gunnar Liden, Apostolis A Koutinas, Anestis Vlysidis, Modeling succinic acid fermentation using a xylose based substrate, Biochemical Engineering Journal 113 (2016) 26-41. doi:10.1016/j.bej.2016.06.011

Chrysanthi Pateraki, Maria Patsalou, Anestis Vlysidis, Nikolaos Kopsahelis, Colin Webb, Apostolis A. Koutinas and Michalis Koutinas, *Actinobacillus succinogenes*: Advances on succinic acid production and prospects for development of integrated biorefineries. Biochemical Engineering Journal 112 (2016) 285-303. doi:10.1016/j.bej.2016.04.005

List of publications - Not related to this thesis

Vasiliki Liakou, **Chrysanthi Pateraki**, Anastasia-Marina Palaiogeorgou, Nikolaos Kopsahelis, Aline Machado de Castro, Denise Maria Guimarães Freire, George-John E. Nychas, Seraphim Papanikolaou, Apostolis Koutinas, Valorisation of fruit and vegetable waste from open markets for the production of 2,3-butanediol. Food and Bioproducts Processing, under revision

Sofia Maina, **Chrysanthi Pateraki**, Nikolaos Kopsahelis, Spiros Paramithiotis, Eleftherios H. Drosinos, Seraphim Papanikolaou, Apostolis Koutinas, Microbial oil production from various carbon sources by newly isolated oleaginous yeasts. Engineering in Life Sciences. 00 (2016) 1–12. doi:10.1002/elsc.201500153

Conference oral presentations - related to this thesis

Chrysanthi Pateraki, Stephen Andersen, Apostolis Koutinas, Korneel Rabaey. Enhanced succinic acid production by *Basfia succiniciproducens* through integrated fermentation with electrolytic membrane extraction. 10th Congress of Chemical Engineering, 1-5 October 2017, Barcelona, Spain

Chrysanthi Pateraki, Georgios Karalias, Dimitrios Skliros, Emmanouil Flemetakis, Apostolis Koutinas. Succinic acid production from pulp and paper industry waste - A transcriptomic approach. Biochemical and Molecular Engineering XX. 16-20 July 2017, Newport Beach, CA, USA

Apostolis Koutinas, Maria Alexandri, **Chrysanthi Pateraki**, Anestis Vlysidis, Harris Papapostolou, Development of an advanced biorefinery consept based on valorization of pulp and paper industry waste streams, 16th European congress on Biotechnology, 13-16 July 2014, Edinburgh, UK

Henrik Almqvist, **Chrysanthi Pateraki**, Apostolis Koutinas, Gunnar Liden, Optimal CO_2 supply for succinate production by *A. succinogenes* using mixed sugars. 10th international Conference on Rebewable Resources and Biorefineries (RRB10). 4-6 June 2014, Valladolid, Spain,

Chrysanthi Pateraki, Maria Alexandri, Dimitris Ladakis, Harris Papapostolou, Anestis Vlysidis, Apostolis Koutinas, Pretreatment of pulp and paper industry by-product stream for the production of lignosulphonates and succinic acid, 247th American chemical society national meeting and exposition, 16-20 March 2014, Dallas, Texas, USA

Chrysanthi Pateraki, Maria Alexandri, Harris Papapostolou, Anestis Vlysidis, Seraphim Papanikolaou, Apostolis Koutinas, Valorization of by-product streams from the pulp and paper industry for succinic acid production. TechConnect World – CleanTech Conference and Showcase. 12-16 May 2013, Washington, USA.

Conference oral presentations - not related to this thesis

Maria Patsalou, **Chrysanthi Pateraki**, Marlen Vasquez, Chryssoula Drouza and Michalis Koutinas, Bioprocess development for the production of succinic acid from orange peel waste. 4th international conference on sustainable solid management 23-25 June 2016, Limassol, Cyprus

Dimitris Ladakis, Anestis Vlysidis, Maria Alexandri, **Chrysanthi Pateraki**, Ioannis Kookos, Seraphim Papanikolaou, Apostolis Koutinas, Succinic acid production using wastes drom pulp and paper industry in continuous fermentation mode. 6th international conference on Engineering for Waste and Biomass Valorisation. 23 - 26 May 2016, Albi, France

Sofia Maina, Aikaterini Papadaki, **Chrysanthi Pateraki**, Nikolaos Kopsahelis, Seraphim Papanikolaou, Apostolis Koutinas. Microbial oil production by newly isolated yeast strains and novel industrial applications based on waste and by-product valorisation. 3rd International Conference on Sustainable Solid Waste Management, 2-4 July 2015, Tinos, Greece.

Chrysanthi Pateraki and Apostolis Koutinas. Optimising the use of fermentation technologies for exploiting food wastes. Total Food, 11-13 November 2014 Norwich, UK. (Invited speaker - Replacement of Apostolis Koutinas)

Conference poster presentations - related to this thesis

Stephen Andersen, **Chrysanthi Pateraki**, Kristof Verbeeck, Apostolis Koutinas, Korneel Rabaey. Direct electrochemical recovery of succinic acid from sugar and industrial by-products from pulp and paper industry. 2nd International Recovery Conference, 5-9 August 2017, New York, USA

Anestis Vlysidis, **Chrysanthi Pateraki**, Maria Alexandri, Harris Papapostolou, Koutinas, Apostolis Koutinas. Examining inhibition kinetics of black liquor on succinic acid fermentation. 9th International Conference on Renewable Resources and Biorefineries (RRB9), 5-7 June 2013, Antwerp, Belgium

Conference poster presentations - not related to this thesis

Chrysanthi Pateraki, Vassiliki Kachrimanidou, Sofia Maina, Aikaterini Papadaki, Erminta Tsouko, Nikolaos Kopsahelis, Seraphim Papanikolaou, Apostolis Koutinas, Effect of nutrient supplements derived from various industrial waste and by-product streams on succinic acid production via fermentation, 12th International Conference on Renewable Resources and Biorefineries (RRB12). 30-31 May & 1 June 2016, Ghent, Belgium.

Dimitris Ladakis, Anestis Vlysidis, Maria Alexandri, **Chrysanthi Pateraki**, Ioannis Kookos, Seraphim Papanikolaou, Apostolis Koutinas, Techno-economic evaluation of an integrated biorefinery scheme producing succinic acid from wood pulp industry wastes, 11th International Conference on Renewable Resources and Biorefineries (RRB11). 3-5 June 2015, York, UK.

Vasilliki Liakou, **Chrysanthi Pateraki**, Natasa Palaiogeorgiou, Serafeim Papanikolaou, Apostolis Koutinas, 2,3-butanediol production from fruit and vegetable waste streams, 11th International Conference on Renewable Resources and Biorefineries (RRB11). 3-5 June 2015, York, UK.

Maria Alexandri, **Chrysanthi Pateraki**, Anestis Vlysidis, Harris Papapostolou, Apostolis Koutinas, An Integrated Biorefinery Concept for the Production of paper and Biochemicals. 2nd Iberoamerican Congress on Biorefineries, 10-12 April 2013, Jaen, Spain

Research visits

Since 2013, I spent two months each year in Petrobras and Federal University of Rio de Janeiro (Rio de Janeiro, Brasil) for the project "Production of hydrolase enzymes and bifunctional monomers (1,3-PDO, 2,3-BDO and fumaric acid) in order to produce petrochemicals", funded by Petrobras

In 2014, I spent one week in AVECOM (Ghent, Belgium) for the project "New tailor-made biopolymers produced from lignocellulosic sugars waste for highly demanding fire-resistant applications -BRIGIT" (Grant Agreement nº: KBBE-2012-6-311935, KBBE.2012.3.4-02: Biotechnology for novel biopolymers

In 2013 and 2014, I spent three months to the LUND University (Lund, Sweden) for the project "New tailor-made biopolymers produced from lignocellulosic sugars waste for highly demanding fire-resistant applications -BRIGIT" (Grant Agreement nº: KBBE-2012-6-311935, KBBE.2012.3.4-02: Biotechnology for novel biopolymers

Acknowledgments

The experimental work of the present thesis was completed in the Laboratory of Food Engineering, Processing and Preservation of Agricultural Products, in the Department of Food Science and Human Nutrition at the Agricultural University of Athens.

This thesis could not have been completed without the contribution of many people that supported me throughout this journey, not only in work but in personal issues.

First, I would like to thank my supervisor, Assistant Professor, Apostolis Koutinas for his continuous support on the research. He gave me motivation and guidance and helped me to widen my research in many perspectives.

I would also like to thank, Associate Professors, Seraphim Papanikolaou and Emmanouil Flemetakis for providing valuable insights on the subject of this thesis.

I would like to thank everyone at the Department of Food Science and Human Nutrition. Ourania Kalantzi and Nikolaos Kopsahelis for all their support. I would like to thank my fellow labmates, Mary, Dimitris, Anestis, Harris, for their collaboration in this project. I would also like to thank my labmates Vaso, Afroditi, Katerina, Sofia and Eri.

I would like to thank my best friends, Anna, Danai, Felix and Nikos for their everyday support, for the laughs and great time we have together.

Last, but not least I would like to thank my parents, Niki and Giorgos, for supporting me through my education and all my life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure 6.5 Final formic acid to succinic acid molar ratios (FA/SA) observed in *A. succinogenes* (A) and *B. succiniciproducens* (B) fermentations at different initial acetic acid concentrations

Figure 6.6 Final acetic acid to succinic acid molar ratios (AA/SA) observed in *A. succinogenes* (A) and *B. succiniciproducens* (B) fermentations at different initial acetic acid concentrations

Figure 6.8 Total sugars (white bar), consumed sugars (grey bar) and final succinic acid concentration (square, ■) achieved by *A. succinogenes* (A) and *B. succiniciproducens* (B) during fermentation on different initial furfural concentrations

Figure 6.9 Productivity (grey bars) and final total consumed sugars to succinic acid conversion yield (square, ■) achieved by *A. succinogenes* (A) and *B. succiniciproducens* (B) in fermentations at different initial furfural concentrations

Figure 6.10 Final formic acid to succinic acid molar ratios (AA/SA) observed in *A. succinogenes* (A) and *B. succiniciproducens* (B) fermentations at different initial furfural concentrations

Figure 6.11 Final acetic acid to succinic acid molar ratios (AA/SA) observed in *A. succinogenes* (A) and *B. succiniciproducens* (B) fermentations at different initial furfural concentrations

Figure 6.12 Effect of initial methanol concentration on specific growth rate of *A. succinogenes* (A) and *B. succiniciproducens* (B) during fermentations in Duran bottles (250 mL) using continuous supply of CO_2

Figure 6.13 Total sugars (white bars), consumed sugars (grey bars) and final succinic acid concentration (square, ■) achieved by *A. succinogenes* (A) and *B. succiniciproducens* (B) during fermentations in Duran bottles (250 mL) using different initial methanol concentrations

Figure 6.14 Productivity (grey bars) and final total consumed sugars to succinic acid conversion yield (square, ■) achieved by *A. succinogenes* (A) and *B. succiniciproducens* (B) in fermentations carried out at different initial methanol concentrations

Figure 6.15 Final formic acid to succinic acid molar ratios (FA/SA) observed in *A. succinogenes* (A) and *B. succiniciproducens* (B) fermentations at different initial methanol concentrations in Duran bottle fermentations.

Figure 6.16 Final acetic acid to succinic acid molar ratios (AA/SA) observed in *A. succinogenes* (A) and *B. succiniciproducens* (B) fermentations at different initial methanol concentrations in Duran bottle fermentations.

Figure 6.17 Total sugars (white bars), consumed sugars (grey bars) and final succinic acid concentration (■ and ●) achieved by *A. succinogenes* 130Z (A) and *B. succiniciproducens* (B) during fermentations in Duran bottles (250 mL) on different initial LS concentrations and

various SSL dilutions. Fermentations carried out via SSL dilution are represented by light grey bars for sugar consumption and circles for succinic acid production.

Figure 6.18 Productivity (grey bars) and final total consumed sugars to succinic acid conversion yield (■ and ●) achieved by *A. succinogenes* 130Z (A) and *B. succiniciproducens* (B) during fermentations in Duran bottles (250 mL) on different initial LS concentrations and various SSL dilutions. Fermentations carried out via SSL dilution are represented by light grey bars for sugar consumption and circles for succinic acid production.

Figure 6.19 Final lactic acid to succinic acid molar ratios (LA/SA) observed in *B. succiniciproducens* cultures carried out in Duran bottles at different initial LS concentrations (■) and various SSL dilutions (●).

Figure 6.20 Final formic acid to succinic acid molar ratios (FA/SA) observed in *A. succinogenes* (A) and *B. succiniciproducens* (B) cultures carried out in Duran bottles at different initial LS concentrations (■) and various SSL dilutions (●).

Figure 6.21 Final acetic acid to succinic acid molar ratios (AA/SA) observed in *A. succinogenes* (A) and *B. succiniciproducens* (B) cultures carried out in Duran bottles at different initial LS concentrations (■) and various SSL dilutions (●).

Figure 6.22 Consumption of C5 and C6 sugars, production of metabolic products and change of dry cell weight (DCW) during fed-batch bioreactor fermentations of *A. succinogenes* (A, C) and *B. succiniciproducens* (B, D) cultivated on mixed commercial sugars using the same sugar ratio as the one measured in SSL. Total sugars (●), xylose (□), glucose (○), galactose (×), arabinose (◁), mannose (▷), dry cell weight (◊), succinic acid (■),acetic acid (▽), formic acid (△), and lactic acid (+)

Figure 6.23 Consumption of C5 and C6 sugars and production of metabolic products during fed-batch bioreactor fermentations of *A. succinogenes* (A, C) and *B. succiniciproducens* (B, D) cultivated on untreated, seven times diluted, SSL. Total sugars (●), xylose (□), glucose (○), galactose (×), arabinose (◁), mannose (▷), dry cell weight (◊), succinic acid (■), acetic acid (▽), formic acid (△), and lactic acid (+)

Figure 6.24 Consumption of C5 and C6 sugars and production of metabolic products during fed-batch bioreactor fermentations of *A. succinogenes* (A, C) and *B. succiniciproducens* (B, D) cultivated on SSL pretreated via ultrafiltration using a membrane with 3 kDa MWCO. Total sugars (●), xylose (□), glucose (○), galactose (×), arabinose (◁), mannose (▷), dry cell weight (◊), succinic acid (■),acetic acid (▽), formic acid (△), and lactic acid (+)

Figure 6.25 Consumption of C5 and C6 sugars and production of metabolic products during fed-batch bioreactor fermentations of *A. succinogenes* (A, C) and *B. succiniciproducens* (B, D) cultivated on SSL pretreated via nanofiltration. Total sugars (●), xylose (□), glucose (○), galactose (×), arabinose (◁), mannose (▷), dry cell weight (◊), succinic acid (■),acetic acid (▽), formic acid (△), and lactic acid (+).

Figure 7.1. Metabolic profile and sampling during the fermentations carried out with glucose (A and B), xylose (C and D), and SSL (E and F) using *A. succinogenes* in bench top bioreactor. Carbon source (●), dry cell weight (◊), succinic acid (□), acetic acid (▽) and formic acid (△).

Figure 7.2. Principal component analysis of relative expression levels during exponential phase of genes involved in glycolysis and pentose phosphate pathways. Glucose (yellow circle), Xylose (blue circle), SSL (brown circle).

Figure 7.3. Heat map of transcriptional changes in *A. succinogenes* genes involved in glycolysis and pentose phosphate pathway metabolic pathways during fermentations in benchtop bioreactors in SSL (S), Glucose (G) and Xylose (X) during lag phase (L), exponential phase (E) and stationary phase (S).

Figure 7.4. Principal component analysis of relative expression levels during exponential phase of genes involved in TCA cycle and pyruvate metabolism pathways. Glucose (yellow circle), Xylose (blue circle), SSL (brown circle).

Figure 7.5. Heat map of transcriptional changes in *A. succinogenes* genes involved in TCA cycle and pyruvate metabolism pathways during fermentations in benchtop bioreactor in SSL (S), Glucose (G) and Xylose (X) during lag phase (L), exponential phase (E) and stationary phase (S).

Figure 7.6. Principal component analysis of relative expression levels during exponential phase of genes involved in TCA cycle and pyruvate metabolism pathways. Glucose (yellow circle), Xylose (blue circle), SSL (brown circle).

Figure 7.7 Heat map of transcriptional changes in *A. succinogenes* genes involved in TCA cycle and oxidative phosphorylation pathways during fermentations in benchtop bioreactor in SSL (S), Glucose (G) and Xylose (X) during lag phase (L), exponential phase (E) and stationary phase (S).

Figure 8.1 Succinic acid production coupled with *in situ* electrochemical extraction - Bioreactor set-up

Figure 8.2 Consumption of xylose and production of metabolic products during fed-batch bioreactor fermentation of *B. succiniciproducens* cultivated on xylose and 5 g/L yeast extract. Xylose (●), succinic acid (■), lactic acid (+), formic acid (△), acetic acid (▽).

Figure 8.3 Xylose and metabolic products observed during fed-batch bioreactor fermentations of *B. succiniciproducens* cultivated on xylose and 5 g/L yeast extract integrated with *in-situ* extraction of metabolic products using membrane electrolysis. (A and C: bioreactor; B and D: extraction cell) Xylose (●), succinic acid (■), lactic acid (+), formic acid (△), acetic acid (▽). The feeding solution used was a concentrated xylose solution.

Figure 8.4 Consumption of xylose and production of metabolic products during fed-batch bioreactor fermentations of *B. succiniciproducens* cultivated on xylose and 5 g/L yeast extract. Glucose (●), succinic acid (■), lactic acid (+), formic acid (△), acetic acid (▽). The feeding medium used was a concentrated xylose solution supplemented with 1% (w/v) yeast extract.

Figure 8.5 Xylose and metabolic products observed during fed-batch bioreactor fermentations of *B. succiniciproducens* cultivated on xylose and 5 g/L yeast extract integrated with *in-situ* extraction of metabolic products using membrane electrolysis. (A and C: bioreactor; B and D: extraction cell). Xylose (●), succinic acid (■), lactic acid (+), formic acid (△), acetic acid (▽). The feeding solution used was a concentrated xylose solution supplemented with 1% (w/v) yeast extract and minerals.

Figure 8.6 Consumption of xylose and production of metabolic products during fed-batch bioreactor fermentations of *B. succiniciproducens* cultivated on xylose and 5 g/L yeast extract. Glucose (●), succinic acid (■), lactic acid (+), formic acid (△), acetic acid (▽). The feeding solution was a concentrated xylose solution supplemented with 1% (w/v) yeast extract and minerals.

Figure 8.7 Consumption of glucose and production of metabolic products during fed-batch bioreactor fermentations of *B. succiniciproducens* cultivated on glucose and 5 g/L yeast extract. Glucose (●), succinic acid (■), lactic acid (+), formic acid (△), acetic acid (▽). The feeding solution contained glucose supplemented with 1% (w/v) yeast extract.

Figure 8.8 Glucose and Metabolic products during fed-batch bioreactor fermentations of *B. succiniciproducens* cultivated on glucose and 5 g/L yeast extract integrated with *in-situ* extraction of metabolic products using membrane electrolysis. (A and C: bioreactor; B and D: extraction cell). Glucose (●), succinic acid (■), lactic acid (+), formic acid (△), acetic acid

(▽). The feeding solution contained glucose supplemented with 1% (w/v) yeast extract and minerals.

Figure 8.9 (A) Biofilm formation on the anionic membrane. (B) *B. succiniciproducens* cells on the biofilm observed with scanning electron microscope (SEM)

Figure 8.10 Consumption of total sugars and production of metabolic products during fed-batch bioreactor fermentations of *B. succiniciproducens* cultivated on ultrafiltrated SSL and 5 g/L yeast extract. Total sugars (●), succinic acid (■), lactic acid (+), formic acid (△), acetic acid (▽). The feeding solution used contained concentrated mixed commercial sugars (72.6% of xylose, 12.2% of galactose, 10.9% of glucose, 4.2% of mannose, and 0.1% of arabinose) supplemented with lignosulphonates and 1% (w/v) yeast extract.

Figure 8.11 Total sugars and metabolic products during fed-batch bioreactor fermentations of *B. succiniciproducens* cultivated on ultrafiltrated SSL and 5 g/L yeast extract integrated with in-situ extraction of metabolic products using membrane electrolysis. (A and C: bioreactor; B and D: extraction cell) Total sugars (●), succinic acid (■), lactic acid (+), formic acid (△), acetic acid (▽). The feeding solution contained concentrated mixed commercial sugars (72.6% of xylose, 12.2% of galactose, 10.9% of glucose, 4.2% of mannose, and 0.1% of arabinose) supplemented with lignosulphonates, 1% (w/v) yeast extract and minerals.

Figure 8.12 Succinic acid production rate of *B. succiniciproducens* in control fed-batch bioreactor fermentations and in fed-batch fermentations with *in-situ* extraction of metabolic products when it is cultivated on SSL (A), glucose (B) and xylose (C). Control fermentation (□), fermentation broth in electrochemical bioreactor (■), extract (●) and fermentation broth in electrochemical bioreactor plus extract (△).

Figure 8.13 Final by-product to succinic acid ratio of *B. succiniciproducens* in control fed-batch bioreactor fermentations and in fed-batch bioreactor fermentations with in-situ extraction of metabolic products when cultivated on SSL (A), glucose (B) and xylose (C). LA:SA (black bars), FA:SA (grey bars), AA:SA (white bars).

Figure 8.14 Extraction rate of the produced acids during fed-batch fermentations with *in-situ* extraction of metabolic products using a membrane electrochemical bioreactor when the carbon source used was SSL (black symbols), glucose (grey symbols) and xylose (white symbols). Succinic acid (A), Lactic acid (B), Formic acid (C), Acetic acid (D).

Chapter 1

Introduction

Nowadays succinic acid is established as a key platform chemical for the bio-economy era according to several reports and increasing industrial interest for commercialisation (Bozell and Petersen, 2010; Jansen and Van Gulik, 2014). Its significance as a platform intermediate is based on the reactivity of the two functional carboxylic groups leading to versatile end-products, the favorable fermentation efficiency, the utilisation of numerous carbon sources and inexpensive renewable resources as feedstocks, and the cost-competitiveness of biotechnological production over petrochemical synthesis.

Various chemical technologies have been developed for the production of succinic acid, including paraffin oxidation (Polly, 1950) and catalytic hydrogenation or electrolytic reduction of either maleic acid or maleic anhydride (Cok et al., 2014; Muzumdar et al. 2004). The paraffin oxidation technology employs a calcium or manganese catalyst to obtain a mixture of dicarboxylic acids. Subsequently, distillation, crystallisation and drying are used to purify the succinic acid, which is produced in relatively low yield and purity through this process. The catalytic hydrogenation technology is a mature industrial process that could be carried out in homogeneous or heterogeneous catalyst systems. Although succinic acid can be obtained in high yield and purity, the operation of the hydrogenation technology is expensive and may cause serious environmental problems.

In the bio-economy era, succinic acid will evolve into a platform intermediate, as replacement for maleic anhydride, for the production of various bulk/intermediate chemicals such as 1,4-butanediol, γ-butyrolactone, tetrahydrofuran, N-methyl-2-pyrrolidone, 2-pyrrolidone, succinimide, succinic esters, maleic acid/maleic anhydride among others (Song and Lee, 2006). Succinate and its derivatives (e.g. adipic acid and 1,4-butanediol) could be applied for the manufacture of biodegradable polymers (e.g. polyamides and polyesters). For instance, its market is expected to grow in the production of polybutylene succinate and polyurethanes such as polyethylene succinate.

The utilisation of succinic acid as platform chemical necessitates its production at a cost around $1 per kg succinic acid as it is required for the production of commodity products by the chemical industry. Microbial bioconversion could lead to more cost-competitive production of succinic acid than petrochemical synthesis due to certain advantages including high carbon source to succinic acid theoretical conversion yield, significant reduction of greenhouse gas emissions and non-renewable energy consumption (Cok et al., 2014; Hermann et al., 2007) and high potential for CO_2 sequestration due to CO_2 fixation involved in the reductive TCA cycle leading to succinic acid production. The market potential and the

advantages provided by bioprocessing have led to investment by several companies (Table 1) in the construction of industrial facilities for fermentative production of succinic acid with varying capacities (Jansen and Van Gulik, 2014; Carus, 2012; Taylor et al., 2015). The current industrial activity for succinic acid production is currently positioned at a Technology Readiness Level of 8 with manufacturing facilities constructed in Europe and North America (Taylor et al., 2015). The market price of bio-based succinic acid is around 2.94 $/kg, while the respective market price of both bio- and fossil-based succinic acid is around 2.5 $/kg (Taylor et al., 2015). The annual production capacity of bio-based succinic acid in the period 2013-2014 was around 38,000 t that constitutes 49% of the total market (Taylor et al., 2015). The bio-based succinic acid market is expected to reach 600,000 t by 2020 with a projected market size of $\$539\times10^6$, but this is regarded as an optimistic scenario because a production cost of 1 $/kg has been considered and the current production cost is much higher (Taylor et al., 2015).

Bioprocessing costs and environmental impact are highly dependent on the selection of the raw material, the upstream pre-treatment stages required to produce a nutrient-complete fermentation medium, the fermentation stage and the downstream separation and purification of succinic acid (especially in the case of high purity grade required for biopolymer formulation). All these stages are highly dependent on the microorganism employed and the fermentation conditions required. Producing bulk bio-based platform chemicals will require the construction of industrial plants with several bioreactors with capacities in the scale of hundred cubic meters per bioreactor. Therefore, the use of facultative anaerobic microorganisms, as in the case of succinic acid production, is preferable because they require reduced bioreactor costs avoiding aeration that increase significantly capital and operating costs when large bioreactor volumes are used. Therefore, the selection of the microorganism depends on both bioprocessing and physiological aspects (e.g. oxygen and specific nutrient requirements, flexibility in raw material utilization, optimum fermentation parameters).

The carbon sources used in current industrial fermentations are mainly purified sugars or glucose syrups from corn. The utilisation of agricultural residues and industrial waste and by-product streams is necessary in order to create sustainable bio-based succinic acid production. Although succinic acid producing bacteria can directly assimilate some industrial waste and by-products streams (e.g. cheese whey and cane molasses), other raw materials (e.g. lignocellulosic residues and starch-rich waste streams) cannot be readily consumed. In the latter case, optimum pre-treatment schemes should be developed upstream the fermentation stage increasing also the complexity of downstream separation stages due to the

Table 1 Industrial facilities for bio-based succinic acid production

Company	Capacity	Start-up	Raw material	Fermentation / Microorganism	Downstream recovery critical stage	Investment made in	Reference
BioAmber (DNP/ard)	3,000 t/y demo plant	2010	Wheat glucose	*E. coli*	Electrodialysis	Europe, Pomacle, France	Carus, 2012; Taylor et al., 2015
BioAmber, Mitsui	30,000 – 50,000 t/y	Under construction	Corn glucose	Low pH culture is targeted using *Candida krusei*	Direct succinic acid separation when low pH conditions are used	Sarnia, Ontario, Canada	Jansen and Van Gulik, 2014; Taylor et al., 2015
BioAmber, Mitsui	70,000 – 200,000 t/y	Two plants to be constructed	-	-	-	North America	Taylor et al., 2015
Reverdia (joint venture between Roquette & DSM)	10,000 t/y	2012	Starch/sugars	Low pH culture is targeted by *S. cerevisiae*	Direct separation of the succinic acid	Cassano Spinola, Italy	Jansen and Van Gulik, 2014; Taylor et al., 2015
Myriant, ThyssenKrupp	1,000 t/y	2013	Glucose	*E. coli*	Ammonia precipitation	Leuna, Germany	Taylor et al., 2015
Myriant	14,000 t/y	2013	Corn glucose	*E. coli*	Ammonia precipitation	Lake Providence, Louisiana, USA	Jansen and Van Gulik, 2014; Carus, 2012; Taylor et al., 2015
Succinity (joint venture between BASF & Corbion-Purac)	10,000 t/y	2013	Glycerol / sugars	*B. succiniciproducens*	Magnesium hydroxide as neutralizer followed by recycling	Montmelo, Spain	Jansen and Van Gulik, 2014; Taylor et al., 2015

need to separate remaining nutrients from the fermentation broth. Therefore, the utilisation of crude renewable resources will eventually lead to sustainable production of bio-based succinic acid only through refining of the original resource in an analogous manner that refining has been applied to corn and petroleum (Koutinas et al., 2014). In this way, the production of value-added co-products will provide the profitability margin required for the development of sustainable bioprocesses.

Chapter 2

State of the art on bio-succinic production

2.1 Succinic acid

Succinic acid or butanedioic acid (Figure 2.1) with a molecular weight of 118.0 g/mol is a di-carboxylic acid and its crystalline form is white and odorless. It is soluble in water, ethanol, ethyl ether, acetone and methanol; slightly soluble in deuterated dimethyl formamide and it is insoluble in toluene and benzene (Anonymous, 2016). Its boiling point is 235 ^{o}C and its melting point is 188 ^{o}C. Succinic acid finds applications in different industrial sectors (with varying market shares) including polybutylene succinate and polybutylene succinate-terephthalate (9%), polyester polyols (6.2%), the food industry as acidulant, flavorant and sweetener (12.6%), the pharmaceutical industry (15.1%), and production of resins, coatings and pigments (19.3%) (Anonymous, 2014).

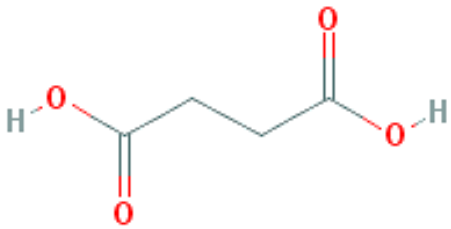

Figure 2.1 Succinic acid chemical structure. *Source: https://pubchem.ncbi.nlm.nih.gov*

Several reviews have been published focusing on various aspects of succinic acid production including both general reviews (Song and Lee, 2006; Cheng et al., 2012a; Cao et al., 2013; Tan et al., 2014) as well as more specific ones focusing on the prospect of *Escherichia coli* (Thakker et al., 2012), comparison of natural versus genetically engineered strains (Beauprez et al., 2010), the utilisation of lignocellulosic biomass or various renewable carbon sources (Tan et al., 2014; Akhtar et al., 2014), the development of wheat-based biorefining (Lin et al., 2012), downstream separation schemes (Cheng et al., 2012b), recent commercialisation attempts (Jansen and Van Gulik, 2014) and metabolic engineering approaches (Cheng et al., 2013).

2.2 Succinic acid producers

Table 2.1 presents information on succinic acid production via fermentation reported for various microbial strains. Fermentative succinic acid production has been accomplished by both wild-type and genetically engineered strains. *Actinobacillus succinogenes*, *Basfia succiniciproducens* and *Mannheimia succiniciproducens* are the most promising wild-type bacterial strains because they consume numerous carbon sources, are facultative anaerobes,

can achieve high fermentation efficiency and are classified as biosafety level 1 microorganisms by DSMZ and ATCC. These strains have been isolated from the rumen. The highest succinate concentration (105.8 g/L) has been produced by *A. succinogenes* FZ53 mutant using glucose with a yield and productivity of 0.82 $g_{SA}/g_{glucose}$ and 1.36 g/L/h, respectively (Guettler et al., 1996). Contrary to other bacterial strains, *B. succiniciproducens* has not been studied to a great extent. Its metabolic fluxes have been recently investigated and two mutant strains have been constructed (Becker et al., 2013).

Highly efficient genetically engineered *E. coli* strains (Jantama et al., 2008) have been developed for succinic acid production, such as *E. coli* strain AFP111/pTrc99A–pyc that produced 99.2 g/L of succinic acid concentration in dual phase fermentations with the highest reported yield of 1.1 $g_{SA}/g_{glucose}$ and a productivity of 1.3 g/L/h (Vemuri et al., 2002). *Corynebacterium glutamicum* ΔldhA-pCRA717 could be a promising microorganism for succinic acid production due to the high productivity (3.17 g/L/h), final concentration (146 g/L) and yield (0.92 $g_{SA}/g_{glucose}$) achieved (Okino et al., 2008). Recent research focuses on the development of genetically engineered yeast strains that can produce succinic acid at low pH in order to reduce the unit operations in downstream separation and purification of succinic acid (Jansen and Van Gulik, 2014; Van De Graaf et al., 2012).

The main advantages of *A. succinogenes* and *B. succiniciproducens* exploitation for succinic acid production are the utilisation of numerous carbon sources, adequate tolerance to inhibitors and sufficient fermentation efficiency even with crude renewable resources. The main disadvantages lie on the fastidious nature of *A. succinogenes* and *B. succiniciproducens* for nitrogen sources (e.g. yeast extract) and vitamins (e.g. biotin), the near neutral optimum pH required and the absence of genetic engineering tools for its genetic manipulation. The implementation of succinic acid production by *A. succinogenes* and *B. succiniciproducens* in integrated biorefineries using complex renewable resources would provide nutrient-complete fermentation media at lower cost than commercial nutrient sources. The use of neutralisers and the neutral pH of the fermentation broth resulting from *A. succinogenes* and *B. succiniciproducens* cultures affect both fermentation and downstream separation costs. Lower pH values of the fermentation broth reduce the downstream separation cost as the pH value affects the dissociation level of succinic acid (pKa_1 = 4.16 and pKa_2 = 5.6) (Jansen and Van Gulik, 2014). Another issue that should be addressed is the supply of CO_2.

Table 2.1 Fermentation efficiency, nutrient composition and operating conditions for different succinic acid producing strains

Fermentation parameters	***A. succinogenes* FZ53**	***M. succiniciproducens LPK7***	***B. succiniciproducens* JF 4016**	***E. coli* AFP 111/pTrc99 A-pyc**	***E. coli* KJ060**	***C. glutamicum* ΔldhA-pCRA717**	***S. cerevisiae* SUC-297**	***Y. lipolytica* Y-3314**
Carbon source	Glucose	Glucose	Glucose	Glucose	Glucose	Glucose	Glucose	Glycerol
Nutrient sources (g/L)	Yeast extract, (5-15), corn steep liquor (10-15, vitamins	Yeast extract (5)	Yeast extract (5), peptone (5), vitamins	Yeast extract, (10), Tryptone (20), biotin, thiamine	$(NH_4)_2HPO_4$, $NH_4H_2PO_4$, thiamine, betaine	Urea (2), yeast extract (2), casamino acid (7), $(NH_4)_2SO_4$ (7), biotin, thiamine	$(NH_4)_2SO_4$, vitamins	Yeast extract (10), peptone (10), uracil, leucine
Gas supply	CO_2 (0.05 – 0.1 vvm	CO_2 (0.25 vvm)	CO_2 atmosphere	Dual - phase cultures	Anaerobic environment	Oxygen deprivation	Air and CO_2	Aerobic shake flasks
pH	7.2-6	6.5	nk[1]	7	7	nk[1]	5	6.8-5.8
Neutralisers	$MgCO_3$, $Mg(OH)_2$	Ammonia solution	$MgCO_3$	NaOH, HCl	$NaHCO_3$, KOH, K_2CO_3	NaOH, bicarbonate	KOH	$CaCO_3$
Yield ($g/g_{glucose}$)	0.82	0.76	0.49	1.1	0.92	0.92	nk	0.36
Productivity (g/L/h)	1.36	1.8	0.53	1.3	0.9	3.17	0.45	0.27
Succinic acid (g/L)	105.8	52.4	20	99.2	86.6	146	43	45.5
By-products[2]	Ace, Prop, Pyr	Mal, Pyr	For, Ace, Lac, EtOH	Ace, EtOH	Mal, Ace, Lac	Ace, Lac, Mal, Pyr	EtOH, Gly, Mal	nk[1]
Reference	Guettler et al., 1996	Lee et al., 2006	Becker et al., 2013	Vemuri et al., 2002	Jantama et al., 2008	Okino et al., 2008	Van De Graaf et al., 2012	Yuzbashev et al., 2010

[1] nk: not known; [2] Ace: acetic acid, For: formic acid, Pyr: pyruvic acid, Pro: propionic acid, Lac: lactic acid, Mal: malic acid, EtOH: ethanol, Gly: glycerol

2.3 The metabolic potential of *A. succinogenes* and *B. succiniciproducens*

A. succinogenes is a Gram-negative, facultative anaerobic, non-motile, non-spore forming, capnophilic, pleomorphic rod, which was isolated from bovine rumen (Guettler et al., 1999) and taxonomically was placed in the *Pasteurellaceae* family, based on 16S rRNA amplification. Its taxonomical order has been formed as follows: Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; *Pasteurellaceae*; *Actinobacillus succinogenes*. *A. succinogenes* is mesophilic and grows well at 37-39 ^{o}C in chemically defined media. The microorganism is capable of consuming a wide range of C5 and C6 sugars as well as various disaccharides and other carbon sources, such as glucose, xylose, arabinose, mannose, galactose, fructose, sucrose, lactose, cellobiose, mannitol, maltose and glycerol (Carvalho et al., 2014; Wan et al., 2008; Zheng et al., 2009; Li et al., 2010; Jiang et al., 2013; Jiang et al., 2014).

B. succiniciproducens is a Gram-negative, facultative anaerobic, non-motile, capnophilic, coccoid to rod shaped, that is catalase-, urease- and indole-negative and oxidase-positive which was isolated from bovine rumen (Kuhnert et al., 2010) and taxonomically was placed in the *Pasteurellaceae* family, based on multilocus sequence analysis (MLSA) using 16S rRNA, rpoB, infB and recN amplification. Its taxonomical order has been formed as follows: Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; *Pasteurellaceae*; *Basfia succiniciproducens*. *B. succiniciproducens* is mesophilic and grows well at 37 ^{o}C. The microorganism is capable of consuming glucose, glycerol, galactose, mannitol, mannose, sucrose, trehalose and xylose (Kuhnert et al., 2010).

The use of more reduced carbon sources than glucose, such as sorbitol, glycerol and mannitol, result in higher succinic acid yields. However, the utilization of C5 sugars, such as xylose and arabinose, result in lower succinate yields (Li et al., 2010). The theoretical yield from C6 (glucose) or C5 (xylose) sugars is similar, but the complexity of the metabolism during xylose degradation, through the pentose phosphate pathway, can result in lower actual yields.

2.4 Metabolic pathway towards succinic acid production

Glucose is transported in the cell with the action of a permease. Phosphorylation of glucose occurs not only through the phosphoenolpyruvate dependent phosphotransferase system (McKinlay et al., 2007), but also through hexokinase activity (Guettler et al., 1999).

Furthermore, glucose-6-phosphate is catabolised to phosphoenolpyruvate (PEP) through the glycolytic pathway, while the oxidative pentose phosphate pathway (OPPP) has limited contribution in the catabolism of glucose. Pentose phosphate pathway (PPP), does not exclusively serves for anabolic precursor supply, through the oxidative branch, but excess carbon is recycled back into the glycolytic chain. Thus, NADPH is formed from NADH through transhydrogenase and/or by the combination of NADH-oxidising malate dehydrogenase and NADP-reducing malic enzyme activity (McKinlay et al., 2007), while NADH is produced through the activity of pyruvate dehydrogenase and formate dehydrogenase. Moreover, NADPH requirements affects the flux distribution between C3 and C4 pathways, mainly due to pyruvate dehydrogenase and formate dehydrogenase activities. The glyoxylate and Entner-Doudoroff pathways are not present in the fermentative metabolism of *A. succinogenes*. The four most important nodes on flux distribution for succinate production in *A. succinogenes* are phospoenolpyruvate (PEP), oxaloacetate (OAA), malate and pyruvate (Figures 2.2 and 2.3) (McKinlay et al., 2008). Significant carbon is diverted towards extracellular succinic acid, formic acid, acetic acid and ethanol formation.

The TCA cycle in *B. succiniciproducens* is closed, contrary to *A. succinogenes* that operates in two brunches but hardly as a cycle. Succinic acid is produced through the reductive brunch of the TCA cycle that functions as a NADH sink, whereas the oxidative brunch contribution is negligible towards succinic acid production and mainly serves for anabolic purposes. Significant carbon is diverted towards extracellular succinic acid, lactic acid, formic acid, acetic acid and ethanol formation (Becker et al., 2013).

Metabolic flux analysis for both microorganisms has established that the major route to succinic acid flows from phosphoenolpyruvate to oxaloacetate, malate, fumarate and finally to succinate catalysed by PEP carboxykinase, malate dehydrogenase, fumarase and fumarate reductase (McKinlay et al., 2007; Becker et al., 2013). PEP could be converted into formate, lactate, acetate and ethanol via the C3 pathway and into succinate via the C4 pathway, with malic enzyme and OAA decarboxylase holding the potential role for the conversion into pyruvate, hence forming reversible shunts between C3 and C4 pathways (McKinlay and Vieille, 2008). PEP carboxykinase (EC 4.1.1.49) is considered as a key enzyme for the

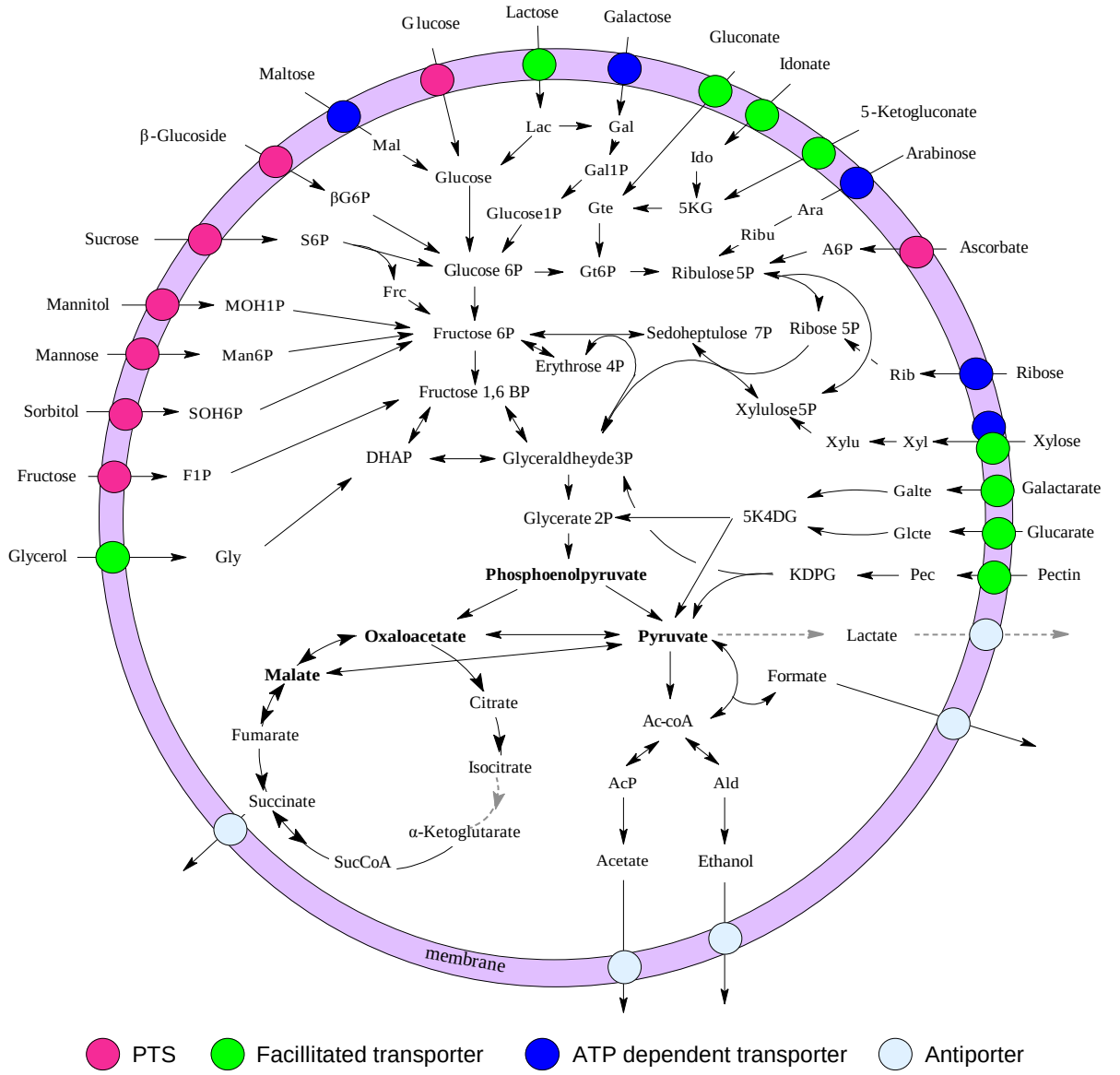

Figure 2.2 *A. succinogenes* and *B. succiniciproducens* metabolic pathways and possible sugar transporters (Grey arrows indicate that these reactions are absent in *A. succinogenes*). 5K4DG: 5-dehydro-4-deoxy-D-glucarate; 5KG: 5-ketogluconate; A6P: Ascorbate-6-phosphate; AcCoA: Acetyl-CoA; AcP: acetyl-phosphate; Ald: Aldehyde; Ara: Arabinose; DHAP: dihydroxyacetone phosphate; E4P: Erythrose-4-phosphate; F1,6P: Fructose-1,6-biphosphate; F1P: Fructose-1-phospshate; F6P: Fructose-6-phosphate; G1P: Glucose-1-phosphate; Gal: Galactose; Gal1P: Galactose-1-phosphate; Galte: Galactarate; Glc: Glucose; Glcte: Glucarate; Gly: Glycerol; Gt6P: Gluconate-6-phosphate; Gte: Gluconate; Ido:Idonate; KDPG: 2-keto-3-deoxy-6-phosphogluconate; Lac: Lactose; Mal: Maltose; Man6P: Mannose-6-phosphate; MOH1P: Mannitol-1-phosphate; Pec: Pectin; R5P: Ribose-5-phosphate; Rib: Ribose; Ribu: Ribulose; Ru5P: Ribulose-5-phosphate; S6P: Sucrose-6-phosphate; S7P: Sedoheptulose-7-phosphate; SOH6P: Sorbitol-6-phosphate; SucCoA: Succinyl-CoA; Xu5P: Xylulose-5-phosphate; Xyl: Xylose; Xylu: Xylulose; βG6P: β-Glucoside-6-phosphate;

production of succinic acid with subsequent ATP formation, since it is capable of catalyzing the conversion of PEP into OAA (ΔG: 5.6 kJ mol-1) through the consumption of CO_2 (McKinlay et al., 2007). Furthermore, malate dehydrogenase (EC 1.1.1.299) catalyses the conversion of oxaloacetate into malate with NADH as a co-factor (ΔG: -32.1 kJ mol-1), while fumarate is generated from malate (ΔG: 3.6 kJ mol-1) with the use of the enzyme fumarase (EC 4.2.1.2). Fumarate reductase (EC 1.3.1.6) is a NADH dependent enzyme which catalyses the conversion of fumarate into succinate, with generation of 2/3 of ATP (ΔG: -71.4 kJ mol^{-1}). Pyruvate kinase (EC 2.7.1.40) occurs in the first reaction of the C3 pathway, involved in the conversion of PEP into pyruvate (ΔG: -30.6 kJ mol-1), producing 1 molecule of ATP [37]. Pyruvate could be converted into lactate, formate, acetate, ethanol and biomass. Pyruvate-formate lyase (EC 2.3.1.54) is considered as an important enzyme converting pyruvate, using CoA as a cofactor, into acetyl-CoA and formate (ΔG: -11.2 kJ mol-1). The values of ΔG presented above have been calculated for pH 7 and standard conditions (http://equilibrator.weizmann.ac.il/).

Theoretically, 1 mole of succinic acid can be produced from the fixation of 1 mole of CO_2 and 0.5 moles of glucose, 0.6 moles of xylose or 1 mole of glycerol. Sufficient carbonate should be provided in order to direct the metabolic flux towards the C4 pathway. In parallel, 2 moles of NADH are oxidised through the reductive pathway of the TCA cycle during the conversion of OAA to malate and fumarate to succinate. This means that additional reducing capacity (i.e. NADH) required in the C4 pathway should be supplied by other parts of the metabolism (e.g. glycolysis, C3 pathway). The maximum glucose to succinic acid conversion yield is 1.12 g/g and is given by the following stoichiometric equation:

$$CH_2O + 1/7\ CO_2 \rightarrow 8/7\ CH_{3/2}O + 1/3\ ATP + 1/7\ H_2O \quad (1)$$

The theoretical yield cannot be achieved due to biomass and by-product formation. The production of 1 mole of acetic acid is accompanied with the generation of 1 mole of ATP. One mole of formate (through pyruvate-formate lyase) is produced along with 1 mole of acetyl-CoA, a precursor of structural molecules and finally biomass. Subsequently formate can be converted into CO_2 and H^+, catalysed by formate dehydrogenase, which can serve as electron donor for the production of succinic acid. The C3 pathway results in the production of NADH that is necessary for the production of succinic acid by the C4 pathway resulting in different glucose to succinic acid theoretical conversion yields depending on the metabolic pathway employed.

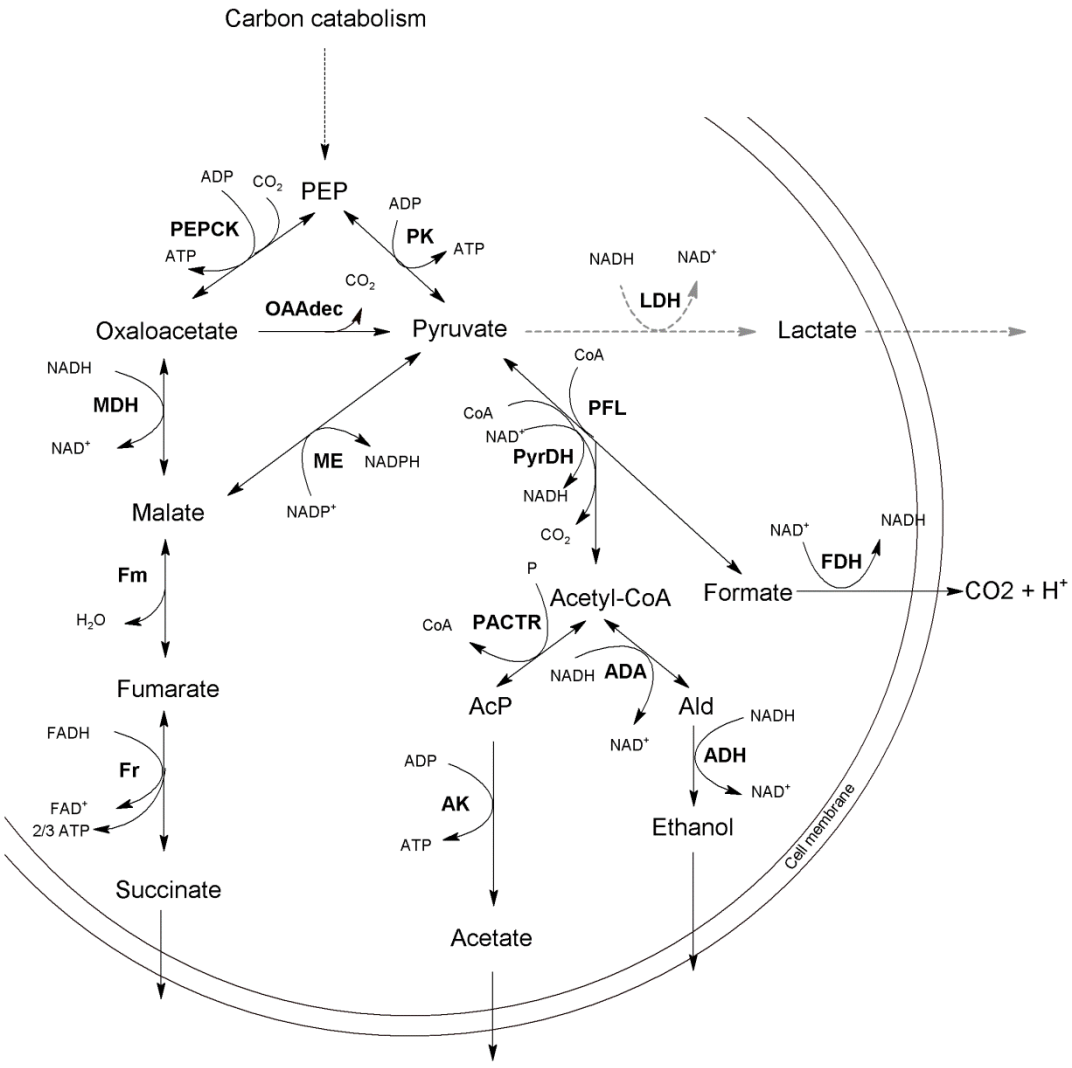

Figure 2.3 Co-factors and enzymes that participate in the C3 and C4 metabolic pathways in *A. succinogenes*

The key factor to achieve higher carbon source to succinic acid conversion yields is to reduce by-product formation. Homo-fermentative succinic acid production by *A. succinogenes* can be achieved by optimising the fermentation process and by genetic manipulation or evolutionary adaptation to create mutants similar to *A. succinogenes* FZ6 and FZ53 (Guettler et al., 1996). Furthermore, the development of continuous fermentations with cell immobilisation could lead to less by-product formation and higher productivities than batch and fed-batch cultures (Yan et al., 2014). *A. succinogenes* cells form biofilms during continuous cultures resulting in reduced NADH requirements for biomass formation and therefore low acetic acid and formic acid concentrations in the effluent (Bradfield and Nicol, 2014).

2.5 Influence of substrate and product inhibition on succinic acid bioprocesses

Succinic acid production via fermentation is hampered by substrate and product inhibition. Inhibition on growth by metabolic product formation could occur by two mechanisms: 1) regulation of homeostasis and 2) membrane breakdown due to osmotic stress. The lipophile, undissociated form of weak acids can enter the cell at neutral pH values. In elevated acid concentrations, pH homeostasis is maintained through the synthesis of excessive ATP and the regulation of H^+-ATPase, which expels protons from the cytoplasm (proton motive force) against the concentration gradient to maintain ΔpH in low levels (between the inner and the outer surface of the cell). High osmotic stress occurs when the medium contains high concentrations of sugars and/or minerals, causing cell shrinkage, subsequent breakdown of the membrane and cell death.

Table 2.2 presents indicative substrate and product inhibition for *A. succinogenes* and *E. coli* strains cultivated mainly on glucose. Substrate inhibition was also tested in the case of *A. succinogenes* CGMCC1593 growth on glucose (Liu et al., 2008a) and sugar cane molasses (Liu et al., 2008b) as carbon sources, demonstrating that inhibition started at 50-60 g/L of glucose concentration and 65 g/L of total sugar concentration, respectively. The growth of the strain *A. succinogenes* 130Z was completely inhibited at initial glucose concentrations higher than 158 g/L (Lin et al., 2008), while the glucose to succinic acid conversion yield was decreased and the duration of the lag phase was increased at glucose concentrations higher than 100 g/L. Formate imposed the most inhibitory effect on succinic acid production, while the inhibitory concentrations of acetate, ethanol, formate, pyruvate and succinate were 46, 42, 16, 74 and 104 g/L, respectively (Lin et al., 2008). The product inhibition of *A. succinogenes* 130Z was compared to 3 mutant strains of *E. coli* (Li et al., 2010) showing that *E. coli* NZN111 is more tolerant than *A. succinogenes* 130Z in the case of succinic, formic and acetic acids.

Table 2.2 Inhibitory product and glucose concentrations on succinic acid production (initial inhibitory concentrations are enclosed in parenthesis). Missing data are either not mentioned or not investigated in the literature-cited references

Microorganism	Glucose (g/L)	Succinic acid (g/L)	Pyruvic acid (g/L)	Lactic acid (g/L)	Formic acid (g/L)	Acetic acid (g/L)	Ethanol (g/L)	Total products (g/L)	Reference
A. succinogenes CGMCC1593	> 100 (50-60)	-	-	-	-	-	-	-	Liu et al., 2008a
A. succinogenes CGMCC1593	> 100 (65)[1]	-	-	-	-	-	-	-	Liu et al., 2008b
A. succinogenes 130Z^T (ATCC 55618)	158 (100)	59.4	59.2	-	10.8 (1.8)	33.7	42	-	Lin et al., 2008
A. succinogenes 130Z^T (ATCC 55618)	> 100 (57.4)	-	-	-	-	-	-	-	Corona-Gonzalez et al., 2008
A. succinogenes 130Z^T (ATCC 55618)	-	40 (9.8)	-	-	-	-	-	- (20)	Corona-Gonzalez et al., 2010
A. succinogenes 130Z^T (ATCC 55618)	-	50 (<40)	-	18 (<9)	35.2 (<8.8)	20 (<10)	-	-	Li et al., 2010
E. coli NZN111	-	80 (<20)	-	18 (<9)	52.8 (<8.8)	60 (<20)	-	-	
E. coli AFP111	-	80 (<20)	-	18 (<9)	35.2 (<8.8)	40 (<20)	-	-	
E. coli BL21	-	>80 (<40)	-	18 (<9)	35.2 (<8.8)	60 (<20)	-	-	

[1] Total sugars from pretreated cane molasses

2.6 Oxido-reduction potential of *A. succinogenes*

Succinic acid is secreted either by diffusion or through a transport protein on the cell membrane. The process involves the oxidation of a substrate and the transfer of electrons to a final electron acceptor. Fumarate is used as an electron acceptor in fumarate respiration, which is converted into succinic acid (Guettler et al., 1999) and excreted through an antiporter (Padan, 2014). Pyruvate is oxidized to release reducing power (i.e. NADH) that can be used in the C4 pathway. Thus, the provision of more reduced carbon sources as well as the supply of reducing power from external sources (e.g. electrically reduced neutral red in an electrochemical bioreactor system) could enhance the production of succinate and ATP under controlled conditions (Li et al., 2010). When H_2 or electricity is not used as additional reducing power, pyruvate should be oxidised in order to produce an electron donor for fumarate reduction resulting in decreased succinate yield and ATP synthesis via electron transport-mediated phosphorylation (Park et al., 1999).

The redox potential or oxido-reduction potential is a measure of the tendency towards reduction or oxidation. Li et al. (2010) investigated the influence of the redox potential on the fermentation profile of *A. succinogenes* NJ113 by addition of potassium ferricyanide as the oxidant agent and dithiotreitol as the reducing agent. At a redox potential level of -350 mV, succinic acid production, yield and productivity increased and by-product formation decreased compared to the rest of the redox potential levels tested (no redox potential regulation, -100, -300, -350, -400, -450 mV). This occurred due to a significantly higher NADH/NAD+ ratio observed at -350 mV (Li et al., 2010).

Xi et al. (2012) reported that biotin concentrations in the range of 2-10 mg/L resulted in enhanced succinic acid production when a chemically defined medium was used. Considering that succinic acid is formed from fumaric acid, the role of biotin in the pathway is to provide cytochrome b and electron transfer exits (Xi et al., 2012). Furthermore, when 5-aminolevulinic acid (a biosynthetic precursor of heme) or heme (an electron carrier that can increase the reduction potential) replaced biotin in the chemically defined medium, similar fermentation results were obtained indicating that biotin biosynthesis could be achieved from 5-aminolevulinic acid.

2.7 Genome analysis of *A. succinogenes* and *B. succiniciproducens*

The genome of *A. succinogenes* was sequenced by McKinlay et al. (2010). Although this is the largest genome of the *Pasteurellaceae* family characterized so far, a genome size of 2.3 Mb (GenBank accession number CP000746) is generally considered as relatively small. The genome contains 2,079 protein coding genes out of 2,199 genes. Compared to other *Pasteurellaceae*, *A. succinogenes* and *M. succiniciproducens* are more closely related. These strains have 78% of the open reading frames (ORFs) in common (1,735), while 488 are found only in *A. succinogenes* and 442 only in *M. succiniciproducens*. Furthermore, 60% (1,252) of 2,081 automated KEGG comparisons were mostly similar to *M. succiniciproducens*, while the GC content was determined by HPLC to be 45.1 mol % (Hong et al., 2004). *A. succinogenes* contains a 39,489 bp prophage genome, which is useful for phage based genetic engineering, but it might be risky for industrial fermentations due to possible lysis of the cells. A USS1 repeat (9 nt sequence) has been shown to be present in *A. succinogenes* (1,690 copies and density of 0.73 USS/bp), which functions for natural competence. Although various *Pasteurellaceae* strains are pathogens, *A. succinogenes* and *M. succiniciproducens* lack pathogenicity, since they do not encode sequences of virulence traits or for the production of leukotoxin, cytolethal toxin and hemolysin. Sequences that function for the production of sialic acid, incorporation of choline into lipopolysaccharide as well as iron uptake, through hemoglobin and transferrin utilisation, have not been identified.

Various genes involved in the transport of a wide range of sugars have been characterized (Figure 2.2). The transport is achieved either through PTS, an ATP-dependent transporter or with the application of a facilitated transporter, while an assumption is often used about the specific function of each sugar transporter. Gene Asuc_0496 encodes a sugar transport protein that possibly functions as glucose facilitator, while Asuc_1504, Asuc_0131 and Asuc_0084 encode possible sugar kinases. Genes that encode glyoxylate pathway enzymes and Entner-Doudoroff enzyme phosphogluconate dehydratase were not present in the genome of *A. succinogenes*, whereas genes that encode for the glycolytic cycle as well as the pentose phosphate pathway are also present. However, although Asuc_0152, Asuc_0374 and Asuc_1471 could be part of operons that encode for 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolases, possibly functioning in glucuronate or galacturonate degradation pathways, the growth substrates triggering activation of these genes still remain unknown. The most important enzymes composing the incomplete TCA cycle are encoded by the following genes: Asuc_0221 (PEP carboxikinase), Asuc_1612 (malate dehydrogenase), Asuc_0956 (fumarase), Asuc_1813-6 (fumarate reductase) and Asuc_1564-5 (succinyl-CoA

synthetase). Asuc_1199 encodes a carbonic anhydrase which is most likely the key enzyme for making CO_2 available, since it catalyses the reaction that converts CO_2 to HCO_3^- and protons. However, genes that encode for PEP carboxylase are absent. The C3 pathway enzymes identified include Asuc_0942-4 encoding for pyruvate dehydrogenase, Asuc_0207 for pyruvate formate-lyase, Asuc_0005 for lactate dehydrogenase and Asuc_1261-6 for the different subunits of formate dehydrogenase. Furthermore, energy for anabolic processes can be gained from reverse fluxes between C4 and C3 pathways, which are particularly affected when CO_2 or H_2 are present. Specifically, malic enzyme and OAA are the nodes where the reverse fluxes between C4 and C3 pathways occur, while the genes encoding for these enzymes are Asuc_0669 and Asuc_0301-3, respectively (McKinlay et al., 2010).

Investigation of the relationship between genome, biochemistry and cell physiology is crucial for understanding the regulatory effects of genes and enzymes, which can lead to the reconstruction of the metabolic network and in silico computation of its integrated functions. Although a limited number of genomic and proteomic studies have been performed up to date (McKinlay et al., 2010, Leduc et al., 2005), it is evident from the relevant literature that a deeper understanding of the biological control mechanisms of *A. succinogenes* growth is required for the development of sustainable succinic acid production. Therefore, research should focus on application of high-throughput experimental technologies combined with mathematical models to identify gene targets for modification and to develop bioprocess engineering strategies for control and optimisation purposes (Koutinas et al., 2012).

The genome of *B. succiniciproducens* was sequenced by DOE - JOINT GENOME INSTITUTE and released on October 2016. The size of the genome is 2.2 Mb with the GenBank accession number, FMUQ01000000. The genome contains 2,028 protein coding genes.

2.8 Genetically modified strains

A wide variety of tools is available to induce genetic diversity and strain development, which can be achieved by targeted genetic manipulation (gene introduction, deletion and overexpression) or through exposure to extreme conditions (e.g. mutagenic chemical agents, UV exposure and radiation). So far, random mutagenesis and directed evolution have been applied in *A. succinogenes* to enhance the production of succinic acid and/or to reduce the formation of by-products. Although suitable shuttle vectors have been constructed and were successful in homologous gene expression in *A. succinogenes* (Jang et al., 2007), there are no focused metabolic engineering studies targeting the improvement of succinic acid production

by *A. succinogenes*. In contrast, several genetically modified *E. coli* strains have been developed to produce succinic acid from various carbon sources (including glucose and xylose) as well as to achieve yields and productivities similar to (or even higher than) natural succinic acid producing bacteria (Vemuri et al., 2002; Jantama et al., 2008; Liang et al., 2013; Liu et al., 2013).

Two mutant strains *A. succinogenes* FZ6 and FZ21 (Table 2.3) with formate lyase and formate dehydrogenase deletions have been constructed (Guettler et al., 1996). Specifically, the mutant FZ6 was used for the production of succinic acid by fumarate alone that was accomplished only in the presence of an external electron donor (e.g. reduced neutral red or H_2) (Park et al., 1999). *A. succinogenes* NJ113 was cultivated in high concentration of ammonium ions (253 mmol/L) resulting in various adapted strains (Table 2.3) (Ye et al., 2010). Batch fermentation was also used for adaptation in osmotic stress (Fang et al., 2011) using the strain *A. succinogenes* mutant YZ0819, which was cultivated in the presence of 0.7 M NaCl. The mutant strain *A. succinogenes* CH050 formed was capable of producing 66 g/L of succinic acid from 95 g/L of glucose, while the mutant YZ0819 produced 48 g/L of succinic acid with a yield of 0.69 g/g. Furthermore, Zheng et al. (2013) reported a new mutation technique based on nitrosoguanidine and UV exposure followed by protoplast fusion. *A. succinogenes* CGMCC1593 was the parental strain and the best succinic acid producing mutant constructed was F3-II-3-F that resulted from the third protoplast fusion (Zheng et al., 2013).

In an attempt to overcome the limitation caused by the lack of suitable shuttle vectors for the development of engineered *A. succinogenes*, an expression vector was constructed (pLGZ901) holding the capacity to carry a foreign gene under the control of a pckA promoter. The vector was successfully introduced into *A. succinogenes* by electroporation (Kim et al., 2004), while the transformation was tested using Tn3 AmpR, Tn10 TetR and Tn9 CmR as selection markers that provide resistance to ampiciclin, tetraciclin and chloramphenicol, respectively. Furthermore, pGZRS-1 was identified as a stable replicon. In a different study, two stable *E. coli-M. succiniciproducens*/*A. succinogenes* shuttle vectors were constructed (pMEx and pMVSCS1) and introduced through optimised electoporation (Jang et al., 2007). The copy numbers of pMEx and pMVSCS1 were 9.9 and 1.7 in *M. succiniciproducens*, while in *A. succinogenes* the copy numbers were 9.9 and 2.5, respectively. Although homologous (fumC) gene expression was successfully tested in both *M. succiniciproducens* and *A. succinogenes*, heterologous (fluorescent proteins) genes were only expressed in *M. succiniciproducens*.

Table 2.3 Mutants and engineered strains of *A. succinogenes*

Genetic modification strategy	Parental strain	Mutant	Glucose (g/L)	Succinic acid (SA) (g/L)	Yield SA (g/g)	Productivity SA (g/L/h)	Formic acid (g/L)	Acetic acid (g/L)	Reference
Adaptation in sodium monofluoroacetate (0.01 - 0.08 mol/L)	*A. succinogenes* 130Z		97	79.8	0.82	nk	7.5	21.4	Guettler et al., 1996
		FZ6	112	92.1	0.82	nk	0	5.2	
		FZ21	110	101	0.92	nk	0	16.4	
Adaptation in ammonium ions (NH_4^+) (0.253 mol/L)	*A. succinogenes* NJ113		45	0	0	0	nk	nk	Ye et al., 2010
		YZ0819	45	33.01	0.73	nk	nk	nk	
		YZ0206	45	31.71	0.71	nk	nk	nk	
		YZ05	45	29.24	0.65	nk	nk	nk	
		YZ03	45	28.11	0.63	nk	nk	nk	
Adaptation in NaCl (0.7 mol/L)	Mutant YZ0819		95	48	0.69	0.69	7.74	11.4	Fang et al., 2011
		Mutant CH050	95	66	0.73	0.74	8.8	12.45	
Genome shuffling by protoplast fusion on mutants derived from nitrosoguanidine (NTG) and UV treatment	*A. succinogenes* CGMCC1593		77.1	55.2	0.72	1.25	0	5.8	Zheng et al., 2013
		VI-10-C	106.7	77.9	0.73	1.62	0	7.1	
		F1-IV-9-D	106	84.2	0.79	1.75	0	7.1	
		F2-III-6-D	110.7	87.3	0.79	1.82	0	6.1	
		F3-II-3-F	121.1	95.6	0.79	1.99	0	6.2	

All genetic alterations of *A. succinogenes* that led to higher succinic acid production have been achieved so far by adaptation and metabolic evolution (Guettler et al., 1996; Ye et al., 2010; Fang et al., 2011; Zheng et al., 2013). Lee and Kim (2015) proposed ten strategies in order to achieve industrial production of a metabolic product. According to the strategies proposed by Lee and Kim (2015), in the case of *A. succinogenes* the following strategies could be elaborated in future studies:

(1) Increasing tolerance to product,
(2) Removing negative regulatory circuits limiting overproduction,
(3) Rerouting fluxes to optimize cofactor and/or precursor availability, and
(4) Diagnosing and optimising metabolic fluxes toward product formation.

Although genetic engineering strategies to enhance succinic acid production in *A. succinogenes* have not yet been reported, shuttle vectors have been successfully constructed and tested for their functionality. Lee et al. (2012) suggested genetic engineering strategies to improve product yield, titer and productivity. One possible solution for *A. succinogenes*, that is a natural succinic acid producer, would be the enhancement of succinic acid titer and yield by gene amplification of the pathways that lead to the desired product or deletion of genes that control pathways leading to the formation of other products. Genes that take part in the reductive pathway of the TCA cycle (PEPCK, MDH, Fm and Fr) could be amplified or genes responsible for by-product formation (AK, PFL) could be knocked out in order to favor the fluxes to the C4 pathway (Figure 2.2).

2.9 Utilisation of renewable resources as nutrient-complete fermentation media

Various industrial waste and by-product streams (e.g. sugar cane molasses, cheese whey, crude glycerol from biodiesel production, wheat milling by-products, sake lees) and agricultural residues (e.g. corn fiber and corncob, sugarcane bagasse, bio-waste cotton) have been evaluated for the production of succinic acid mainly by *A. succinogenes* (Table 2.4). Production of succinic acid requires significant quantities of complex nitrogen sources such as yeast extract. The reduction of succinic acid production cost necessitates the utilisation of low-cost nitrogen sources supplied either by separate renewable resources, such as corn steep liquor (CSL), or by the same renewable resource that also provides the carbon source (e.g. wheat milling by-products, waste bread). For instance, the use of whey as carbon source achieved succinic acid yield of 0.72 $g_{SA}/g_{lactose}$ in the presence of yeast extract, which was only slightly reduced (0.71 $g_{SA}/g_{lactose}$) in the presence of CSL (Lee et al., 2003). The

Table 2.4 Bio-based succinic acid production in fermentations utilising different raw materials and microbial strains

Carbon source	Strain	Nitrogen - nutrient sources (g/L)	Type of fermentation, working volume	SA concen. (g/L)	SA productivity (g/L/h)	Yield ($g_{SA}/g_{total\ sugars}$)	SA:LA:FA:AA (mol/mol)*	Ref.
Representative succinic acid production from pure carbon sources by A. succinogenes								
Glucose	*A. succinogenes* 130Z	YE (6) / CSL (10)	CO_2 sparging, continuous, 0.158 L	48.5	nk	0.84	1:0:0:0.38	Bradfield and Nicol, 2014
Glucose	*A. succinogenes* CGMCC 1593	YE (10) / CSL (6) / Vit	CO_2 sparging, fed-batch, bioreactor, 3 L	60.2	1.3	0.75	1:0: 0.13:0.31	Yan et al., 2014
Glycerol	*A. succinogenes* 130Z	YE (5-10) / Vit	CO_2 sparging, batch, bottle reactors, 0.07 L	26.7	0.23	0.96	1:0:0.15:0.14	Vlysidis et al., 2011
Glycerol	*A. succinogenes* 130 Z	YE (10)	CO_2 sparging, fed-batch, bioreactor, 1.5 L	49.6	0.62	0.92	1:0:0.39:0.16	Carvalho et al., 2014
Sucrose	*A. succinogenes* NJ113	YE (10) / CSL (5)	CO_2 sparging, fed-batch, bioreactor, 1.5 L	60.4	2.16	0.72	1:0:0.55:0.29	Jiang et al., 2014
Cellobiose	*A. succinogenes* NJ113	YE (10) / CSL (5)	CO_2 sparging, batch, bottles, 0.03 L	38.9	1.08	0.66	1:0:0:0.69	Jiang et al., 2013
Representative succinic acid production from crude renewable resources by A. succinogenes								
Corn fiber	*A. succinogenes* FZ6 (mutant)	YE (10), Biotin (10 µg)	CO_2 sparging, batch, vials, 0.01 L	70.6	0.70	0.88	1:0:0.01:0.08:f	Guettler et al., 1996
	A. succinogenes NJ113	YE (10) / CSL (5)	CO_2 sparging, batch, bioreactor, 4.5 L	35.4	0.98	0.72	nk	Chen et al., 2010

Corncob	*A. succinogenes* CICC 11014	YE (11)	CO_2 sparging, batch anaerobic bottles, 0.025 L	23.6	0.49	0.58	nk	Yu et al., 2010
Corn stover	*A. succinogenes* CGMCC 1593	CSL (20)	CO_2 sparging, batch SSF[a], bioreactor, 2 L	47.4	0.99	0.72[b]	1:0.06:0.06:0.44	Zheng et al., 2010
Corn straw	*A. succinogenes* CGMCC1593	YE (15)	CO_2 sparging, fed-batch, bioreactor, nk	53.2	1.21	0.82	1:0:0:0.22	Zheng et al., 2009
Corn stalk	*A. succinogenes* CGMCC 2650 or BE-1	YE (30) / Urea (2)	CO_2 sparging, batch, nk	17.8	0.56	0.66	nk	Li et al., 2010
Wheat milling by-products	*A. succinogenes* 130Z	YE (2.5)	CO_2 sparging, batch, bioreactor, 0.5 L	62.1	0.91	1.02	nk	Dorado et al., 2009
Waste bread	*A. succinogenes* 130Z	Bread hydrolysate (200 mg/L free amino nitrogen)	CO_2 sparging, batch, bioreactor, nk	47.3	1.12	nk	nk	Leung et al., 2009
Cotton stalk	*A. succinogenes* 130Z	YE (30) / Urea (2)	CO_2 sparging, batch SSF[a], flasks, nk	63	1.17	0.64	nk	Li et al., 2013
Cane molasses	*A. succinogenes* CGMCC 1593	YE (10)	CO_2 sparging, fed-batch, bioreactor, nk	55.2	1.15	nk	1:0:0.16:0.32	Liu et al., 2008b
Cane molasses	*A. succinogenes* GXAS137	YE (8.8)	CO_2 sparging, fed-batch, bioreactor, 0.8 L	64.3	1.07	0.76	1:0:0:0.39	Shen et al., 2014
Sugarcane bagasse cellulose	*A. succinogenes* NJ113	YE (10) / CSL (5)	CO_2 sparging, batch bioreactor, 1.5 L	20	0.61	0.65	1:0:0:1.28	Jiang et al., 2013
Sugar cane bagasse	*A. succinogenes* NJ113	YE (10) / CSL (5)	CO_2 sparging, batch, bioreactor, 1.5 L	23.7	0.99	0.79	1:0:0:0.37	Xi et al., 2013
Sugarcane bagasse	*A. succinogenes* CIP 106512	YE (2)	CO_2 sparging, batch, bioreactor, 1.5 L	22.5	1.01	0.43	nk	Borges and Pereira, 2011

Macroalgal hydrolysate	*A. succinogenes* 130Z	YE (16.7)	CO_2 sparging, batch bioreactor, 1.5 L	33.0	1.27	0.75	1:0.18:0.28:0.54:g	Morales et al., 2015
Rapeseed meal	*A. succinogenes* 130 Z	YE (15)	CO_2 sparging, fed-batch SSF[a], bioreactor, 1.2 L	23.4	0.33	0.115[c]	1:0:0:0.71	Chen et al., 2011
Whey	*A. succinogenes* 130Z	YE (5) / Pep (10)	CO_2 sparging, batch, bioreactor, 1.2 L	21.3	0.43 [b]	0.44	1:0.02:0.68:0.78:h	Wan et al., 2008
Sake lees hydrolysate	*A. succinogenes* 130Z	SLH / YE / biotin	CO_2 sparging, batch bioreactor, 1.5L	52.3	1.74	0.85	1:0:0:0.30	Chen et al., 2012
Representative succinic acid production from pure carbon sources by various strains								
Glucose	*A. succiniciproducens* ATCC 53488	YE (5) / Pep (10) / $(NH_4)_2SO_4$ (5)	CO_2 sparging, batch, bioreactor, nk	32.2	1.19	0.90	1:0:0:0.52	Nghiem et al., 1997
Galactose	*A. succiniciproducens* ATCC 29305	YE (2.5) / Pep (2.5) / $(NH_4)_2SO_4$ (5)	CO_2 sparging, batch bioreactor, 1 L	15.3	1.46	0.90	1:0:0:0.60	Lee et al., 2008
Glucose				45.4	2.84	0.92	1:0:0:0.24	
Xylose	*E. coli* AFP184	CSL (33) / $(NH_4)_2SO_4$ (3)	Dual phase, batch, bioreactor, 8 L	29.2	1.79	0.69	1:0:0:0.45	Berglund et al., 2007
Fructose				27.7	1.54	0.46	1:0:0:0.34	
Glucose	*E. coli* AFP111	$(NH_4)2HPO_4$ (8) / NH_4Cl (0.2) / $(NH_4)_2SO_4$ (0.8) / Vit	Dual phase, fed-batch, bioreactor, 3 L	101	1.18	0.78	1:0:0:0.07	Ma et al., 2011
Representative succinic acid production from crude renewable resources by various strains								
Corn stalk	*E. coli* SD121	YE (10) /Tryp (20) / $(NH_4)_2SO_4$●7H2O (3)	Dual phase, batch, bioreactor, 1L	57.8	0.96	0.87	1:0:0:0.29:j	Wang et al., 2011

	A. succiniciproducens ATCC 29305	CSL (20) / Tryptophane (0.02)	CO_2 sparging, fed-batch, bioreactor, nk	34.7	1.02	0.91	nk	Samuelov et al., 1999
	A. succiniciproducens ATCC 29305	CSL (20) / Tryptophane (0.02)	CO_2 sparging, continuous, bioreactor, nk	19.8	3	0.64	nk	
Whey	*M. succiniciproducens* MBEL55E	CSL (7.5)	CO_2 sparging, batch, bioreactor, 1L	13.4	1.18	0.71	1:0.06:1.10:0.73	
	M. succiniciproducens MBEL55E	YE (2.5)	CO_2 sparging, batch, bioreactor, 1L	13.5	1.21	0.72	1:0.05:1.11:0.74	Lee et al., 2003
	M. succiniciproducens MBEL55E	CSL (5)	CO_2 sparging, continuous , bioreactor, 0.5L	10[d]	3.9[d]	0.69[d]	1:0:0.80:0.79	
Cane molasses	*E.coli* AFP111/pTrcC-cscA	$(NH_4)_2HPO_4$ (8) / NH_4Cl (0.2) / $(NH_4)_2SO_4$ (0.7) / Vit	Dual phase , fed-batch, bioreactor, 1.5L	37.3	1.04	0.79	1:0:0:0.17:k	Ma et al., 2014
Cane molasses	*E.coli* KJ122-pKJSUC-24T	$(NH_4)_2HPO_4$ (19.9) / $NH_4H_2PO_4$ (7.5) / Vit	CO_2 sparging, batch, bioreactor, 7.5 L	55.8	0.77	0.96	1:0:0:0.18	Chan et al., 2012
Softwood hydrolysate	*E. coli* AFP184	YE (15) / CSL (15) / $(NH_4)_2SO_4$ (3.3)	Dual phase, batch, bioreactor, 0.7 L	42.2	1.00	0.72	nk	Hodge et al., 2009
Pre-treated wood hydrolysate	*M. succiniciproducens* MBEL55E	YE (5)	CO_2 sparging, batch, bioreactor, 1 L	11.73	1.17	0.56	1:0.23:0.45:0.59	Kim et al., 2004
Pre-treated wood hydrolysate	*M. succiniciproducens* MBEL55E	YE (5)	CO_2 sparging, continuous, bioreactor, 0.5 L	7.98	3.19	0.55	nk	

Nitrogen source: YE: Yeast extract, CSL: Corn steep liquor, Tryp: Tryptone, Pep: Peptone, Vit: Vitamin supplementation; *mol/mol ratio of fermentation by-products SA : Succinic acid, LA : Lactic acid, FA : Formic acid, AA : Acetic acid; nk: not known; [a] Simultaneous saccharification and fermentation; [b] Yield: g SA/g substrate; [c] Yield: gsuccinic acid / gdry matter; [d] Maximum value observed during continuous fermentation at different dilution rates; [e] Also propionic acid (1.9 g/L) and pyruvic acid (2.3 g/L); [f] Also propionic acid (3 g/L); [g] Also ethanol: (2.5 g/L); [h] Also ethanol (3 g/L); [i] Only by-products: ethanol (16.4 g/L) and glycerol (14.9 g/L); [j] Also ethanol (1.62 g/L); [k] Also pyruvic acid (1.2 g/L)

utilisation of agri-industrial waste and by-product streams may also supply other nutrients, such as minerals and vitamins.

Sugar cane molasses has been employed, after pretreatment with sulfuric acid, in fed-batch cultures for succinic acid production by *A. succinogenes* CGMCC1593 leading to the production of 55.2 g/L at a productivity of 1.15 g/L/h (Liu et al., 2008b). Shen et al. (2014) identified the optimum concentrations of total sugars of cane molasses (85 g/L), yeast extract (8.8 g/L), and $MgCO_3$ (63.1 g/L) that led to the production of 64.3 g/L of succinic acid concentration at 60 h fed-batch fermentation. As comparison, the succinic acid concentration (37.3 g/L and 55.8 g/L) and productivity (1.04 g/L/h and 0.77 g/L/h) achieved by genetically engineered *E. coli* strains were approximately in the same range (Ma et al., 2014; Chan et al., 2012).

Crude glycerol is a highly promising industrial by-product stream for succinic acid production because glycerol is a more reduced carbon source than C5 and C6 sugars. Vlysidis et al. (2011) reported the production of 26.7 g/L of succinate concentration at a yield and productivity of 0.96 g/g and 0.23 g/L/h, respectively. Limited glycerol consumption during cell growth by *A. succinogenes* could be improved by the supplementation of external electron acceptors such as dimethylsulfoxide that led to the production of 49.6 g/L of succinic acid concentration with a productivity of 0.96 g/L/h and a yield of 0.64 $g_{SA}/g_{glycerol}$ in fed-batch cultivation (Carvalho et al., 2014).

Wheat milling by-products have been utilised for the production of succinic acid employing a two-stage bioprocess (Dorado et al., 2009). Initially, amylolytic and proteolytic enzymes were produced via solid state fermentations on bran-rich wheat milling streams using the fungal strains *Aspergillus awamori* and *Aspergillus oryzae*. Crude fermented solids were subsequently used to hydrolyse the starch and protein contained in wheat milling by-products. The hydrolysates were used as the sole fermentation feedstock for the production of 50.6 g/L succinic acid using the strain *A. succinogenes*. The utilization of 20% (v/v) inoculum at the beginning of fermentation led to the production of 62.1 g/L succinic acid.

Enzymatic hydrolysates of sake lees (pretreated with 0.5% sulfuric acid) supplemented with 2.5 g/L of yeast extract and 0.2 mg/L biotin led to the production of 36.3 g/L succinic acid with a productivity of 1.21 g/L/h and a yield of 0.59 $g_{SA}/g_{glucose}$ (Chen et al., 2012).

Lignocellulosic biomass has also been used for succinic acid production. Pretreatment of lignocellulosic biomass should be carried out via combined thermo-chemical and

enzymatic treatment in order to produce C5 and C6 sugars. Chen et al. (2010) utilised corn fiber (containing 31.6% hemicellulose, 21.7% cellulose and 15.4% starch) hydrolysates produced via sulfuric acid pretreatment followed by $CaCO_3$ neutralisation and activated carbon absorption (targeting the removal of furfural) for the production of 35.4 g/L succinic acid with a yield of 0.72 g_{SA}/g_{sugars} and a productivity of 0.98 g/L/h using the strain *A. succinogenes* NJ113. Hydrolysates from waste corncob produced via dilute acid pretreatment were used as xylose and arabinose rich media (constituting around 90% of total sugars) supplemented with yeast extract (11 g/L) and $MgCO_3$ (38 g/L) as neutralisation agent for the production of 23.6 g/L succinic acid with a yield of 0.58 g_{SA}/g_{sugars} and a productivity of 0.49 g/L/h (Yu et al., 2010).

Cereal straws are abundant renewable resources with 35 – 45% cellulose, 20 – 30% hemicelluloses and 8 – 15% lignin (Zheng et al., 2009). Corn straw hydrolysate has been demonstrated to be more efficient for the production of succinic acid compared to hydrolysates derived from rice and wheat straw (Zheng et al., 2009). Fed-batch fermentation with the strain *A. succinogenes* CGMCC1593 cultivated on corn straw hydrolysates, rich mainly in glucose and xylose, produced by combined alkali pretreatment followed by enzymatic hydrolysis led to the production of 53.2 g/L of succinic acid concentration with a yield of 0.82 g/g and a productivity of 1.21 g/L/h (Zheng et al., 2009). Cotton stalks pretreated by steam explosion followed by $NaOH/H_2O_2$ treatment were employed in simultaneous saccharification and fermentation at 40°C for the production of succinic acid (63 g/L) by *A. succinogenes* 130Z with a productivity of 1.17 g/L/h and a conversion yield of 0.64 g/g (Li et al., 2013).

Sugarcane bagasse hydrolysates containing glucose (8 g/L), arabinose (5 g/L), xylose (4 g/L) and cellobiose (25.7 g/L) led to complete consumption of all sugars and production of 20 g/L of succinic acid concentration with a yield of 0.65 g/g and a productivity of 0.61 g/L/h using the strain *A. succinogenes* NJ113 (Jiang et al., 2013). Ultrasonic pretreatment of sugarcane bagasse followed by hydrolysis with dilute acid led to the production of 23.7 g/L of succinic acid concentration with a yield and productivity of 0.79 g/g and 0.99 g/L/h, respectively (Xi et al., 2013).

Chen et al. (2011) demonstrated that rapeseed meal could be employed as a renewable resource for succinic acid production providing both carbon and nitrogen sources. The rapeseed meal hydrolysate was produced via pre-treatment with dilute sulfuric acid followed by hydrolysis by a commercial pectinase preparation. Fed-batch fermentations with *A.*

succinogenes ATCC 55618 were carried out with simultaneous saccharification with the pectinase formulation and a rapeseed meal concentration of 20.5% (w/v, on a dry basis) leading to the production of a succinic acid concentration of 23.4 g/L with a yield of 0.115 $g_{SA}/g_{dry\ matter}$ and a productivity of 0.33 g/L/h (Chen et al., 2011).

The pulp and paper industry produces significant quantities of spent liquors that contain high concentrations of sugars derived mainly from hemicellulose degradation. Alexandri et al. (2016) reported the production of succinic acid by *A. succinogenes* and *B. succiniciproducens* in batch cultures using crude and pretreated spent sulphite liquor produced by the sulphite pulping process. The spent sulphite liquor contains predominantly xylose with lower quantities of galactose, glucose, mannose and arabinose. Besides *A. succinogenes*, *Escherichia coli* AFP 184 can also consume xylose (Donnelly et al., 2003).

Waste and by-product streams from the food-industry could be employed for the production of succinic acid. Around 47.3 g/L of succinic acid with productivity of 1.12 g/L/h were produced by *A. succinogenes* cultivated on waste bread hydrolysates produced via hydrolysis of starch and protein contained in waste bread by crude enzymes produced via solid state fermentation (Leung et al., 2012). Spent yeast from breweries and wineries could be employed for nitrogen and other nutrient supplementation after autolysis or enzymatic hydrolysis in order to release the intracellular nutrients. Jiang et al. (2010) reported that spent brewer's yeast hydrolysate supplemented with vitamins could successfully replace the addition of 15 g/L of yeast extract, resulting in the production of 46.8 g/L of succinic acid concentration with a yield of 0.69 $g_{SA}/g_{glucose}$. Besides spent yeast, the corn steep liquor derived from corn refining could be employed as nutrient-rich supplement that could replace yeast extract (Xi et al., 2013).

The high carbohydrate content of macroalgae (up to 60 % dry matter) could be used for succinic acid production. Fermentation of algal hydrolysates (Morales et al., 2015), with initial glucose of around 45 g/L and mannitol around 7.5 g/L with *A. succinogenes* 130Z resulted in 33.78 g/L of succinic acid with a yield of 0.63 $g_{SA}/g_{consumed\ sugars}$ and a productivity of 1.5 g/L/h.

2.10 Effect of pH regulators and osmotic stress

Cellular maintenance and regulation of intracellular enzymatic activities are processes known to be highly pH dependent. Therefore, the pH level is often a key parameter in fermentation processes with most bacterial cultures requiring near neutral pH values for optimal performance. Succinic acid production via fermentation results also in the production

of other organic acids (e.g. formic, acetic acids) necessitating the use of neutralising agents to prevent acidification of the medium. At near neutral pH values, these organic acids are present in their dissociated forms resulting in the production of the respective salts via neutralisation during fermentation. Wang et al. (2012) reported an optimum pH of 7.5 leading to maximum succinic acid production via fermentation of *A. succinogenes* ATCC 55618, while the utilisation of NaOH or KOH as individual neutralising agents led to decreased production of succinic acid due to severe cell flocculation. Inhibition of growth was observed when NH_4OH or $Ca(OH)_2$ were used as neutralising agents (Wang et al., 2012). A combination of 5 M NaOH and 40 g/L of $MgCO_3$ prevented cell flocculation and resulted in 27.9% higher succinic acid concentration (59.2 g/L) than the fermentation where NaOH was used as the sole neutralising agent.

Liu et al. (2008a) reported stable succinic acid production at a pH range of 6 to 7.2, whereas $MgCO_3$ was the best neutralising agent when compared to $CaCO_3$, Na_2CO_3, NaOH and NH_4OH in fermentations carried out with *A. succinogenes* CGMCC1593 using glucose as carbon source. Cell flocculation was observed at 12 h when NaOH or Na_2CO_3 were used, while the utilisation of $MgCO_3$ did not cause any cell flocculation. The use of $CaCO_3$ decreased the production of succinic acid, while NH_4OH resulted in total inhibition of growth and acid formation. Chloride, sulphate and phosphate ions were found not to inhibit the microbial growth. Chloride salts were used to determine which cation caused the inhibitory effect. Although sodium and calcium ions are known to be essential for cell growth, initial concentrations higher than 0.2 mol/L NaCl and 0.1 mol/L $CaCl_2$ are inhibitory to microbial growth and succinic acid production. When $MgCl_2$ was used in a concentration range of 0 – 0.3 mol/L, growth and succinic acid production remained stable.

Li et al. (2011) demonstrated that $MgCO_3$ is the most efficient neutraliser among Na_2CO_3, $NaHCO_3$, $Mg(OH)_2$, $Ca(OH)_2$, $CaCO_3$, NaOH and $NH_3.H_2O$ regarding *A. succinogenes* NJ113 cell growth, glucose utilisation, succinic acid production and glucose to succinic acid conversion yield. The neutralisers $Ca(OH)_2$, $CaCO_3$ and $NH_3.H_2O$ suppressed cell growth resulting in low succinic acid production. Li et al. (2011) reported that addition of mixed neutralisers of $Mg(OH)_2$ and NaOH at a ratio of 1:1 could result in similar succinic acid production efficiency as in the case that only $MgCO_3$ is used as neutraliser. This could be attributed to the strong alkalinity and solubility of NaOH and the necessity of the cofactor Mg^{2+} for the PEP carboxykinase. McKinlay et al. (2015) reported that a $NaHCO_3$ concentration of 25 mM leads to 1.3 – 1.4 times higher *A. succinogenes* growth rate than any other $NaHCO_3$ concentration used when a defined medium (i.e. phosphate-buffered medium

containing glutamate, cysteine and methionine as the required amino acids, NH_4Cl as the main nitrogen source, as well as vitamins and minerals) was used for succinic acid production. In the studies presented above (Liu et al., 2008a; Wang et al., 2012; Li et al., 2011), the most effective pH regulator was $MgCO_3$ providing CO_2 and Mg^{2+} ions that serve as cofactors for PEP carboxykinase, the first enzyme in the reductive branch of the TCA cycle.

The fluidity and permeability of the cell membrane requires the presence of Ca^{+2} ions for energy and transfer regulation (Li et al., 2011; Norris et al., 1996). Elevated concentrations of Ca^{+2} potentially interfere with cell membrane regulation and acid accumulation, while K^+ are crucial for maintenance of intracellular osmotic pressure as well as exchange of acids and bases. Furthermore, Na^+ is involved in intracellular pH regulation (Padan, 2014) and nutrient uptake, whereas high concentrations may result in hyperosmotic stress. Apart from the influence of $MgCO_3$ on succinic acid production, Mg^{+2} ions do not interrupt the stability of the membrane and cell flocculation is not observed. Lee et al. (1999) reported that the maximum cell concentration of *Anaerobiospirillum succiniciproducens* decreased when NaCl concentration was greater than 4 g/L. Moreover, Fang et al. (2011) investigated the effect of three osmoprotectants in *A. succinogenes* NJ113 fermentations performed with high NaCl concentrations. *A. succinogenes* NJ113 was strongly inhibited in NaCl concentration higher than 0.3 mol/L. However, proline was demonstrated as an excellent osmoprotectant and it was more effective compared to trehalose and glycine betaine.

2.11 Supply of CO_2

The CO_2 is more soluble than O_2 in water at 37°C because it reacts with water and in reality dissolved CO_2 is in equilibrium with bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}). The concentration of dissolved CO_2 that is available for succinic acid production when gaseous CO_2 is supplied is dependent on the components of the fermentation broth, the temperature, the agitation, the flow rate of CO_2 and the CO_2 partial pressure. The supply of carbonate (e.g. $MgCO_3$) or bicarbonate salts is applied in many studies focusing on succinic acid production with *A. succinogenes* because their supply together with or without gaseous CO_2 results in higher dissolved CO_2 concentrations (Zou et al., 2011). The presence of carbonates and bicarbonates offer an indirect CO_2 donor because the consumption of CO_2 will lead to conversion of carbonates and bicarbonates into CO_2 in order to maintain the equilibrium. The diffusion of carbonates and bicarbonates through cell membranes requires ATP consumption (Xi et al., 2011). However, supplying high concentration of insoluble carbonates is not practical from an industrial point of view. Samuelov et al. (1991) reported that the affinity for

CO_2 of the enzymes responsible for its fixation is low. This means that high CO_2 partial pressures are required to divert the metabolic flux towards succinic acid production.

Zou et al. (2011) investigated the effect of dissolved CO_2 concentration, gaseous CO_2 partial pressure as well as the supplementation of $MgCO_3$ on the production of succinic acid by *A. succinogenes* ATCC 55618. When gaseous CO_2 was supplied as the sole CO_2 donor, the dissolved CO_2 concentration was never high enough to maximize succinic acid production. The supplementation of 40 g/L of $MgCO_3$ and 101.33 kPa of CO_2 partial pressure (achieved by supplying gas with 100% CO_2 content) resulted in the highest succinic acid concentration (60.4 g/L), productivity (0.84 g/L/h) and yield (0.58 $g_{SA}/g_{glucose}$). Xi et al. (2011) reported that when *A. succinogenes* NJ113 was cultivated at a stirring speed of 200 rpm, a CO_2 partial pressure of 0.1 MPa, pH of 6.8, temperature of 37 ^{o}C and 150 mM $NaHCO_3$, the optimum CO_2 fixation rate of 0.57 g/L/h could be achieved leading to a succinic acid concentration of 51.6 g/L with a yield of 0.76 g/g.

Anthropogenic energy-related CO_2 emissions could be used for succinic acid production targeting carbon capture and recycling of CO_2 rather than simple carbon capture and storage (Quadrelli et al., 2011). The availability of CO_2 may increase due to its market price of around 60 - 450 $/t and an annual estimated carbon capture and storage potential of 3.6 Gt (Quadrelli et al., 2011). The cost associated with the transportation of CO_2 should be minimized as it could be significant contribution to the total cost when the capture site is far from the recycling/storage site. An alternative solution could be precipitation of CO_2 with a Mg^{2+} solution for the production of $MgCO_3$ that could be used in fermentative succinic acid production (Quadrelli et al., 2011).

2.12 Type of bioreactor and operation mode

A. succinogenes was used for the production of succinic acid in different types of bioreactors and operation modes, including utilisation of immobilised biocatalysts, integrated fermentation and separation systems and batch, fed-batch and continuous modes of operation. Fed-batch operation with maintenance of a glucose concentration at 10-15 g/L during feeding could enhance succinic acid concentration and productivity, but increase the succinic acid to acetic acid ratio, as compared to maintaining 30 – 35 g/L glucose concentration during feeding (Liu et al., 2008a). Although the majority of the developed processes include batch or fed-batch fermentations, continuous systems have been also employed for the production of succinic acid. Bradfield and Nicol (2013) investigated continuous succinic acid production by

biofilm formation using perlite packing in an external recycle bioreactor to achieve high productivity (6.35 g/L/h at a dilution rate of 0.56 h^{-1}) and constant succinic acid yield of 0.69 $g_{SA}/g_{consumed\ glucose}$ that was independent of fermentation conditions. In a subsequent publication, Bradfield and Nicol (2014) reported the production of 48.5 g/L of succinic acid concentration with a yield of 0.91 g/g during continuous fermentations of *A. succinogenes* using glucose and CO_2 feeding into a biofilm reactor operated with external-recycle. It was observed that the succinic acid to acetic acid ratio (2.4 – 5.7 g/g) was increased with increasing glucose consumption (10 – 50 g/L), while the formic acid to acetic acid ratio was gradually decreased to almost zero with increasing glucose consumption. Urbance et al. (2004) reported the production of 10.4 g/L and 40 g/L of succinic acid concentrations in continuous and repeated batch biofilm fermentations employing a plastic composite support.

Yan et al. (2014) studied the production of succinic acid by immobilised *A. succinogenes* cells in spiral cotton fiber packed in a modified bioreactor. The fibrous-bed bioreactor was used in 4 cycles of fed-batch fermentations leading to the production of 98.7 g/L of succinic acid concentration with a yield of 0.89 $g_{SA}/g_{glucose}$ and a productivity 2.77 g/L/h. Li et al. (2011) employed fed-batch fermentation integrated with in situ product removal by an expanded-bed adsorption system leading to increased succinic acid concentration (up to 145.2 g/L) with average yields and productivities of 0.52 $g_{SA}/g_{glucose}$ and 1.3 g/L/h, respectively.

2.13 Downstream separation and purification of succinic acid

Downstream separation and purification of succinic acid mainly focuses on the separation of pure crystals from various impurities (e.g. proteins, colour) and by-products generated during fermentation. The separation of metabolic by-products (mainly acetate and formate in the case of *A. succinogenes*) is crucial in order to produce succinic acid crystals of high purity, even in the range of 99.5% (Glassner and Datta, 1992). Thus, in an attempt to develop a cost effective technology several methods have been evaluated for the purification of succinic acid (Figure 2.4). Precipitation of organic acids with calcium hydroxide is the conventional separation process employed by the traditional fermentation industry. Following treatment with calcium hydroxide, calcium succinate is recovered by filtration and converted into succinate with the addition of sulfuric acid. The resulting solution is treated with

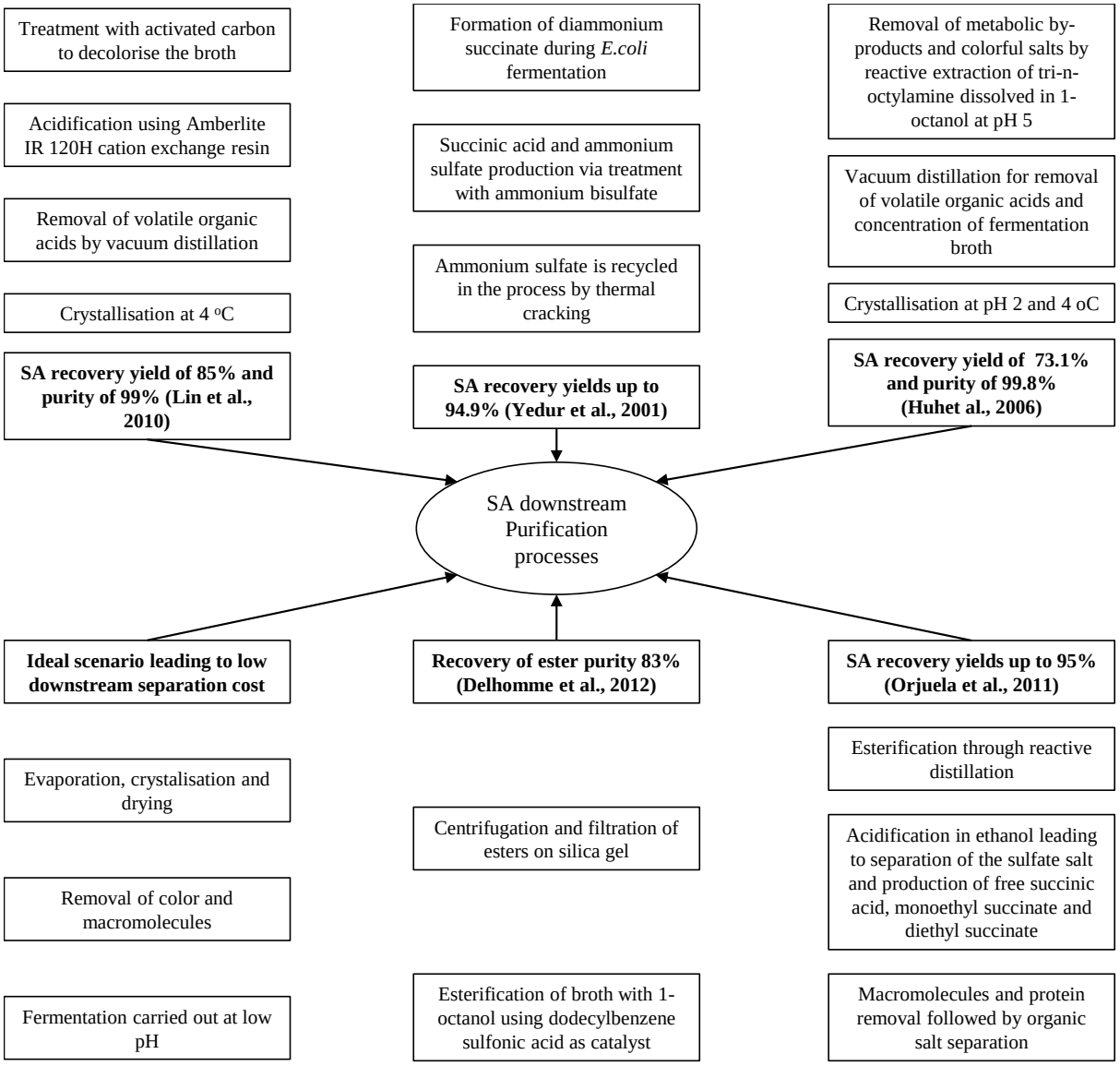

Figure 2.4 Representative downstream separation processes for succinic acid purification

activated carbon and succinic acid is separated using ion exchange resins (Song et al., 2006). The crystals of succinic acid are purified via evaporation to concentrate the solution followed by crystallisation (Song et al., 2006). However, the purification of succinic acid by precipitation with calcium hydroxide presents significant disadvantages, such as the equimolar production of calcium sulfate which is considered a waste stream as well as the high demand for calcium hydroxide and sulfuric acid that cannot be recovered and recycled.

An alternative process employing diammonium succinate produced via neutralisation with an ammonia-based solution during fermentation of *E. coli* resulted in a succinic acid recovery yield of up to 94.9% (Yedur et al., 2001). Diammonium succinate is converted into succinic acid and ammonium sulfate via treatment with ammonium bisulfate and/or sulfuric

acid. The ammonium sulfate could be thermally cracked into ammonia and ammonium bisulfate, which could be reused in the succinic acid production process. The purity of succinic acid crystals could be increased by removing sulfates, which may co-crystallize with succinic acid, via methanol purification. However, ammonia or ammonium based neutralisers do not lead to efficient succinate production by *A. succinogenes* (Liu et al., 2008b).

The relatively high pH values (6.2 – 7.2) employed during fermentation results in the conversion of succinic acid, as well as other organic acids produced as by-products, into ionised succinate salts. Other compounds present in fermentation broths, including carbohydrates, proteins and amino acids, mainly remain non-ionised (Song et al., 2006). Electrodialysis is a technology where separation between ionised and non-ionised compounds is achieved through an ion exchange membrane. As in the case of most organic acids produced via fermentation, bipolar membrane electrodialysis has been employed for the separation of succinic acid from fermentation broths (Fu et al., 2014). The divalent cations (e.g. Mg^{2+}) in the succinate salts produced during *A. succinogenes* fermentation should be replaced by sodium ions in order to minimise operational limitations of bipolar membrane electrodialysis. The sodium hydroxide produced by this process, besides succinic acid separation, could be recycled in the fermentation. However, the high capital and operating costs, the complexity of fermentation broths, the effect of other acids on purity and the operation efficiency of this process need further optimisation in order to ensure industrial implementation (Fu et al., 2014).

Reactive extraction is a promising process for the separation of carboxylic acids from fermentation broths (Jun et al., 2007). The efficiency of reactive extraction is dependent on the amine and solvent used and the stoichiometry of the amine–acid complexes formed. Huh et al. (2006) employed a reactive extraction system comprising of tri-n-octylamine combined with 1-octanol followed by evaporation, crystallisation and drying to achieve succinic acid separation with purity of 99.8% (w/w) and yield of 73.1% (w/w). In this case, the reactive extraction targeted the selective removal of by-products (e.g. acetic acid, pyruvic acid, maleic acid) and salts from diluted *M. succiniciproducens* fermentation broth (Huh et al., 2006).

A downstream process based on treatment with activated carbon, acidification, vacuum distillation, crystallization and drying for the purification of succinic acid crystals has been evaluated leading to relatively high purity (90 – 97 %) and yield (61 – 75%) in the case of simulated broths and lower purity (45%) and yield (28%) in the case of actual fermentation broths (Luque et al., 2009). Lin et al. (2010) reported high purity (99%) and separation yield

(89.5%) of succinic acid crystals via transformation of the salt forms of succinate and other by-products into the free acids via treatment with a commercial cation-exchange resin Amberlite IR 120H, evaporation of the resulting broth to concentrate the succinic acid and remove the other acids, and crystallisation of succinic acid.

Orjuela et al. (2011) employed acidification and esterification in ethanol as a novel approach for the recovery of succinic acid. The fermentation broth was initially treated for cell, macromolecules and protein removal followed by separation of the salts of organic acids that were subsequently placed in ethanol along with a slight stoichiometric excess of sulfuric acid. This process led to separation of the sulfate salt and the succinate was recovered as free succinic acid, monoethyl succinate and diethyl succinate in ethanol, a mixture that was subsequently esterified via reactive distillation (Orjuela et al., 2011). The recovery of succinate obtained with this process was higher than 95% for both simulated succinate salt solutions and actual fermentation broth mixtures.

Succinic acid could be ideally converted directly in the fermentation broth into less miscible products. Budarin et al. (2007a,b) demonstrated that the water insoluble diethyl succinate could be generated by aqueous phase esterification of succinic acid with ethanol that separates easily from the aqueous fraction as a second layer in good purity. The catalyst dodecylbenzene sulfonic acid could esterify succinic acid from fermentation broths with 1-octanol in two-phase systems at a final ester purity of 83% and conversion yield of 85% (Delhomme et al., 2012).

Commercial implementation of succinic acid production requires low number of unit operations for succinic acid purification that could be achieved through implementation of fermentations carried out at low pH, where the organic acids will be present in their undissociated forms. Bioamber is targeting the production of bio-based succinic acid at low pH using recombinant yeast strains (Rush and Fosmer, 2014).

2.14 Techno-economic analysis for succinic acid production

The cost-competitiveness of succinic acid production has been evaluated when different carbon sources were used including pure sugars, such as sucrose (Efe et al., 2013) and glucose (Claypool et al., 2013), and crude renewable resources, such as crude glycerine from biodiesel production processes (Posada et al., 2012; Vlysidis et al., 2011). Efe et al. (2013) carried out a conceptual design of a succinic acid production process with plant capacity of 30,000 t using

hypothetical low pH fermentation and cane sugar as carbon source. The downstream separation process was based on cell removal via centrifugation, succinic acid adsorption using ZSM-5 zeolite followed by desorption using hot water, a flash drum, evaporation, crystallisation and drying resulting in succinic acid crystals with 99.5% purity. The minimum selling price, considering a zero net present value at the end of the life cycle of the plant (10 years), was estimated at 2.26 $/kg at a discount rate of 10%. The contribution of the downstream process was estimated at 0.36 $/kg succinic acid. The total capital investment was estimated at more than $146.6 million with around 11% attributed to the downstream separation process. The bioreactors are the major cost contributor to the total capital investment. Efe et al. (2013) suggested that heat integration and process improvement could significantly decrease the downstream separation cost.

Posada et al. (2012) carried out techno-economic evaluation of succinic acid production using pure glycerol (98% w/w) and a downstream separation stage based on reactive extraction with tri-n-octylamine and 1-octanol as extraction and diluent agents, respectively. The unitary production cost for succinic acid varied in the range 2.01-2.95 $/kg considering a plant production capacity of around 460 kg/h (Posada et al., 2012). Vlysidis et al. (2011) evaluated the design of a small scale succinic acid production plant integrated in an existing biodiesel plant with an annual capacity of 430 t of succinic acid production. The unitary product cost and the minimum selling price were estimated at 2.3 and 3.64, respectively, for an interest rate of 7% and 20 years of plant lifetime (Vlysidis et al., 2011).

Orjuela et al. (2013) carried out process design and techno-economic evaluation for the downstream separation of succinic acid based on dissolution and acidification of succinate salt in ethanol, followed by reactive distillation to produce succinate esters. A capital investment of $75 million and a net processing cost of $1.85 per kg succinic acid were estimated at 54,900 t per year plant capacity and 100 g/L succinic acid concentration in the fermentation stage.

The raw material used, the fermentation stage and the downstream separation process contribute the major costs to succinic acid production. The fermentation stage contributes the highest expenditure to the fixed capital investment. Process integration in existing industrial facilities could reduce logistics related costs. The utilisation of waste and by-product stream could reduce the raw material costs depending on the pretreatment cost of the raw material. Succinic acid production usually requires expensive nutrient supplement, such as yeast extract, that could contribute a considerable cost in the operating cost. The production of

succinic acid crystals with purity higher than 99% is essential in order to reach high yields in the subsequent chemical conversion of succinic acid to various products. For instance, the polymerization of succinic acid to PBS requires a succinic acid purity of at least 98%, and hence, efficient purification processes need to be developed to reach this target (Wang et al., 2014).

The cost of utilities is a critical factor in all cost estimates as significant quantities of electricity and steam are required for the sterilisation process, the agitation of the bioreactors and the downstream separation process. However, utility requirements can be reduced by implementing heat integration techniques. Kastritis et al. (2012) developed an optimum design for a heat exchange network in a biodiesel plant co-producing succinic acid so as to minimise the annual operational cost of the plant. The total annual cost was reduced by 17.2% and the operational cost by 46.4% by implementing heat integration methodologies (Kastritis et al., 2012).

2.15 Biorefinery concepts including succinic acid production

The fermentation efficiency is the most important parameter that decides the cost of manufacture of metabolic products. The higher the final succinic acid concentration and the volumetric productivity achieved, the lower the capital investment required. The higher the carbon source to succinic acid conversion yield achieved, the lower the raw material requirements. Furthermore, by-product formation and neutral pH conditions during fermentation lead to more downstream stages for succinic acid purification meaning higher operating costs and capital investment. Besides fermentation efficiency, refining of renewable resources will lead to the production of versatile end-products with diversified market outlets improving the profitability margin of succinic acid production. Table 2.5 presents potential biorefinery concepts for simultaneous production of succinic acid and various value-added co-products.

In 2021, the worldwide production of crude glycerine is projected at around 3×10^6 t/y based on the expected biodiesel production from edible vegetable oils of 30×10^6 t/y (Anonymous, 2012). If it is assumed that 5% of crude glycerine is used for succinic acid production then around 0.14×10^6 t/y could be produced at a glycerol to succinic acid conversion yield of 0.92 g/g (taken from Table 2.4). The production of succinic acid could be also integrated in biorefineries based on the valorization of oilseed meals (Figure 2.5). This concept involves the utilization of crude glycerine and the carbohydrate content of oilseed

meals as carbon sources for fermentative succinic acid production combined with the extraction of value-added co-products from oilseed meals. A fraction of the protein content in the oilseed meal could be used as substitute for yeast extract. Kachrimanidou et al. (2014) have demonstrated the production of antioxidant-rich fractions, protein isolate and poly(3-hydroxybutyrate) from sunflower meal. The protein content in rapeseed and sunflower meals is around 35 – 40% and 20 – 40%, respectively (Koutinas et al., 2014). Protein isolates could be used as higher value animal feed, whereas their hydrolysates could be used as food additives. Furthermore, rapeseed and sunflower meals contain around 19% and 35% of carbohydrates, respectively (Koutinas et al., 2014). Considering the conventional use of rapeseed and sunflower meals as animal feed, if 5% of the worldwide production in 2012/2013 of rapeseed meal (35.8×10^6 t/y) and sunflower meal (14.9×10^6 t/y) are used for succinic acid production (Anonymus, 2013a), it is estimated that around $0.16 – 0.33\times10^6$ and $0.12 – 0.25\times10^6$ t of succinic acid per year could be produced from the cellulose and hemicelluloses contents of these two meals, respectively, allowing for a mixed sugar to succinic acid conversion yield of 0.43 – 0.88 g/g (taken from Table 2.4).

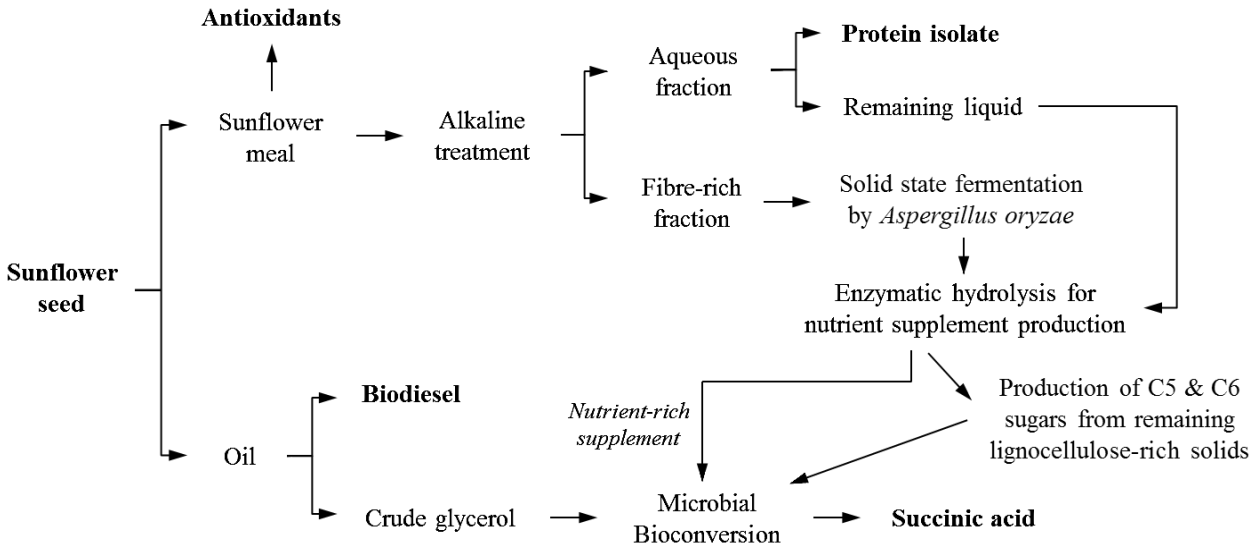

Figure 2.5 Integrated biorefinery for succinic acid production based on the utilisation of biodiesel industry by-products

Table 2.5 Potential production of succinic acid based on agri-industrial side streams and residues

Industrial plant	Availability of waste stream (million t)	Practical carbon source to succinic acid conversion yield (taken from Table 2.4) (g/g)	Potential succinic acid production from by-product steam (million t)[1]	Potential added-value co-products
Biodiesel	3 for crude glycerine	0.92	0.14	-
Biodiesel or edible oil production processes	35.8 for rapeseed meal	0.43 – 0.88 (only the carbohydrate content is considered)	0.16 – 0.33	Phenolic-rich extract with antioxidant properties and protein isolates
	14.9 for sunflower meal		0.12 – 0.25	
Pulp and paper	Around 210×10^6 m^3/y of thick liquor containing an average sugar concentration of 145 g/L	0.43 – 0.88	0.65 – 1.34	Phenolic-rich extract with antioxidant properties and lignin derivatives (e.g. lignosulphonates)
Wheat straw	74	0.43 – 0.88	1 – 2.1 [2]	Waxes and bioenergy/chemicals from lignin
Wineries	18.7 considering prunings, stalks and pomace	0.43 – 0.88	0.04 – 0.09 [3]	Bioactive compounds, pectins, tannins, ethanol and tartrate salts
Wheat and rye losses during processing and consumption in EU27	23.95	1.02 (based on wheat milling by-products)	0.5 [4]	Lipids for biodiesel or oleochemical production
DDGS production from corn ethanol	31.6	0.43 – 0.88	0.4 – 0.8 [5]	Lipids and proteins

[1] assuming 5% raw material utilisation; [2] a moderate polysaccharide content of 60% has been assumed; [3] a10% cellulose/hemicellulose content has been assumed; [4] assuming 40% starch content; [5] assuming 53% carbohydrate content

By-product streams from the current pulp and paper industry are mainly composed of lignin derivatives, phenolic compounds, C5 and C6 sugars, small amounts of cellobiose and various minerals, impurities and derivatives of lignocellulose degradation (e.g. acetic acid, furfural). Phenolic compounds and higher molecular weight lignin derivatives (e.g. lignosulphonates produced by the sulphite pulping process) could be separated by liquid-liquid extraction and membrane filtration to avoid the degradation of sugars (Figure 2.6). Alexandri et al. (2016) has demonstrated the extraction of phenolic-rich fractions with strong antioxidant activity from spent sulphite liquor produced by the sulphite pulping process of hardwood *Eucalyptus globulus*. Phenolic-rich extracts and lignosulphonates could be used in various applications including utilisation of antioxidants in polymer formulation and utilization of lignosulphonates in conventional cement production or novel polymer production. If 5% of the remaining sugars in thick liquors are used for succinic acid production then around 0.65×10^6 – 1.34×10^6 t/y of succinic acid could be produced taking into consideration that the average sugar concentration in thick spent sulphite liquor or black liquor is around 145 g/L and the quantity of thick liquor produced is around 210×10^6 m^3/y (Koutinas et al., 2014).

Wheat straw production in the EU will be approximately 74×10^6 t by 2020 (Kretschmer et al., 2012). Only 17.5% of total straw production can be used as feedstock in the bio-economy sector, after allowing straw usage for soil improvement (75%), power generation (2.5%) and animal husbandry (5%) (Kretschmer et al., 2012). If it is assumed that wheat straw contains a moderate polysaccharide content of 60% (Buranov and Mazza, 2008), then it could be estimated that 1 – 2.1×10^6 t/y of succinic acid could be produced from only 5% of total wheat straw utilisation. Prior to pre-treatment of the lignocellulosic content of wheat straw, it could be processed for the extraction of value-added components (Deswarte et al., 2007). For instance, Deswarte et al. (2007) have developed a process based on supercritical CO_2 extraction of waxes from straw that could be used as soaps, detergents, lubricating grease, wax coatings, surfactants, cosmetics and metal chelators among others, allowing in the same time for minimal change in the nature of the lignocellulosic material. Lignin could be also used either for energy generation or material and chemical production.

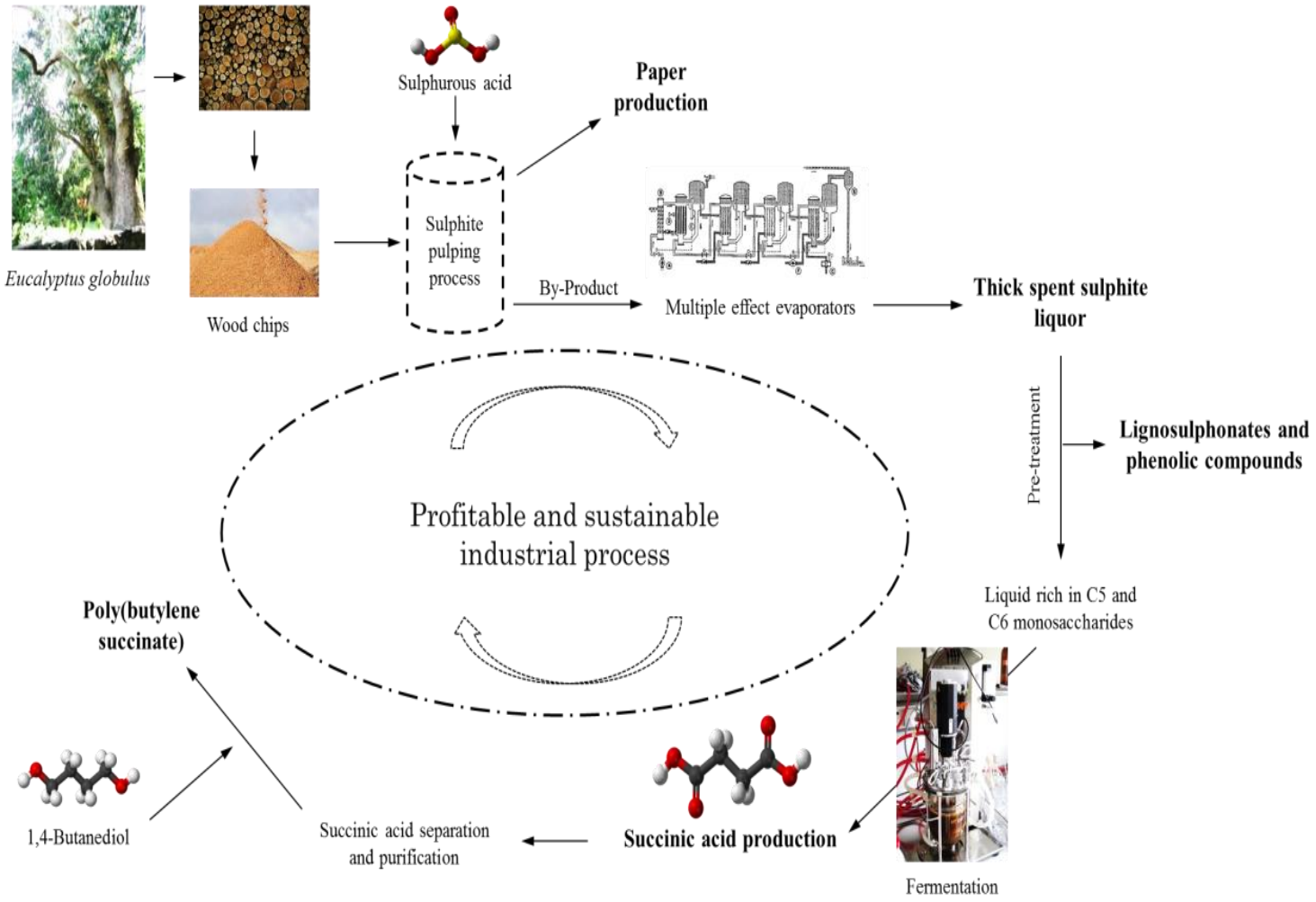

Figure 2.6 Integrated biorefinery based on the utilisation of spent sulphite liquor

Wine production generates many side streams, such as prunings from vineyards and stalks, pomace and lees from wineries. Prunings, stalks and pomace/marc contain cellulose and hemicellulose together with free sugars from pomace/marc depending on the vinification process. These streams also contain bioactive compounds, pectins and tannins that could be extracted as value-added co-products. The wine lees are rich sources of yeast cells, tartrate salts, ethanol and antioxidants. Dimou et al. (2015) developed a process leading to the extraction of antioxidants, ethanol and tartrate salts as value-added co-products followed by the conversion of yeast cells into a nutrient rich hydrolysate via enzymatic hydrolysis that could be used as nutrient supplement in various fermentation processes. The annual production of wine grape in EU is around 24×10^6 t leading to the production of 14×10^6 t of prunings, 1.2×10^6 t of stalks, 3.5×10^6 t of pomace/marc and 1×10^6 t of wine lees. In the case of holistic utilisation of winery side streams, at least 70% of side streams should be utilized for soil improvement. If it is assumed a conservative 10% cellulose/hemicellulose content and only 5% usage potential of prunings, stalks and pomace/marc, then the production of 0.04 – 0.09×10^6 t/y of succinic acid can be estimated. If it is assumed that 5% of the total wine lees is yeast cells, then around 50,000 t of yeast extract would be available for succinic acid production. It should be also stressed that technologies could be developed for the utilization of CO_2 produced during wine production as supplement in fermentative succinic acid production. Figure 2.7 presents a potential biorefinery concept targeting the production of succinic acid and various value-added co-products from winery by-products.

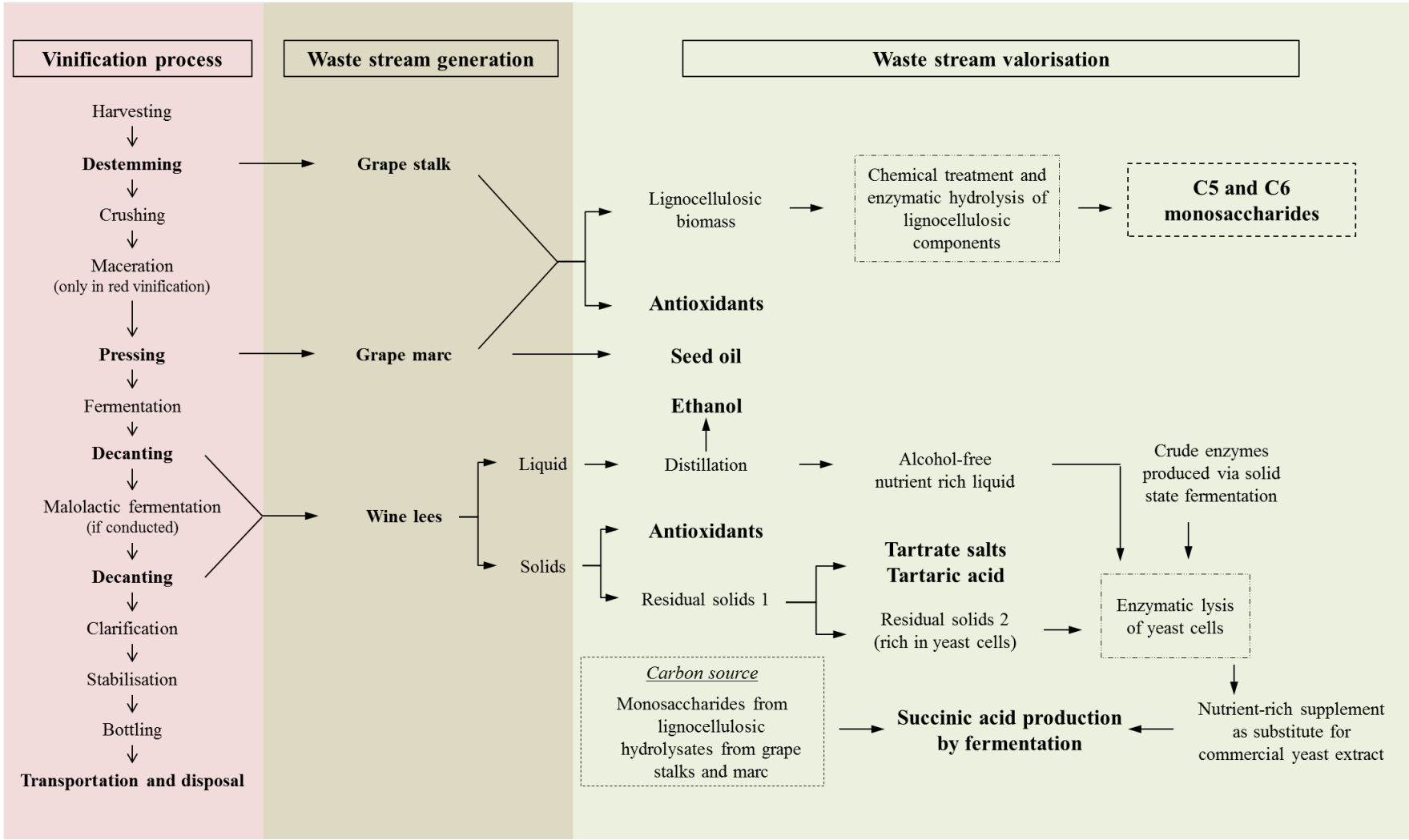

Figure 2.7 Integrated biorefinery based on the utilization of winery by-products

Bakery and confectionery industries generate by-products that are rich in starch, sucrose, lipids and proteins. Lipids can be extracted and used for the production of biodiesel or oleochemicals. Hydrolysis of starch to glucose and proteins to peptides and amino acids could be used as a nutrient-rich fermentation medium for the production of succinic acid. The losses and wastes of wheat and rye in Europe generated at the industrial bread baking and the consumption stages of the whole value chain are 7.45×10^6 and 16.5×10^6 t/y (Gustavsson et al., 2011). If it is assumed that these waste streams contain 40% starch then it is estimated that 0.5×10^6 t/y of succinic acid could be produced from only 5% of wheat and rye losses and wastes, assuming a glucose to succinic acid conversion yield of 1.02 g/g (taken from Table 2.4). If it is assumed a 10% protein content in the aforementioned waste streams then around 2.4×10^6 t/y of protein would be available as nitrogen source in fermentation processes.

Bioethanol production is currently mainly produced from corn and wheat in the USA and Europe, respectively. The dry milling of cereal grains generates distillers dried grains with soluble (DDGS) as by-product stream that contains carbohydrates, lipids, protein, minerals and vitamins (Liu and Rosentrater, 2012). The main conventional use of DDGS is animal feed. The production of DDGS in the USA amounted to 31.6×10^6 t in 2012 (Anonymus, 2013b). The average total carbohydrate content in DDGS is around 53%, which means that if only 5% of total DDGS production is used as fermentation feedstock then around $0.4 – 0.8\times10^6$ t/y of succinic acid could be produced. DDGS could be also used for the extraction of lipids and proteins.

Chapter 3

Objectives

The pulp and paper industry generates huge quantities of SSL and the current exploitation of SSL focuses only on lignosulphonates that are utilised in low-value applications, such as plasticisers for concrete production. The conventional process employed for the separation of lignosulphonates is based on SSL treatment with sodium or calcium hydroxide leading to lignosulphonate precipitation and simultaneous destruction of the sugar fraction. The eventual depletion of fossil resources raises significant environmental concerns, rendering inevitable the substitution of conventional petroleum-derived products by bio-based products derived from renewable resources. Under this prospect, bio-based chemicals (e.g. succinic acid) are envisaged to replace petroleum derived chemicals.

In the future bio-economy era, biorefinery concepts using renewable resources for the production of various products should be developed and gradually evolve into mature processes in a similar manner as petroleum refineries evolved since the beginning of the 20th century. Under this viewpoint, this thesis focused on the exploitation of SSL, separating LS for current or novel applications and utilising the remaining fraction rich in sugars for the production of bio-based succinic acid. A deep understanding of the bacterial metabolism is crucial in order to determine the bottlenecks of bacterial fermentation when crude feedstocks are used. Furthermore, an integrated system for succinic acid production and separation could enhance the sustainability of the whole bioprocess. The main objectives of the experimental work are presented below:

- Selection of the most efficient bacterial strain for the production of succinic acid using SSL as carbon source.
- Evaluation of the effect of initial sugar concentration on succinic acid production and bacterial growth when the strains *A. succinogenes* and *B. succiniciproducens* were used.
- Evaluation of the effect of initial concentration of metabolic products (*i.e.* succinic acid, acetic acid, formic acid and lactic acid) on succinic acid production and bacterial growth using the strains *A. succinogenes* and *B. succiniciproducens*. The critical inhibitory concentration for each organic acid was determined.
- Evaluation of the effect of initial concentration of specific SSL components (*i.e.* lignosulphonates, methanol, acetic acid and furfural) on succinic acid production and bacterial growth using the strains *A. succinogenes* and *B. succiniciproducens*. The critical inhibitory concentration for each SSL component was determined.
- Evaluation of SSL pretreatment via ultrafiltration or nanofiltration for the separation of LS in the retentate stream and sugars in the permeate stream.

- Succinic acid production via fed-batch fermentations using the strains *A. succinogenes* and *B. succiniciproducens* cultivated on commercial mixed sugars, SSL, ultrafiltrated SSL and nanofiltrated SSL.
- Analysis of RNA expression of the main metabolic pathways that contribute to succinic acid production and basic metabolic regulations of the bacterial cells.
- Application of an integrated fermentation coupled with organic acid extraction through an electrochemical cell using an anion selective membrane. This technology was evaluated in this thesis at a preliminary level and it was shown that it could evolve into a breakthrough succinic acid production technology.

Chapter 4

Materials and methods

4.1 Bacterial strains, pre-culture conditions and microorganism preservation

The bacterial strains *Actinobacillus succinogenes* 130Z (DSM-22257), *Basfia succiniciproducens* JF 4016 (DSM-22022), *Xylanibacter oryzae* KB3^T (DSM-17970), *Ruminococcus flavefaciens* 17 (DSM- 25089) and *Succinivibrio dextrinosolvens* 0554 (DSM-3072) were purchased from the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures. The genetically engineered *Escherichia coli* AFP184 was purchased from ATCC (Catalog No. 8739).

The pre-culture medium for *Actinobacillus succinogenes* 130Z, *Basfia succiniciproducens* JF 4016 and *Xylanibacter oryzae* KB3^T was tryptic soy broth (TSB), with pH of 7.3 ± 0.5, that consists of (per L): dextrose (2.5 g), enzymatic digest of casein (17 g), enzymatic digest of soybean meal (3 g), NaCl (5 g) and Na_2HPO_4 (2.5 g). The pre-culture medium for *Escherichia coli* AFP184 was luria bertani (LB) medium, with pH of 7.0 ± 0.5, that consists of (per L): tryptone (10 g), yeast extract (5 g), and NaCl (10 g). Pre-culture medium for *Ruminococcus flavefaciens* 17 was prepared under CO_2 atmosphere and consisted of (per L): glucose (3 g), cellobiose (2 g), tryptone (5 g), yeast extract (2 g), K_2HPO_4 (0.24 g), KH_2PO_4 (0.24 g), $(NH_4)_2SO_4$ (0.8 g), NaCl (0.48 g), $MgSO_4 \cdot 7\ H_2O$ (0.1 g), $CaCl_2 \cdot 7H_2O$ (0.064 g), resazurin (1 mg), Na_2CO_3 (4 g), iso-butyric acid (0.1 mL), iso-valeric acid (0.1 mL), 2-methyl-butyric acid (0.7 mL) and cystein-HCl·H_2O (0.5 g). The final pH was adjusted to 7.0 ± 0.5. The pre-culture medium for *Succinivibrio dextrinosolvens* 0554 was prepared under CO_2 atmosphere and consisted of (per L): glucose (0.5 g), cellobiose (0.5 g), maltose (0.5 g), soluble starch (0.5 g), glycerol (0.5 g), trypticase peptone (2 g), yeast extract (0.5 g), K_2HPO_4 (0.3 g), KH_2PO_4 (0.23 g), $(NH_4)_2SO_4$ (0.23 g), NaCl (0.46 g), $MgSO_4 \cdot 7\ H_2O$ (0.1 g), $CaCl_2 \cdot 7H_2O$, (0.06 g), resazurin (1 mg), Na_2CO_3 (4 g), $Na_2S \cdot 9H_2O$ (0.25 g), acetic acid (1.7 mL), propionic acid (0.6 mL), butyric acid (0.4 mL), n-valeric acid (0.1 mL), iso-butyric acid (0.1 mL), iso-valeric acid (0.1 mL), 2-methyl-butyric acid (0.1 mL), cystein-HCl·H_2O (0.25 g). The final pH was adjusted to 6.75 ± 0.5.

Incubation of *A. succinogenes* 130Z, *B. succiniciproducens* JF 4016, and *E. coli* AFP184 was conducted at 37 °C and *X. oryzae* KB3^T at 30 °C in conical flasks with cotton plug in an orbital shaker using an agitation speed of 180 rpm. Incubation of *R. flavefaciens* 17 and *S. dextrinosolvens* 0554 was conducted at 37 °C in hungate bottles (with serum cup) in an orbital shaker using an agitation speed of 180 rpm.

All cultures were collected at 12-16 h (late exponential phase) for inoculation to the main fermentation media or for the preparation of stock solutions. All microorganisms were

preserved in cryopreservation vials at –80°C in a medium containing liquid culture and pure glycerol in 50% v/v ratio.

4.2 Commercial supplements

Glucose, xylose, galactose, mannose, arabinose, yeast extract, $NaHCO_3$, $NaH_2PO_4 \cdot H_2O$, Na_2HPO_4, NaCl, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, succinic acid, lactic acid, formic acid, acetic acid, furfural, methanol were purchased from Sigma.

4.3 Spent Sulphite Liquor and extracted lignosulphonates

The spent sulphite liquor (SSL) and the extracted lignosulphonates (LS) used in this study were kindly provided by Sniace S.A. (Torrelavega, Spain). The SSL (Figure 4.1) was produced via the sulphite pulping process of *Eucalyptus globulus* and the composition is presented in Chepter 6. The SSL was used as fermentation medium for succinic acid production either intact or after membrane pretreatment. The SSL was pretreated by ultrafiltration or nanofiltration as described in the next sections.

Figure 4.1 Spent sulphite liquor (SSL) provided by Sniace S.A. (Torrelavega, Spain)

4.3.1 Ultrafiltration of SSL

Ultrafiltration of SSL was carried out in Amicon stirred ultrafiltration cells (model 8400, Merck Millipore, Cat.No. 5124). The capacity of the apparatus was 400 mL and it was filled with 10 times diluted SSL. Nitrogen sparging was applied in the system creating a transmembrane pressure of 3 bars. Regenerated cellulose membranes (Merck Millipore, Cat.No. PLBC07610, PLCC07610 and PLGC07610) were used with three different molecular weight cut offs (MWCO), namely 3 kDa, 5 kDa and 10 kDa. The diameter of each membrane was 76 mm. The flux of the permeate generated through the membrane with 10 kDa MWCO was 35.7 L/(m^2.h) throughout filtration, whereas when the membranes with MWCO of 5 kDa and 3 kDa were used the flux of the permeate through the membrane was 12.6 L/(m^2.h) for both membranes. After filtration, the membranes were washed thoroughly with 0.1 M NaOH and stored at 22°C in ultrapure water for a short period or at 4°C in 10% of ethanol solution for a longer storage period.

4.3.2 Nanofiltration of SSL

Nanofiltration of SSL was carried out by the company AVECOM NV (Ghent, Belgium) using a vibratory shear-enhanced processing filtration unit (V-SEP, New Logic Research, Emeryville, CA). The V-SEP filtration module uses oscillatory vibration to create high shear at the surface of the filter membrane leading to high throughputs and minimal reject volumes due to reduced membrane fouling. The membrane (thin film non-polyamide membrane, NF-500) used in the V-SEP filter for nanofiltration of SSL had MWCO of 500 Da. The surface area of the membranes in the V-SEP module was 0.045 m². The initial volume of SSL used was 32 L of 7 times diluted SSL. The filtration unit was initially operated in a closed circuit mode for 1 h, before the experiment was carried out, meaning that the filtrate was returned to the influent vessel so that there was no up-concentration of the influent. This preliminary membrane test was performed in order to determine the membrane flux under steady-state conditions. Nanofiltration of SSL using the membrane with 500 Da MWCO was carried out at a stable flow rate of the concentrate at approximately 680 L/h. The temperature during filtration was in the range of 54-57^{0}C. The initial pressure was 20.8 bar and was gradually increased to 31 bar during filtration. The flux at the beginning of the experiment was 44 L/(m^2.h) that was gradually reduced to 10.7 L/(m^2.h) when the filtration stopped. The maximum volumetric recovery achieved was 65.5%. The total permeate (20.95 L) obtained contained 27.2 g/L total sugars and 4.1 g/L LS.

4.4 Evaluation of bacterial growth in microplate reader incubator

Preliminary experiments where growth parameters were evaluated were conducted in the microplate reader. Experimental runs evaluating the bacterial growth in varying initial substrate concentration and initial fermentation product concentrations were conducted in a microplate reader (Thermo Labsystems Microtiter 96 Plate). Figure 4.2 presents the microplate reader incubator used in this study. In this way we could identify the critical concentrations of substrate, individual organic acids and mixed organic acids that inhibit bacterial growth.

Figure 4.2 Thermo Labsystems Microtiter 96 Plate

The culture medium used in this set of experiments contained (per L): xylose (10 g), yeast extract (5 g), $NaHCO_3$ (10 g), $NaH_2PO_4 \cdot H_2O$ (1.16 g), Na_2HPO_4 (0.31 g), NaCl (1 g), $MgCl_2 \cdot 6H_2O$ (0.2 g), and $CaCl_2 \cdot 2H_2O$ (0.2 g). The main fermentation products were added individually in increasing initial concentrations. Succinic acid (0 – 60 g/L), formic acid (0 – 20 g/L), acetic acid (0 – 40 g/L) and lactic acid (0 – 60 g/L) were added in the culture medium before inoculation. Also the synergistic inhibition effect was evaluated by the addition of increasing initial concentrations of a mixture of organic acids ranging from 0 to 37 g/L in a ratio (g/g) of SA:FA:AA:LA equal to 1:0.45:0.59:0 for *A. succinogenes* and 1:0.12:0.29:0.22 for *B. succiniciproducens*. The culture medium for the evaluation of the effect of initial substrate concentration contained increasing initial sugar concentration of 10 – 220 g/L. The substrate was composed of different monosaccharides at the following ratio: 72.6% xylose, 12.2% galactose, 10.9% glucose, 4.2% mannose, and 0.1% arabinose. The monosaccharide

ratio was selected so as to simulate the SSL sugar composition that was evaluated in this study as a carbon source for succinic acid production.

Incubation of *A. succinogenes* 130Z and *B. succiniciproducens* JF 4016 in a 96 well microplate was performed at 37^{o}C with a 10 sec orbital agitation of 960 rpm every 10 min. Growth was monitored by measuring optical density at 660 nm at 10 min intervals in a continuous kinetic mode. Concentrated solutions of each substrate were prepared separately and the pH was adjusted to 7 with 0.1 M NaOH or 7% (v/v) HCl in all solutions before reaching the final volume. All solutions were sterilised separately at 121^{o}C for 20 min and the microplates were either sterile or sterilized under UV light exposure for 1 h. The inoculum was 10 % (v/v) in a 200 μL total fermentation volume. To avoid evaporation and to maintain anaerobic conditions 50 μL of mineral oil was added after inoculation. Experimental values were the average of six replicates.

4.5 Set-up of Duran bottle fermentations

Small scale fermentations in 0.5 L Duran bottles (Figure 4.3**)** with a working volume of 0.25 L were conducted to evaluate the ability of *A. succinogenes* 130Z and *B. succiniciproducens* JF 4016 to consume different monosaccharides alone or in a mixture and their efficiency to produce succinic acid in increasing initial concentrations of sugar mixture. Furthermore, the ability to grow and produce succinic acid in the presence of the main inhibitors contained in SSL was evaluated. Finally, fermentations in different dilutions of SSL and in the presence of extracted LS were conducted so as to evaluate the critical inhibitory concentration.

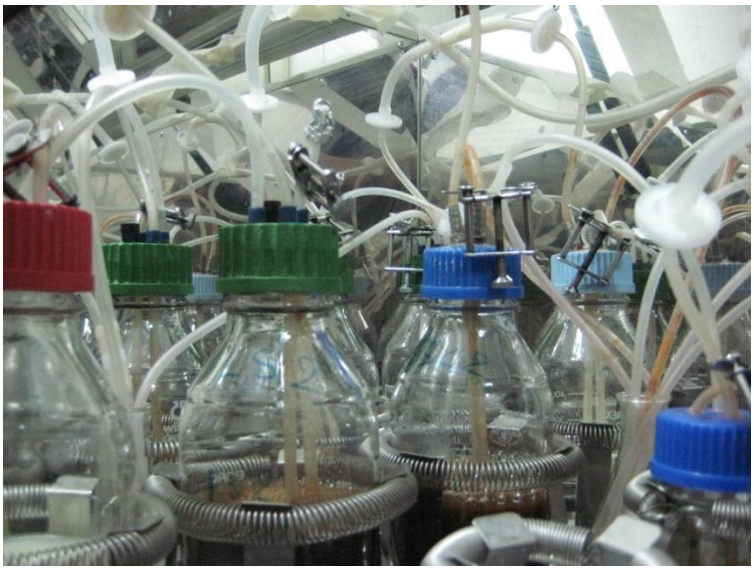

Figure 4.3 Duran bottle fermentations in orbital shaker incubator

Increasing initial concentrations (0 – 70 g/L) of a monosaccharide mixture that contained 72.6% of xylose, 12.2% of galactose, 10.9% of glucose, 4.2% of mannose, and 0.1% of arabinose were used in order to simulate the monosaccharide content of spent sulphite liquor. Different dilutions (1:10, 1:7 and 1:5) of concentrated SSL were evaluated as carbon sources. SSL treated via ultrafiltration of 10 kDa, 5 kDa and 3 kDa and nano-filtration of 500 and membrane cut-offs were also evaluated as carbon sources. Inhibitory compounds that are found in SSL were individually added to the fermentation broth in order to evaluate the critical concentration. Acetic acid (10 g/L, 12 g/L and 17 g/L) , furfural (0.5 g/L, 1 g/L, 2 g/L, 3 g/L), methanol (0.5 g/L, 2 g/L, 4 g/L, 8 g/L 10 g/L, 15 g/L), lignosulphonates (36 g/L, 72 g/L ,155 g/L) were added in increasing initial concentrations. The fermentation broth was enriched with 5 g/L yeast extract, 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 a few drops of antifoam. An equal amount to the total sugar concentration of $MgCO_3$ was also added in each experiment in order to maintain pH at levels of 6.4 - 7.0. The fermentation medium was sterilized at 121^oC for 20 min. The sugars were sterilized separately from the rest of the medium.

Agitation was controlled at 170 rpm in a rotary shaker and temperature was kept constant at 37^oC. Continuous sparging of CO_2 was supplied with a flow rate of 0.1 vvm. The CO_2 gas was filter sterilized using 0.2 μm whatman filter units. Dual-phase fermentations were carried out with *E. coli* AFP184 with an initial aerobic growth phase (with air gas sparging at 1 vvm flow rate) of 6 h and 8 h, and then a transition to anaerobic fermentation (switch to CO_2 gas sparging at 0.1 vvm flow rate). Duran bottles were equipped with a gas outlet tube that was placed in 70% (v/v) aqueous ethanol solution throughout the whole experiment.

4.6 Lab-scale fermentations using different feedstocks

Bench-top bioreactors were used for the optimisation of succinic acid fermentation of *A. succinogenes* 130Z and *B. succiniciproducens* JF 4016 using SSL. Fed-batch fermentations were carried out using mixed sugars, SSL and membrane treated SSL in order to investigate the limitations of the substrate and select the optimum substrate for succinic acid production. The commercial sugars employed were glucose, xylose or mixed C5 and C6 sugars formulated using commercial sugars following the ratio of sugars contained in SSL (*i.e.* 72.6% of xylose, 12.2% of galactose, 10.9% of glucose, 4.2% of mannose, and 0.1% of arabinose). The medium also contained 5 g/L of yeast extract, 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 5 g/L of $MgCO_3$.

The SSL-based media were untreated SSL, ultrafiltrated SSL and nanofiltrated SSL. The feeding solution was prepared with: 1) commercial mixed sugars at the same ratio as in SSL when the fermentation was carried out with mixed sugars, 2) untreated SSL when the fermentation was carried out with untreated 7 times diluted SSL, and 3) commercial mixed sugars in which extracted lignosulphonates were added in order to simulate the composition of concentrated ultrafiltrated or nanofiltrated SSL. The extracted LS were used in order to create a simulated ultrafiltrated or nanofiltrated SSL that could be produced after concentration via evaporation. The commercial mixed sugars were used to create a sugar concentration of 400 g/L in order to avoid dilution of the fermentation broth due to feeding. The total sugar concentration in the feeding solutions was 400 g/L, except for the fermentation where the thick SSL was used as feeding solution in which the total sugar concentration was 176.5 g/L. All feeding solutions contained also 1 % (w/v) yeast extract. The feeding solution was added continuously in order to maintain low sugar concentration (5 - 20 g/L) throughout fermentation.

Figure 4.4 Bench-top bioreactor Labfors 4, Infors HT

All fermentations were carried out at 1 L working volume in Labfors 4, Infors HT bioreactor with 2.5 L total volume capacity. Figure 4.4 presents an image of the bioreactor used in this study to evaluate different carbon sources. Temperature was always controlled at 37^{o}C and agitation speed at 100 rpm. Continuous sparging of CO_2 at a flow rate of 0.5 vvm

was applied. Inoculum size was 10% (v/v). Fermentation pH was automatically controlled at 6.6 by supplying 10 M NaOH. The fermentation medium was heat sterilized at 121^{o}C for 20 min. The sugars were sterilised separately from the rest of the medium.

4.7 Bench-top lab scale fermentations for RNA expression

Batch fermentations were carried out with glucose, xylose and ultrafiltrated SSL as carbon sources to study the RNA expression in the metabolism of *A. succinogenes* 130Z. Initial sugar concentration was 30 g/L. The medium also contained 5 g/L yeast extract, 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 $CaCl_2 \cdot 2H_2O$. The SSL-based media were ultrafiltrated with 3 kDa MWCO membrane. All fermentations were carried out at 2 L working volume in Labfors 4, Infors HT bioreactor with 3.4 L total volume capacity. Temperature was always controlled at 37^{o}C and agitation speed at 100 rpm. Continuous sparging of CO_2 at a flow rate of 0.5 vvm was applied. Inoculum size was 10% (v/v). Fermentation pH was automatically controlled at 6.6 by supplying 10 M NaOH. The fermentation medium was heat sterilized at 121^{o}C for 20 min. Sugar solution was sterilized separately from the rest of the medium. The samples were taken under sterile conditions and transferred immediately to ice. They were washed with sterile solution of 150 mM NaCl prepared with ddH_2O and the biomass pellet was stored at -80^{o}C until further treatment.

4.8 Integrated succinic acid production and separation using an electrochemical bioreactor

Figure 4.5 presents an image of the electrochemical bioreactor used in this study, while Fed-batch fermentations were carried out in a bench-top bioreactor (Bioengineering, RALF Advanced, 6.7 L), with an initial working volume of 3 L, at 37 ^{o}C, an agitation speed of 200 rpm using two Rushton impellers and continuous sparging of 0.1 vvm of CO_2 through a sterile filter of 0.2 μm pore size. A 10% (v/v) inoculum was used. The initial carbon source employed during all fermentations was glucose, xylose or ultrafiltered SSL. The SSL was treated via ultrafiltration using membranes with 3 kDa MWCO in an Amicon stirred ultrafiltration cell (model 8400, Merck Millipore, Cat.No. 5124) as described at section 4.4.1. Yeast extract was initially added in the medium at a concentration of 5 g/L. The following minerals were also supplemented: 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 $CaCl_2 \cdot 2H_2O$.

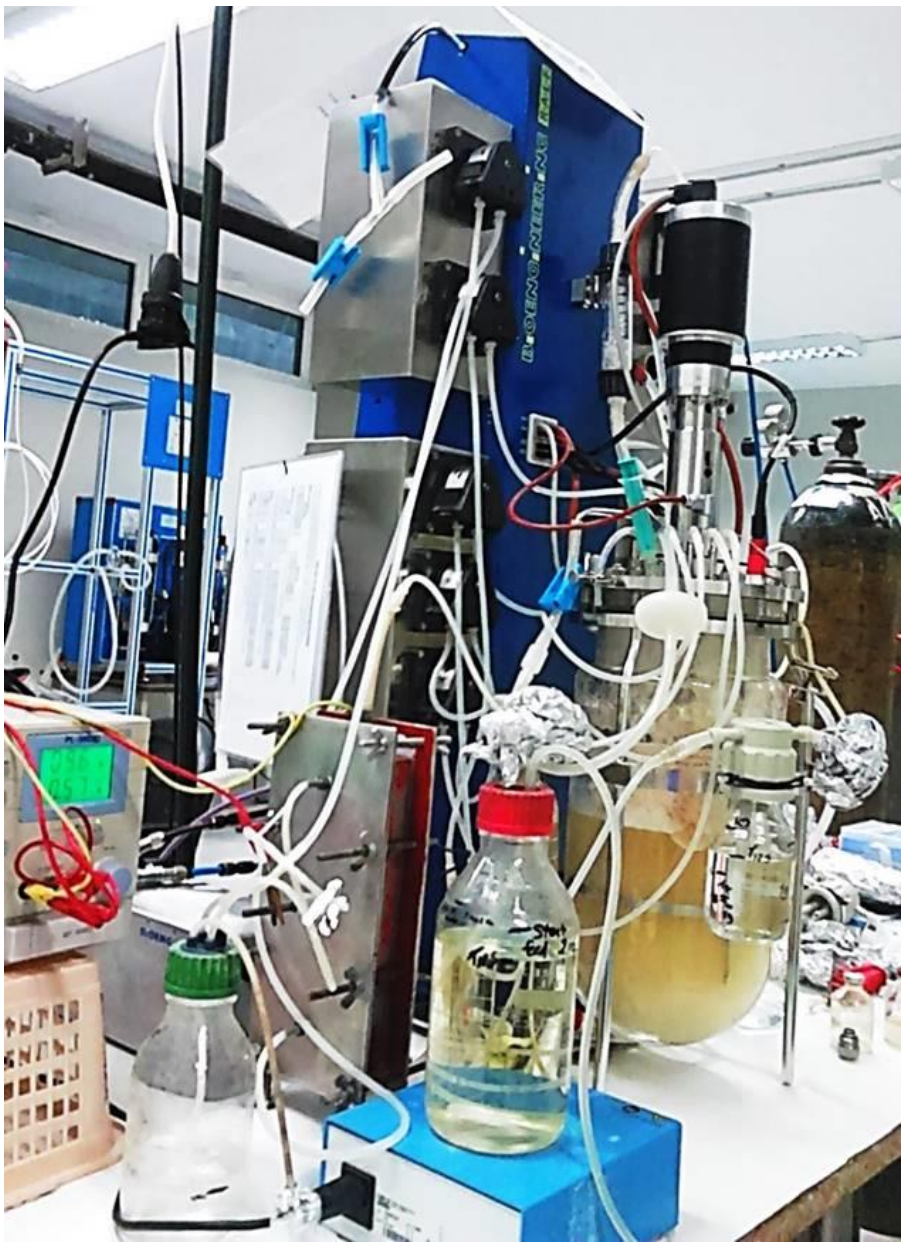

Figure 4.5 Integrated succinic acid production and separation using an electrochemical bioreactor

The feeding solution was prepared with: 1) commercial sugars (glucose or xylose), 2) commercial mixture of sugars (xylose, glucose, galactose, mannose and arabinose) supplemented with extracted lignosulphonates in order to simulate the composition of concentrated ultrafiltrated SSL in the ratio 400 g/L mixed sugars and 40 g/L of lignosulphonates. All feeding solutions contained also 1 % (w/v) yeast extract, unless stated otherwise. The feeding solution was added continuously in order to maintain a sugar concentration of 5 - 30 g/L throughout fermentation. Fermentation pH was automatically controlled at 6.6 by supplying 10 M NaOH. The carbon source (commercial sugars and SSL), the nitrogen source and the mineral supplements were sterilised separately at 121°C for 15

min. The electrochemical cell contained two chambers similar to Andersen et al. (2015). The internal dimensions of each chamber were 20 cm (l) × 5 cm (w) × 2 cm (d) cm and were separated by a single anion exchange membrane (AEM) with operating surface dimensions of 20 cm (l) × 5 cm(w) × 0.3 cm (thickness) (AMI 7001, Membrane International, New Jersey, United States). The cathode was a stainless steel mesh cathode (20 cm × 5 cm), and the anode an iridium oxide-coated titanium anode (20 cm × 5 cm), IrO2/TaO2: 0.65/0.35 (Magneto, The Netherlands). Two spacers (ElectroCell A/S, Denmark) were placed between the surface of the electrodes and the anion exchange membranes to prevent electrode contact with the anion exchange membrane and promote convection at the membrane surface. The fermentation chamber was filled with a mineral solution (part of the fermentation medium) and a 10 mM solution of K_2HPO_4 was used to fill the extraction chamber during sterilisation. The electrolysis cell was sterilised separately from the bioreactor at 121 ^{o}C for 15 min, and connected under aseptic conditions after cooling. A peristaltic pump with two compartments recycled the fermentation broth and the extract solution at 3 L/h in parallel flow across the membrane. The electrolysis cell was connected to the fermentation broth which was recycled until it was homogenised without power supply with a hydraulic retention time of 1 h that was maintained constant throughout the experiments. The solution of the extract chamber was recycled in an external vessel with a total liquid volume of 500 mL and a hydraulic retention time of 0.17 h that was maintained constant throughout the experiments. The temperature of the extraction vessel was controlled at 37 °C using a water bath. A power supply unit (PL-3003D, Protek, United States) was used to provide electricity to the cell. The electric current was controlled at 0.57 mA and the voltage was monitored throughout the experiments. The power supply was switched on when *A. succinogenes* cells were inoculated in the bioreactor.

4.9 Analytical methods

4.9.1 Growth determination

To eliminate excess of $MgCO_3$ in each sample, an HCl solution of 7% (v/v) was added into each sample.

4.9.2 Determination of carbon source and extracellular metabolites

The concentration of monosaccharides as well as succinic acid, lactic acid, formic acid, acetic acid, ethanol, methanol and furfural were determined using an HPLC Shimadzu UFLC XR unit with an RI detector equipped with an Aminex HPX-87H (7.8 x 300 mm) column.

The temperature of the column was 65°C and the mobile phase was a 10 mM H_2SO_4 aqueous solution with 0.6 mL/min flow rate. The concentration of monosaccharides was also determined with a Shodex SP0810 column at 60 °C with 1 mL/min flow rate of pure water as the mobile phase. Figures 4.6 and 4.7 present the standard curves of glucose and succinic acid that were used for the determination of the concentration of each component determined by HPLC analysis with the column Aminex HPX-87H. The standard curves are represented by the following equations:

Glucose: Y = (6.9313e-006)X + (0.0252232)), R^2: 0,9997919

Xylose: Y = (7.31234e-006)X + (0.00620701), R^2: 0,9999437

Arabinose: Y = (7.07778e-006)X + (0.0225372)), R^2: 0,9999375

Succinic acid: Y = (9.67609e-006)X + (0.0391408)), R^2: 0,9997305

Lactic acid: Y = (9.17263e-006)X + (0.0587179)), R^2: 0,9977983

Formic acid: Y = (2.0262e-005)X + (0.0410526)), R^2: 0,9999238

Acetic acid: Y = (1.44313e-005)X + (0.0411117)), R^2: 0,9999296

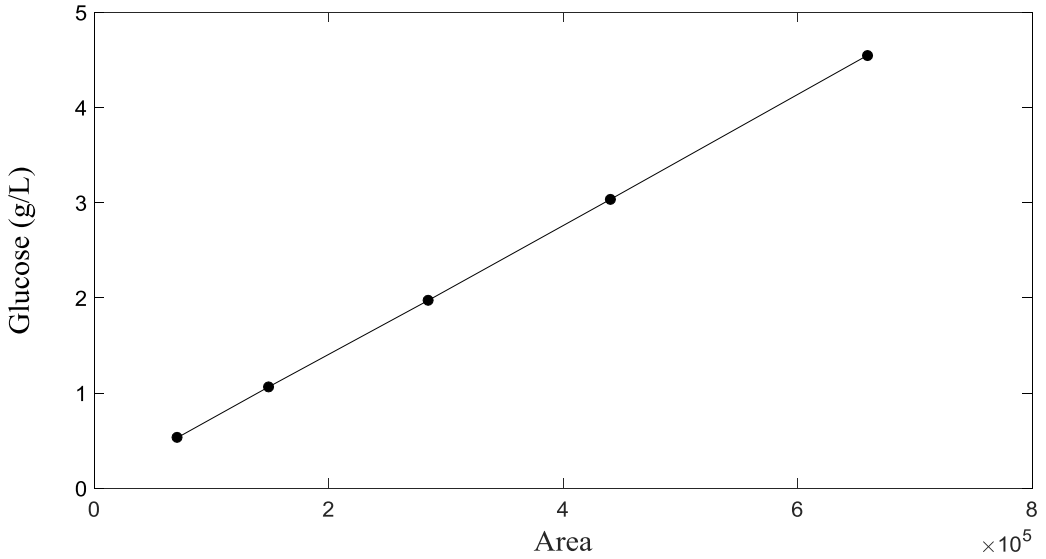

Figure 4.6 Calibration curve of glucose with Aminex HPX-87H

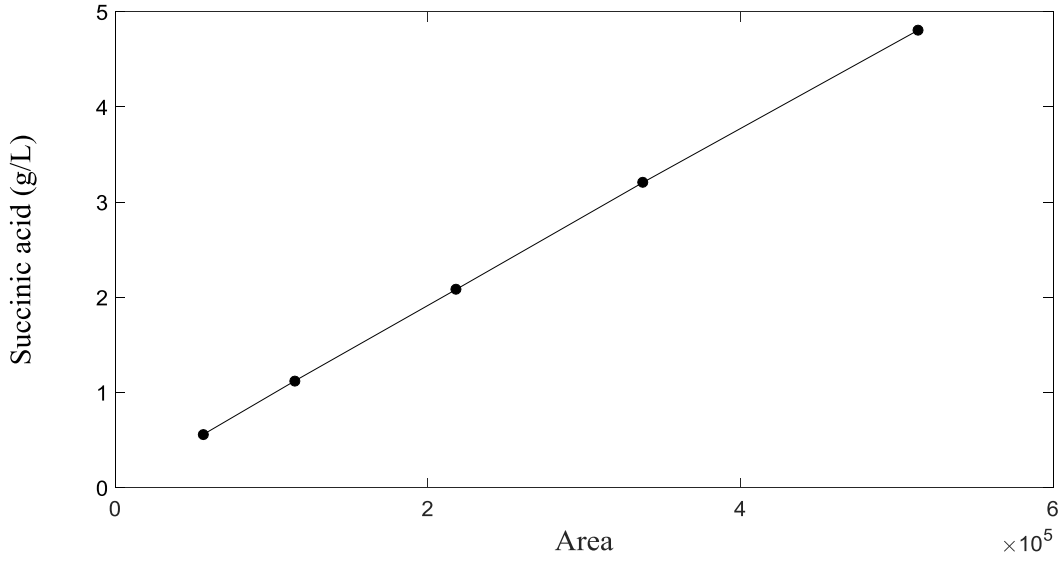

Figure 4.7 Calibration curve of succinic acid with Aminex HPX-87H

4.9.3 Determination of lignosulphonates and physical parameters

The lignosulphonate and the phenolic content of the SSL were determined spectrophotometrically (Jasco V-530), according to the protocol UNE EN 16109:2012. LS absorbance was measured at 232.5nm. Phenolics were determined by scanning from 340nm to 220 nm and measuring the absorbance of the peak at about 255 nm and the minimum valley. The density of SSL was determined gravimetrically by measuring the weight of a specific volume and the dry matter was determined by drying at 100±5°C for 24h a pre-weighted sample, until constant weight was obtained in a four decimals balance.

4.10 RNA extraction, purification and cDNA construction

The samples were taken with a sterile syringe into 15 mL sterile falcon tubes from the bioreactor close to flame in order to secure aseptic conditions. All samples were placed and maintained in ice immediately after collection. The cells were collected by centrifuge for 10 min at 4^{o}C. The supernatant was kept at -20^{o}C for further analysis. The pellet was washed three times under aseptic conditions with sterile solution of NaCl 150 mM, transferred to 1.5 mL Eppendorf tubes and stored at -80 ^{o}C until further treatment.

RNA extraction was performed using a standard TRIzol^{TM} (Thermo Fisher Scientific, Waltham, MA, USA) protocol. Trizol reagent is a mono-phasic solution of phenol and

guanidine iso-thiocyanate. Specifically, 250 μL of trizol reagent were added to the pellet and were homogenised with the use of appropriate sticks. Another 250 μL of trizol reagent were added and it was homogenised with the rest of the reagent by 2 min vortex. RNA and DNA isolation from the cell membranes occurred after centrifuge at 12,000 rpm and 4 ^{o}C for 12 min. The supernatant, with the DNA and RNA, was transferred to a separate Eppendorf tube. The samples were left for 5 min at room temperature and 100 μL of chloroform were added, for RNA isolation. The samples were shaken vigorously for 15 sec and left at room temperature for another 2-3 min. The samples were centrifuged at 12,000 rpm and 4 ^{o}C for 15 min. The supernatant was transferred to a separate Eppendorf tube. 250 μL of isopropanol were added, for RNA precipitation, and the samples were shaken vigorously for 15 sec and left at room temperature for 10 min. The samples were centrifuged at 12,000 rpm and 4 ^{o}C for 15 min and the supernatant was discarded. Another 250 μL of isopropanol was added, when needed. 100μL of 75% ethanol was added and the samples were homogenised using vortex. The samples were centrifuged at 12,000 rpm and 4 ^{o}C for 10 min and the supernatant was discarded. The supernatant was discarded and the samples were vacuum dried for 30-45 min, until the pellet was dry. The pellet was dissolved in 40-50 μL of ddH_2O. The quantity of RNA was determined using nanodrop measurement and the quality of RNA was determined at an agarose gel (3%). This method resulted in at least 25 μg of RNA per sample

To clean the samples from remaining DNA, a treatment with DNAse was followed. The sample was diluted to a final concentration of 100 ng/25 μL. To the same tube, 5 μL DNAse buffer x5, 5 μL DNase RQ1 (Promega, Madison, WI, USA), in two doses, and 0.5 μL RNaseOUT (Thermo Fisher Scientific, Waltham, MA, USA), were added. The remaining volume was filled with ddH_2O to reach a final volume of 50 μL. The samples were incubated for 30 min at 37 ^{o}C. To deactivate the DNAse, 1 μL of the treated sample was mixed with 1 μL of DNAse stop solution and 8 μL ddH_2O. The samples were incubated for 10 min at 75 ^{o}C. The quality of the DNA lysis was determined with PCR at a total reaction volume of 25 μL. Briefly in the same tube, 1 μL of the treated sample, 5 μL of buffer with $MgCl_2$ (5x), 1 μL dNTP's, 0.75 μL of forward primer 10 μM, 0.75 μL of reverse primer 10 μM and 1 μL taq polymerase, were added. The PCR program was the following: 95 ^{o}C for 2 min (95 ^{o}C for 30 sec, 60 ^{o}C for 30 sec, 72 ^{o}C for 15 sec) for 28 cycles, 72 ^{o}C for 5 sec and then drop to 4 ^{o}C. The quality of the DNA lysis was determined at an agarose gel (3%).

Afterwards the samples were further purified. 100 μL of ddH_2O were added to the sample that was treated with DNAse to reach a final volume of 150 μL. 1 volume phenol/chloroform/isoamyl alcohol (25:24:1) was added and the sample was vortexed

vigorously. The samples were centrifuged at 13,000 rpm at room temperature for 5 min. The supernatant (aqueous phase) was transferred to a separate Eppendorf tube. 1 volume of chloroform was added and the sample was vortexed vigorously. The samples were centrifuged at 13,000 rpm at room temperature for 5 min. The supernatant (aqueous phase) was transferred to a separate Eppendorf tube. 1/2 volume of 3M sodium acetate (pH 5.2) and 2 volumes of ethanol 100% were added and the samples were shaken vigorously. The samples were left overnight at -80 ^{o}C. The samples were centrifuged at 13,000 rpm and 4 ^{o}C for 30 min and the supernatant was discarded. The pellet was washed with 150 µL 75% ethanol and then centrifuged at 13,000 rpm and 4 ^{o}C for 15 min. The supernatant was discarded and the samples were vacuum dried for 30-45 min, until the pellet was dry. The pellet was dissolved in 20-25 µL of ddH_2O. The quantity of RNA was determined using nanodrop measurement.

Approximately 1µg of RNA was used per cDNA synthesis by using Superscript II (Thermo Fisher Scientific, Waltham, MA, USA) enzyme. Specifically, first-strand cDNA was reverse transcribed from 1µg of RNA, the samples were denatured at 65^{o}C for 5 min followed by quick cooling on ice in a 12µl reaction mixture containing 10µl of RNA, 1µl of random primers ((50ng/µL) and 1µl of 10mM dNTPs. After the addition of 4 µl of 5x First-Strand buffer (Thermo Fisher Scientific, Waltham, MA, USA), 1µl (40 U) of RNaseOUT (Thermo Fisher Scientific, Waltham, MA, USA) RNase inhibitor and 2 µl of 0.1M dithiothreitol (DTT), the reaction was incubated at 25^{o}C for 2 min before the addition of 1 µL (200 U) of SuperScript II reverse transcriptase. The reaction was incubated again at 25^{o}C for an additional 10 min, followed by incubation at 42^{o}C for 50 min and a final heat-inactivation at 70^{o}C for 15 min. cDNAs were then stored at -20^{o}C. The quality of cDNA (Figure 4.8) was determined at an agarose gel (3%).

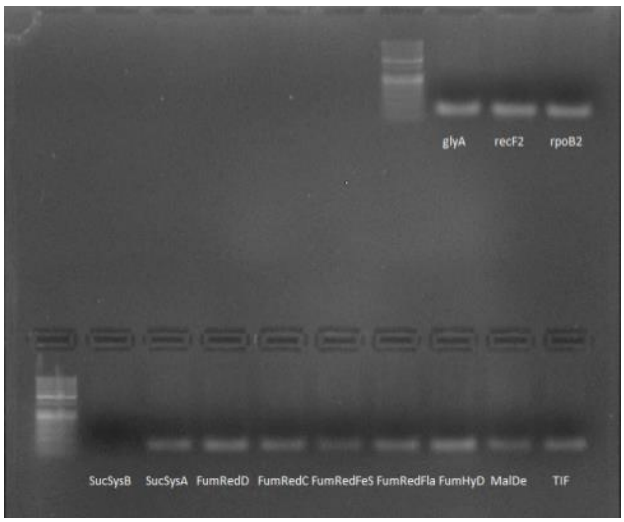

Figure 4.8 Agarose gel (3%) of cDNAs of different genes

Primers were selected to have length of 20 bases, optimum melting temperature (Tm) at 60±1 ^{o}C, 40-60 GC% content and give an amplicon with size around 70 bp and maximum melting temperature (Tm) at 85 ^{o}C. All primers were evaluated for their function to one DNA region using blastn. The following table shows the primers that were used for the RT qPCR analysis. Quantitative real time PCR was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Select Master Mix (Applied Biosystems, Austin, TX, USA), gene-specific primers at a final concentration of 0.2 μM each, and 1 μL of the cDNA as template. PCR cycling started with the initial polymerase activation at 95^{o}C for 10 min, followed by 40 cycles at 95^{o}C for 15 seconds and 60^{o}C for 1 min. The expression levels of *A. succinogenes* recombinase F (*recF*) and the β-subunit of RNA polymerase (*rpoB*) were used as housekeeping genes (HK) to normalise cDNA templates. Relative transcript levels of the gene of interest were calculated as primer Efficiency$^{-\Delta Ct}$, where ΔCt is the difference between the geometrical mean of the two HK genes' Cts and the Ct of the gene of interest, while primer efficiency is the mean of each primer's efficiency, which was calculated by employing the linear regression method on the Log (Fluorescence) per cycle number data (ΔRn), using the LinRegPCR software (Ramakers et al., 2003).

Table 4.1 Selected primers (forward and reverse) with optimum melting temperature 60 ± 1^{o}C, 40 – 60 GC% content resulting to an amplicon size around 70 bp for RT PCR analysis.

Gene	EC number	Function	Forward	Reverse
recombinase F		Housekeeping	TGACACTGCTTAACGGCGGT	CGGTCGATGGTGAAACAACC
β-subunit of RNA polymerase			GCAGGTTTCGAAGTGCGAGA	CCTTCAGGCGTTTCGATCG
PTS system, glucose subfamily, IIA sub	2.7.1.199	Glucolysis / Gluconeogenesis	GCGAAATCAGTATTAACGCCGA	CCCACGCGTTTTTCGATATG
Glucose-6-phosphate isomerase	5.3.1.9		AACGGTCAACACGCGTTCTATC	GGTGCAATGAAATCGCAAGG
Fructose-1,6-bisphosphatase, class II	3.1.3.11		GGCGATATGCAAGCACGTTT	CCAACCTTTCGATTTTCAGGC
Inositol phosphatase/fructose-16-bisphosphatase	3.1.3.11		ACGAGGACGAAATGCACGTTAT	CGTTGACGTCGATATTGGATGA
6-phosphofructokinase	2.7.1.11		GGAAGAACACGGTTTCCCTTGT	TGTAGTCGGTGCCCGGAATAT
Fructose-bisphosphate aldolase, class II	4.1.2.13		TTGCCGTAAATATCTGGAACGC	TTCTTCACCACCGGTAATACCG
Triose-phosphate isomerase	5.3.1.1		TCGCGAAAAAATTCTCCGC	CCTCTTCGGTTTCACCGATACA
triosephosphate isomerase	5.3.1.1		GGCATTCTGAACGTCGTCATG	TGCTTCAAAGACGCAAGCACT
Triose-phosphate isomerase	5.3.1.1		CCGGTTATGCCGATGAAAAAC	TTGTCGCCTTCTTCACCGAA
Glyceraldehyde-3-phosphate dehydrogenase, type I	1.2.1.12		TGTTGATATCGCAGTGGAAGCA	CGGCGGTAATATGTTTACGTGC
Phosphoglycerate kinase	2.7.2.3		ACCGAAGGCGAATTCAAACC	CGCCAAGCTCCGCATTATT
Phosphoglycerate mutase	5.4.2.12		GAACGGTTTGGAACGAACAGG	TTTCACGCCTTCTTCCGTCA
Phosphopyruvate hydratase	4.2.1.11		CGGCAGCATCAAAAGGCTTA	CGGCATAGAATATTGACCCGG

Gene	EC number	Function	Forward	Reverse
Putative low affinity D-xylose-proton symporter	1.3.1.74	Pentose phosphate pathway	AAATACACCACGAGTTGGCCC	GCACATATTCGCGGCAAAAT
High affinity ATP-dependent xylose transporter (XylHGF)			CCAGTTCGCCCACTTTGGTAT	AATGTACCTGCGTTGCAAAACC
Xylulose kinase	2.7.1.17		AAGCTCTTCCTATTTCCCGCC	TGCAGCATCCCACCAATCTT
Ribulose-phosphate 3-epimerase	5.1.3.1		ACTTCCATCACCTTTCACGGC	AACCTTGATCGCGAATCAGCT
Ribose 5-phosphate isomerase B	5.1.3.1		TTCTTACCTGCGGAACCGGTA	ATGACAAACCGCCGCTCTTAC
Ribose 5-phosphate isomerase B	5.1.3.1		TATACCCCGATGTTGCGGAA	CGCAGGTTAAAATGGCACGT
Ribose 5-phosphate isomerase A	5.3.1.6		TGGTGACGGATAACGGCAAT	TTCCATTTCCGGCGGATT
Transaldolase	2.2.1.2		TGATTTCACCGTTCGTCGGT	TTCTGCCGGTGCATATTCTTG
Glucose-6-phosphate 1-dehydrogenase	1.1.1.49/1.1.1.363		CGTTTCTTCGAGGAACACCAGA	CCAACAGATTTTGCACGGTTTC
6-Phosphogluconolactonase	3.1.1.31		AATATTCACCGTATTCGCGGC	CGCACTTAATTCCGCTTCAAAG
6-Phosphogluconate dehydrogenase	1.1.1.44/1.1.1.343		CTTGCTCGCCTCCAAAATCA	AGTCGAATTGCTCGGATGCTT
Ribokinase	2.7.1.15		GTGTGATCAATCGCAAATGACG	CAAATGCCTGTTTCATGGTGC
Ribose-phosphate pyrophosphokinase	2.7.6.1		GCGAAATCCAAGTGCAGATCA	TGCGCAAGTGGACTGAATGA
S-(hydroxymethyl)glutathione dehydrogenase / alcohol dehydrogenase	1.1.1.284 1.1.1.1		AAGTATTGGTGCGAATTGTGGC	CGAATCCTGTCCGGAAAGTGT
Acetaldehyde dehydrogenase / alcohol dehydrogenase	1.2.1.10 1.1.1.1		CGGAAGTCAATTCGCTGGTAGA	CCTGTTCCTGATTGAGTCCCCT

Gene	EC number	Function	Forward	Reverse
Phosphoenolpyruvate carboxykinase (ATP)	4.1.1.49	TCA cycle	GACATTCTCGGCCTGTTTCG	CGACCAACACGTCCGCATAT
DCU antiporter			CTGCGCCGTAATCCGAAATA	CCCGCCAATACCGTGAATG
Malate dehydrogenase	1.1.1.37		ACATTCAGGTGTGACGATTTTACCTT	TTAATTCCTGCTCGGTAAATTCCA
Fumarate hydratase	4.2.1.2		TATGCCAACGCGGATTTAGG	ATTTCATCGCAGGCTTGTGC
Fumarate reductase flavoprotein subunit	1.3.5.4		TCTACAACGGCAACGATGCA	TTTAGCTGGTTGGAATGTGGTAATTT
Fumarate reductase iron-sulfur subunit	1.3.5.4		ACCGTTTGGAACCGGAGCT	CATCATACCGCAAGAACCGC
Ffumarate reductase subunit C	1.3.5.4		ATCACCCCGACTTGGTGGA	GGAACGGCTGTCGCTTCAC
Fumarate reductase subunit D	1.3.5.4		GCATTATTGCTTTCGCCCATC	CCAGGCCGGAAAAATAGTTAGC
Succinyl-CoA synthetase subunit alpha	6.2.1.5		GCGGAAAAAATCAAAGCGCT	GCCGAACCTATATCCGCCAG
Succinyl-CoA synthetase subunit beta	6.2.1.5		ATTGCAAACGGATGCTCAGG	CTTTTTTTACTGCGGCGCAG
Bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	4.2.1.3		CACCGGAAGCATGCAAAAAG	TGCTTGATGGCAAAATACGGA
2-Oxoglutarate dehydrogenase, E2 subunit, dihydrolipoamide succinyltransferase	2.3.1.61		AGATCTCCGGTGCAAATCTGC	ATGTCCGCGCGATCAAAGT
Dihydrolipoamide dehydrogenase	1.8.1.4		CTATTCAACGCTTGGCGGAGT	TTGCCACGTGCAATAAGGCT
2-Oxoglutarate dehydrogenase, E1 subunit	1.2.4.2		GCCTTGCCCGCGATAATACT	CCTTGACTAAACGCGCGCT
Pyruvate dehydrogenase subunit E1	1.2.4.1		CGGACTTCGCCCAGTTGTTA	CAATACGCGAACAAAGGCCA

Gene	EC number	Function	Forward	Reverse
Pyruvate kinase	2.7.1.40	Pyruvate metabolism	GGTAGGCGGTCCTTTGTCAAA	TTAATGCGTCCGCCGATAAA
D-Lactate dehydrogenase	1.1.1.28		TTTATACGAGGCAAAACCCGC	TCCCGGATTAAAACTGTTGGTG
Pyruvate dehyd rogenase E1 component	1.2.4.1		AACAAGGCAAAGTCGATGCG	TGCACGATCTGCTTTAATGTCG
Dihydrolipoamide dehydrogenase	1.8.1.4		CTATTCAACGCTTGGCGGAGT	TTGCCACGTGCAATAAGGCT
Pyruvate dehydrogenase E2 component	2.3.1.12		AGATCTCCGGTGCAAATCTGC	ATGTCCGCGCGATCAAAGT
Formate acetyltransferase	2.3.1.54		AGCGGCATTGATGGCTTTC	GCAACAGACAGTCCGGCAATA
Phosphate acetyltransferase	2.3.1.8		GTTACAATACGACGCGGCAGTT	TACCTGCAACCGGCGAATT
Acetate kinase	2.7.2.1		CGACCTTAACCAAAAAATCCGG	TGTCTTCCGCAAAGCGACA
Acylphosphatase	3.6.1.7		GAAAAATCAAGCCGACGGTTC	CTGCTCCAGCCAGTCTCTGAAT
Iron-containing alcohol dehydrogenase	1.1.1.1		CGATTCGCCGTTTAAGCAAA	AAATCTTCCGGTTTCACGCC

Gene	EC number	Function	Forward	Reverse
F0F1 ATP synthase subunit epsilon	3.6.3.14	Oxidative phosphorylation	CATGCCGATGTGGATCACAA	TCGTAAGCTCGCAGCTTTGC
F0F1 ATP synthase subunit epsilon	3.6.3.14		ACCGTTGTAAGTGCGGAAGAAA	CCTTCAGAACCGGTAGCTTGG
ATP synthase F1, gamma subunit	3.6.3.14		CATGTCGCAAAAACCGAGTGT	CCCAAACCTGTTGTCTTTCGTC
ATP synthase F1, alpha subunit	3.6.3.14		ATCTCGCCGAAGGTATGGAAG	CAGTAAACCGCGACCTACCG
ATP synthase F1, delta subunit	3.6.3.14		TAGCTCGCCCTTATGCCAAAG	TTCATGCCATTTTTCCACCG
F-type H+-transporting ATPase subunit b	3.6.3.14		TGGCCGCCTATTATTAAAGCG	TTCTTGCAGCTTCAGCCGAT
ATP synthase F0, C subunit	3.6.3.14		CTTCTTGCTTTCGCTGCACTG	CAGGTTGACGAGCTGAAGATTC
ATP synthase F0, A subunit	3.6.3.14		AAAGGTTTTGGCGGATTGGT	TTGACCGGAACAAGTGTCCAG
NADH dehydrogenase	1.6.99.3		GGAAACCGATGCCGTAAGCTA	GCAATGGAACCCTGTTCGAA
cytochrome d ubiquinol oxidase subunit I	1.10.3.14		AAGCGCAGATTAAATCAGCAGC	CACACGGAACGACCAGAATACA
cytochrome d ubiquinol oxidase subunit II	1.10.3.14		ATTTTACCCGCCCTGGTTGTAA	AAACCCGAACGGTTCGCTT
inorganic pyrophosphatase	3.6.1.1		AATTCCGGCAAACTCGGATC	AACGGTCAACAAACAGCGTACC

Chapter 5

Succinic acid production using commercial substrates

5.1 Introduction

Succinic acid can be produced by a wide spectrum of sugars (including pentoses and hexoses) using natural and genetically engineered microorganisms. Spent sulphite liquor (SSL), a by-product stream from the pulp and paper industry constitutes a potential feedstock for succinic acid production. Glucose, xylose, galactose, mannose and arabinose are the monosaccharides found in lignocellulosic hydrolysates and also in spent sulphite liquor. The major monosaccharide found in the SSL derived via sulphite pulping of *Eucalyptus globulus* (hardwood) is xylose (ca. 72% w/w). Among the microorganisms that have been reported in the literature to produce succinic acid as the major extracellular metabolite, six bacterial strains (Ueki et al., 2006, O' Herrin and Kenealy, 1993, Nghiem et al., 2004, Guettler et al., 1999, Kuhnert et al., 2010) were screened on commercial xylose and later on spent sulphite liquor as carbon sources.

Succinic acid is produced by the reductive branch of the tricarboxylic acid cycle. One of the most important nodes in succinic acid production is the conversion of PEP to OAA through phosphoenolpyruvate-carboxykinase (*pepck*) with simultaneous fixation of CO_2. Oxaloacetate decarboxylase (*OAA dec*) and malate decarboxylase (*Malate dec*) are two enzymes that control the carbon flux between the TCA cycle and pyruvate metabolism. The contribution of these two enzymes on final succinic acid production is equally important. Finally, the conversion of PEP to pyruvate, which is catalysed by pyruvate kinase (*PK*), contributes to carbon flux distribution.

The pyruvate metabolism leads to three extracellular products. Lactic acid production is catalysed by lactate dehydrogenbase (*LDH*) and NADH is oxidised to NAD^+. Pyruvate formate lyase (*PFL*) catalyses the tranformation of 1 mol of pyruvate to one mol of formic acid and one mol of acetyl-CoA (the precurson of structural components). The antagonistic pathway that leads to acetyl-CoA production from pyruvate is catalysed by pyruvate dehydrogenase complex (*PyrDH*), which consists of three enzymes pyruvate dehydrogenase E1 (*PyrDH* E1), dihydrolipoamide dehydrogenase (*DihydrolDH*) and pyruvate dehydrogenase E2 (*PyrDH* E2). Acetic acid is produced through acetate kinase (*AK*) along with one mole of ATP (McKinlay et al., 2010).

The reducing equivalents from different carbon sources are shown in Figure 5.1. Succinic acid production is favoured by reductive environment, thus more reduced carbon sources, like glycerol, are most likely to improve succinic acid production. Succinic acid production is also affected by the availability of CO_2 as well as the production of energy

storage molecules (ATP). Additional reducing capacity (*i.e.* NADH) required in the C4 pathway should be supplied by other parts of the metabolism (e.g. glycolysis, C3 pathway). *A. succinogenes*, like other *Pasteurellaceae* spp., shows auxotrophy for some nutrients, such as the amino acids glutamate, cysteine and methionine (Guettler et al., 1999).

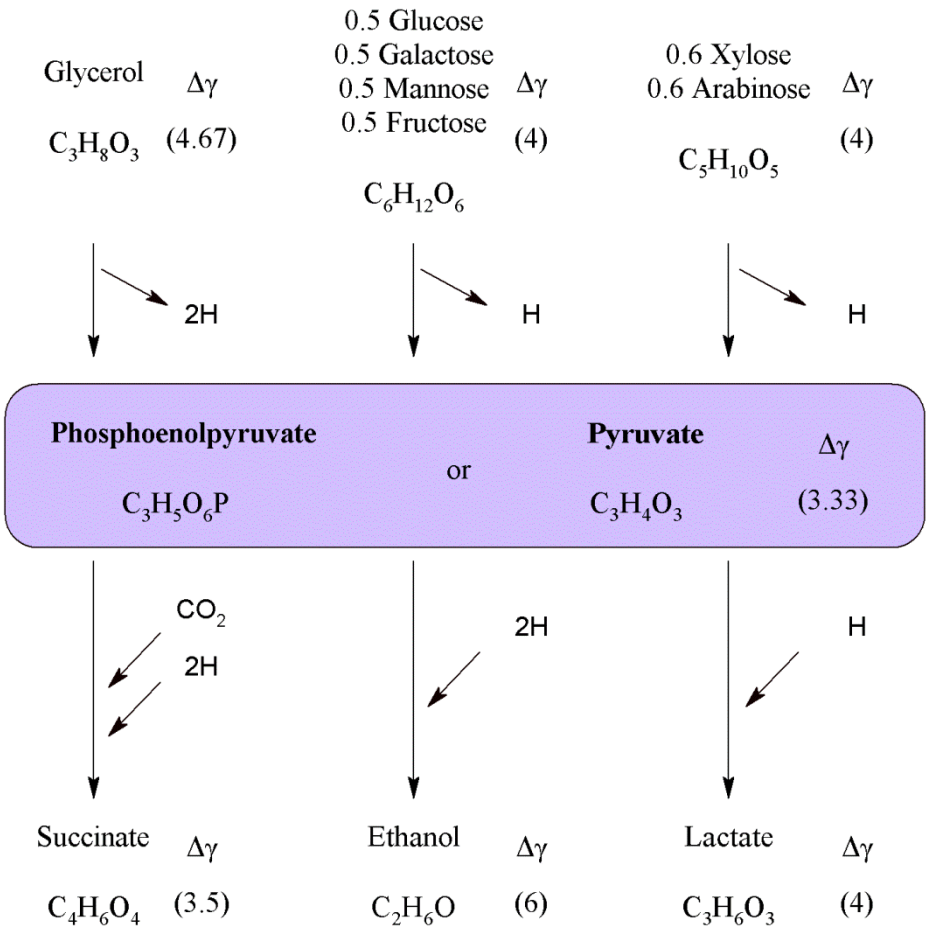

Figure 5.1 Reducing equivalents on catabolic and anabolic processes

The most promising bacterial strains were selected to further evaluate succinic acid production utilising xylose, glucose, galactose, mannose and arabinose. Furthermore, the effect of initial substrate concentration on succinic acid production via fermentation was evaluated using *Actinobacillus succinogenes* 130Z and *Basfia succiniciproducens* JF 4016 cultivated in a mixture of the previously mentioned monosaccharides in the same ratio as they are present in the spent sulphite liquor used in this study. Subsequently, the effect of the main extracellular metabolites concentration on bacterial growth was evaluated.

5.2 Microorganism screening for succinic acid production

Xylanibacter oryzae KB3^T was isolated from rice field soil in Japan. It is a Gram-negative, non-motile, non-spore-forming, short to filamentous rod (Ueki et al., 2006). Its taxonomical order has been formed as follows: Bacteria; FCB group; Bacteroidetes/Chlorobi group; Bacteroidetes; Bacteroidia; Bacteroidales; *Prevotellaceae*; *Xylanibacter oryzae*. The strain is able to consume various carbon sources including arabinose, ribose, xylose, fructose, galactose, glucose, mannose, rhamnose, cellobiose, lactose, maltose, sucrose, trehalose, carboxymethylcellulose (CMC), soluble starch, xylan, pectin, salicin and pyruvate and produced acetate, propionate and succinate with a small amount of malate. Succinic acid production on 10 g/L of xylose was 0.56 g/L with a yield of 0.05 g/g and a productivity of 0.02 g/L/h (Table 5.1). The major extracellular product was acetic acid (0.96 g/L).

Ruminococcus flavefaciens was isolated from bovine rumen. It is a gram-positive anaerobe. Its taxonomical order has been formed as follows: Bacteria; Terrabacteria group; Firmicutes; Clostridia; Clostridiales; *Ruminococcaceae*; *Ruminococcus flavefaciens*. Succinic acid production on 10 g/L of xylose was 1.83 g/L with a yield of 0.18 g/g and a productivity of 0.04 g/L/h (Table 5.1). The major extracellular product was acetic acid and lactic acids (3.86 g/L and 2.77 g/L, respectively).

Succinivibrio dextrinosolvens was isolated from bovine rumen. It is a gram-negative anaerobe and it is often the predominant when the diet of the animal is rich in starch. Its taxonomical order has been formed as follows: Bacteria; Proteobacteria; Gammaproteobacteria; Aeromonadales; Succinivibrionaceae; *Succinivibrio*; *Succinivibrio dextrinosolvens*. Succinate and acetate were the major products when it was cultivated on glucose while formate and lactate were produced in smaller amounts (O' Herrin and Kenealy, 1993). Succinic acid production on 10 g/L of xylose was 0.56 g/L with a yield of 0.05 g/g and a productivity of 0.02 g/L/h (Table 5.1). The major extracellular product was acetic acid (4.14 g/L).

Escherichia coli AFP 184 is an engineered strain with mutations in *pfl*, *ldhA* and *ptsG* and it is able to ferment xylose (Nghiem et al., 2004). Its parental strain AFP 111 (Donnelly et al., 2000) has the ability to produce succinic acid but not to consume xylose. *E. coli* AFP 184 requires aerobic conditions for cell growth, but succinic acid production requires CO_2 environment. A crucial factor for the fermentation efficiency is the transition time from aerobic to anaerobic phase as it affects final succinic acid concentration and yield (Vemuri et al., 2002). Biomass growth and enzyme generation for succinate production occur during the

aerobic phase (2-8 hours are required for the biomass to grow properly) (Lu et al., 2009; Andersson et al., 2007). *E. coli* AFP 184 was cultivated on xylose as the carbon source in dual phase fermentations with air flow rate of 1 vvm for the first 6 and 8 hours and transition to anaerobic environment later on by continuous CO_2 sparging (0.5 vvm). *E. coli* AFP 184 achieved 4.55 g/L of succinic acid concentration with a yield of 0.50 g/g and a productivity of 0.14 g/L/h when cultivated on 10 g/L of xylose. Acetic acid was also detected at final concentration of 2.42 g/L (Table 5.1). *E. coli* AFP 184 lacks the genes for the production of lactate (*ldhA*) and formate (*pfl*) and no other by-products were formed.

Actinobacillus succinogenes 130Z is a Gram-negative, facultative anaerobic, non-motile, non-spore forming, capnophilic, pleomorphic rod, which was isolated from bovine rumen (Guettler et al., 1999) and taxonomically was placed in the *Pasteurellaceae* family, based on 16S rRNA amplification. Its taxonomical order has been formed as follows: Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; *Pasteurellaceae*; *Actinobacillus succinogenes*. The main fermentation product when cultivated under anaerobic conditions is succinic acid, while formate and acetate are also formed. Succinic acid production on 10 g/L of xylose was 3.94 g/L with a yield of 0.45 g/g and a productivity of 0.16 g/L/h (Table 5.1).

Table 5.1 Succinic acid production by six bacterial strains cultivated on 10 g/L of xylose in Duran bottles with continuous sparging of CO_2 or dual aeration conditions in the case of *E. coli*

Microorganism	Concentration (g/L)	Yield (g/g)	Productivity (g/L/h)	By-products SA:LA:FA:AA* (mol/mol)
X. oryzae KB3^T	0.56	0.05	0.02	1:0.66:-:3.37
R. flavefaciens ATCC 19208	1.83	0.18	0.04	1:3.65:-:14.54
S. dextrinosolvens ATCC 19716	0.56	0.05	0.02	1:1.98:-:4.15
E. coli AFP184 (6 h growth)	4.55	0.55	0.16	1:-:-:0.79
E. coli AFP184 (8 h growth)	4.66	0.50	0.14	1:-:-:1.02
A. succinogenes 130Z	3.94	0.45	0.16	1:-:1.65:1.45
B. succiniciproducens JF 4016	5.51	0.50	0.25	1:-:0.82:0.69

* LA: lactic acid, FA: formic acid, AA: acetic acid

Basfia succiniciproducens JF 4016 is a gram-negative, coccoid, non-motile bacterial strain that is catalase-, urease- and indole-negative, facultative anaerobic and oxidase-positive,

isolated from the bovine rumen (Kuhnert et al., 2010). Its taxonomical order has been formed as follows: Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; *Pasteurellaceae*; *Basfia succiniciproducens*. The main fermentation product when cultivated under anaerobic conditions is succinic acid, while lactate, formate and acetate are also formed. Succinic acid production on 10 g/L of xylose was 5.51 g/L with a yield of 0.50 g/g and a productivity of 0.25 g/L/h (Table 5.1).

B. succiniciproducens JF 4016 was selected for further investigation as it was the most efficient bacterial strain for succinic acid production. Although the cultivation of *E. coli* AFP184 on xylose led to slightly higher succinic acid concentration and yield than *A. succinogenes* 130Z, the later was selected because it does not need oxygen for growth leading to lower production cost and capital investment. In the case of *A. succinogenes*, succinic acid is produced through the reductive branch of TCA cycle (McKinlay et al., 2005), while in *B. succiniciproducens* (Becker et al, 2013) both the reductive and oxidative branches of TCA cycle contribute in the formation of succinic acid. Becker et al. (2013) proved using C^{13} that the contribution of the oxidative branch of the TCA cycle in *B. succiniciproducens* to the production of succinic acid is very low. Thus the higher succinic acid concentration and yield achieved by *B. succiniciproducens* than *A. succinogenes* cannot be entirely attributed to the contribution of the oxidative branch of *B. succiniciproducens* TCA cycle.

5.3 Fermentations on commercial substrates

A. succinogenes and *E. coli* have been widely studied for succinic acid production using various raw materials, while *B. succiniciproducens* is a relatively new bacterial strain with only a few publications on succinic acid production. Both *A. succinogenes* and *B. succiniciproducens* were selected for the evaluation of succinic acid production in different carbon sources. Initially, the ability and efficiency of the bacterial strains to consume glucose, xylose, galactose, mannose and arabinose were evaluated. Subsequently, the effect of initial carbon source concentration was evaluated. Fermentations were also carried using a mixture of monosaccharides the ratio of which was selected in order to resemble the concentration ratio that is found in spent sulphite liquor (*i.e.* 72.6% xylose, 12.2% galactose, 10.9% glucose, 4.2% mannose and 0.1% arabinose).

5.3.1 Bacterial cultivations using glucose, xylose, galactose, mannose and arabinose

Initial experiments were carried out in Duran bottles at a working volume of 250 mL with continuous supply of CO_2. Glucose, xylose, galactose, mannose and arabinose at initial

concentration of around 10 g/L were the carbon sources that were evaluated for succinic acid production via fermentation by *A. succinogenes* and *B. succiniciproducens*. *A. succinogenes* consumes all monosaccharides except for galactose (alone or in a mixture with other sugars), despite the fact that the genes for galactose catabolism (Asuc_1896-9, 1817, 0049-51) have been identified in its genome (see supplement 1 in McKinlay et al., 2010). The bacterial strain *B. succiniciproducens* consumes galactose with sufficient yield (0.46 g/g) and productivity (0.68 g/L/h). The highest yield and productivity for both microorganisms was observed when glucose was used (Table 5.2). Specifically, 0.6 g/g conversion yield and 0.74 g/L/h productivity were achieved by *A. succinogenes*, while 0.74 g/g and 1.04 g/L/h were achieved by *B. succiniciproducens* in the case of glucose. Xylose degradation is a more energy consuming pathway than glucose, thus xylose consumption resulted in conversion yields of 0.44 g/g and 0.5 g/g for *A. succinogenes* and *B. succiniciproducens*, respectively. The productivity observed in the case of xylose was rather low for both strains, namely 0.16 g/L/h and 0.25 g/L/h, respectively. In the case of *B. succiniciproducens*, besides glucose, high yield (0.72 g/g) and productivity (0.95 g/L/h) were also observed in the case of mannose.

For energy balance purposes both strains produce simultaneously succinic acid, formic acid and acetic acid. *B. succiniciproducens* produces lactic acid contrary to *A. succinogenes*. However, in the fermentations presented in Table 5.2, lactic acid production was not observed by *B. succiniciproducens*. This could be attributed to the low xylose concentration used in these experiments. The molar ratios of FA:SA (1.663 mol/mol) and AA:SA (1.453 mol/mol) in the case of *A. succinogenes* acquired the highest values when xylose was used as carbon source. In the case of *B. succiniciproducens* the highest molar ratios of FA/SA (0.985 mol/mol) and AA/SA (1.471 mol/mol) were observed in the case of galactose (Table 5.2). Glucose and mannose resulted at the lowest by-product to succinic acid ratios in both microorganisms (Table 5.2). The experimental results presented in Table 5.2 indicate that succinic acid and by-product formation by both strains depend on the sugar used and thus the cultivation of both strains in a mixture of sugars, as in the case of SSL, it is expected to influence significantly their succinic acid production efficiency.

Table 5.2 Fermentation efficiency of *A. succinogenes* and *B. succiniciproducens* cultivated in Duran bottles using glucose, xylose, galactose, mannose and arabinose as carbon sources

Microorganism	Substrate	DCW_{max} (g/L)	Succinic acid (g/L)	Yield (g/g)	Productivity (g/L/h)	FA/SA (mol/mol)	AA/SA (mol/mol)
Actinobacillus succinogenes	Glucose	1.51 ± 0.16	5.6 ± 0.45	0.60 ± 0.01	0.74 ± 0.06	0.744 ± 0.008	0.840 ± 0.031
	Xylose	1.16 ± 0.18	3.9 ± 0.54	0.44 ± 0.02	0.16 ± 0.02	1.663 ± 0.113	1.453 ± 0.069
	Galactose	-	-	-	-	-	-
	Mannose	2.02 ± 0.04	4.1 ± 0.22	0.43 ± 0.04	0.18 ± 0.01	1.466 ± 0.105	0.779 ± 0.083
	Arabinose	1.30 ± 0.16	3.8 ± 0.19	0.51 ± 0.003	0.15 ± 0.01	1.131 ± 0.346	1.209 ± 0.024
Basfia succiniciproducens	Glucose	2.48 ± 0.03	6.8 ± 0.10	0.74 ± 0.03	1.04 ± 0.02	0.711 ± 0.169	0.580 ± 0.000
	Xylose	1.88 ± 0.06	5.5 ± 0.41	0.50 ± 0.05	0.25 ± 0.02	0.824 ± 0.031	0.690 ± 0.098
	Galactose	2.21 ± 0.02	4.4 ± 0.41	0.46 ± 0.04	0.68 ± 0.06	0.985 ± 0.177	1.471 ± 0.020
	Mannose	1.76 ± 0.38	6.2 ± 0.52	0.72 ± 0.13	0.95 ± 0.08	0.677 ± 0.005	0.671 ± 0.006
	Arabinose	1.95 ± 0.10	6.6 ± 0.26	0.69 ± 0.66	0.66 ± 0.03	0.765 ± 0.015	0.759 ± 0.006

* Lactic acid production was not observed

5.3.2 Effect of initial carbon source concentration on succinic acid production

Substrate and product inhibition are crucial factors that have to be considered in industrial scale applications in order to maximize the succinic acid production efficiency. When the carbon source concentration is high, growth and subsequently product formation is inhibited. On the other hand, low concentrations of the carbon source, is an important factor that should also be considered to prevent inhibition by starvation or hyposmotic stress. The critical concentrations of tolerance for each microorganism are unique. Therefore, the identification of optimal and critical concentrations of carbon source facilitates bioprocess design so as to achieve production stability. The effect of initial carbon source concentration on growth was evaluated first in a microtiter well plate at a scale of 200 μL final culture volume and at a later stage, the effect of initial carbon source concentration on other fermentation parameters (final product concentration, yield and productivity) was evaluated in Duran bottle fermentations at a scale of 250 mL final fermentation volume. All fermentations were carried out using the ratio of mixed sugars that is present in the SSL used in this study.

5.3.2.1 Microtiter plate cultures

The maximum specific growth rate in microtiter plate experiments (Figure 5.2) was found for initial mixed sugar concentration in the range of 20 to 50 g/L for both *A. succinogenes* and *B. succiniciproducens*. Initial mixed sugar concentrations higher than 120 g/L caused significant inhibition in cell growth.

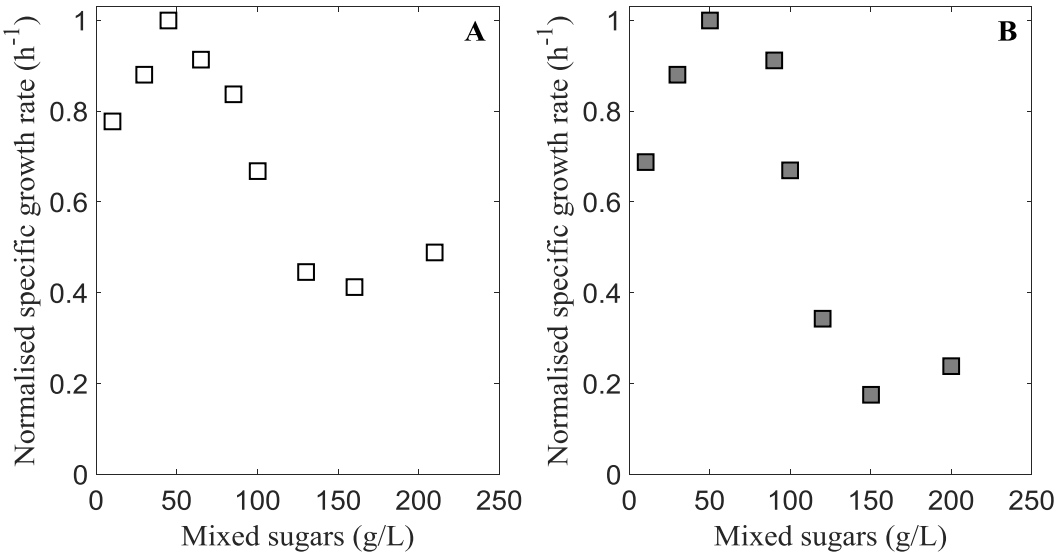

Figure 5.2 Effect of initial mixed sugar concentration on specific growth rate of *A. succinogenes* (A) and *B. succiniciproducens* (B) in total culture volume of 200 μL. Standard deviation is less than 0.5 % between six biological replicates

Bacterial membrane is permeable to water which affects the decrease or increase of the cytoplasmic volume, depending on the substrate concentration that causes efflux or influx of water. Low initial sugar concentrations in both bacterial strains did not inhibit bacterial growth as much as high initial sugar concentrations, which is in agreement with previous findings on hypo- and hyperosmotic stress. According to Csonka (1989) hypo-osmotic stress is not as severe as hyperosmotic stress because the increase in cell volume is small and bacterial cells can tolerate the pressure that is generated. On the other hand, hyperosmotic stress which occurs when the medium contains high sugar concentrations results in cell shrinkage and subsequent breakdown of the membrane. The effect of hyperosmotic stress on bacterial cell growth depends on the concentration and not the nature of the solutes as the solutes are impermeable to the membrane. Glycerol or ethanol that can be diffused through the bacterial cell membrane do not cause cell lysis (Csonka, 1989). When hyperosmotic stress is not severe, as in the range of 60-100 g/L of initial mixed sugar concentration for both bacteria (Figure 5.3), a prolonged lag phase is observed (4-6 h for *A. succinogenes* and 4-5 h *B. succiniciproducens*). Also an increase in ATP levels of the cells has been observed by Ohwada and Sagisaka (1988) during sudden plasmolysis. Osmotic stress can be controlled by the addition of osmoprotectants such as proline, glycinebetaine and choline. Proline acts as an excellent osmoprotectant on *A. succinogenes* NJ113 cells in order to overcome hyperosmotic stress by NaCl (Fang et al., 2011).

5.3.2.2 Duran bottle fermentations

Bacterial cell growth (Figure 5.3) during *A. succinogenes* and *B. succiniciproducens* cultures in Duran bottles (250 mL) occurred at a wide range of initial total mixed sugar concentration using the same ratio of mixed sugars present in SSL. The specific growth rate of *A. succinogenes* was in the range of 0.08 - 0.49 h^{-1} at initial total sugar concentration in the range of 2 - 32 g/L, which was significantly lower than *B. succiniciproducens* where the specific growth rate was in the range of 0.14 - 1.01 h^{-1} at initial total sugar concentration of 2 - 19 g/L. Substrate inhibition on cell growth was obvious at initial total sugar concentration of around 68 g/L for *A. succinogenes* and 45 g/L for *B. succiniciproducens*.

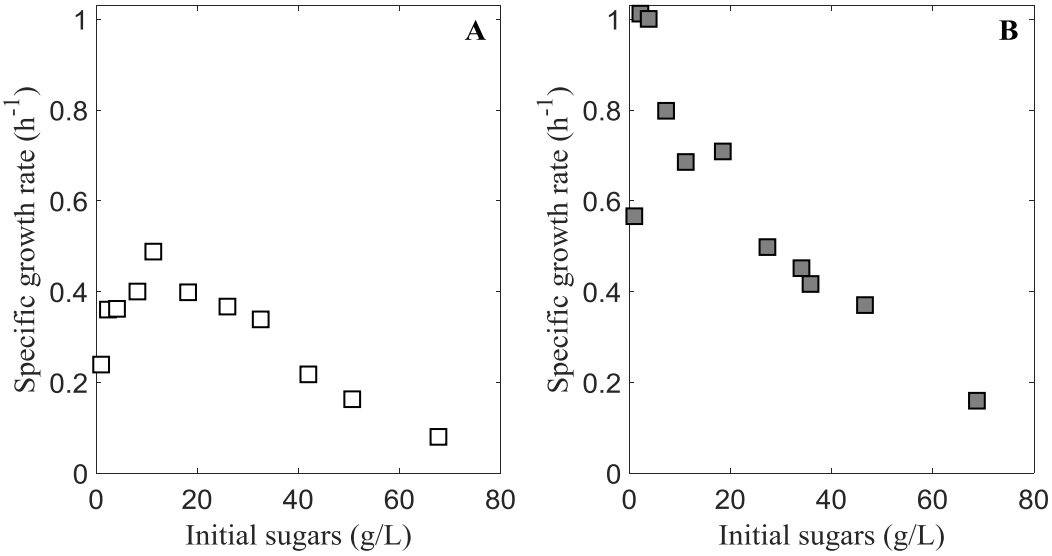

Figure 5.3 Effect of initial substrate concentration on specific growth rate of *A. succinogenes* (A) and *B. succiniciproducens* (B) during fermentations in Duran bottles (250 mL) using continuous supply of CO_2

Succinic acid production (Figure 5.4) during *A. succinogenes* and *B. succiniciproducens* cultures in Duran bottles was evaluated up to a total mixed sugar concentration of around 68 g/L. Final succinic acid concentrations higher than 15 g/L were observed at initial total sugar concentrations in the range of 32 - 50 g/L in the case of *A. succinogenes* and 27 - 46.5 g/L in the case of *B. succiniciproducens*. The highest final succinic acid concentration produced by *A. succinogenes* was 27.4 g/L at initial total sugar concentration of 50.5 g/L. Unconsumed sugars remained at all initial sugar concentrations in the case of *A. succinogenes* due to the fact that 12.2% of the total sugars consisted of galactose that cannot be consumed by this strain. The highest final succinic acid concentration produced by *B. succiniciproducens* was 26 g/L at initial total sugar concentration of 35.8 g/L. The bacterial strain *B. succiniciproducens* consumed all sugars up to a total sugar concentration of 34.4 g/L. Substrate inhibition occurred at sugar concentrations higher than 60 g/L in the case of *A. succininogenes* and 45 g/L in the case of *B. succiniciproducens*.

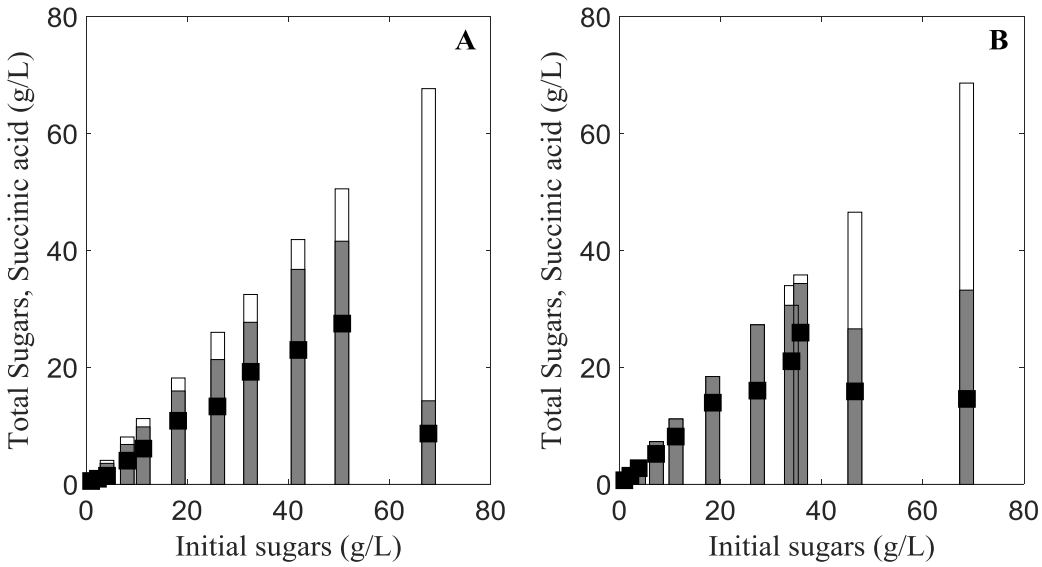

Figure 5.4 Total sugars (white bars), consumed sugars (grey bars) and final succinic acid concentration (square, ■) achieved by *A. succinogenes* 130Z (A) and *B. succiniciproducens* (B) during fermentations in Duran bottles (250 mL) on different initial mixed sugar concentrations.

Figure 5.5 presents the productivities and total consumed sugar to succinic acid conversion yields for both bacterial strains cultivated in Duran bottles on different mixed sugar concentrations. For both microorganisms, the yield was in the range of 0.6 - 0.7 g/g at all initial sugar concentrations except for initial sugar concentrations of 2.36 g/L (yield: 0.5 g/g) and 4.1 g/L (yield: 0.4 g/g) for *A. succinogenes* and 68.6 g/L (yield: 0.44 g/g) for *B. succiniciproducens*. The highest productivities were 0.6 g/L/h at 26 g/L initial total sugar concentration for *A. succinogenes* and 0.66 g/L/h at 36 g/L initial total sugar concentration for *B. succiniciproducens*. Initial total sugar concentration in the range of 26 - 50 g/L maintained the productivity at 0.45 - 0.6 g/L/h in the case of *A. succinogenes*, whereas the productivity remained at 0.55 - 0.66 g/L/h in the case of *B. succiniciproducens* when the initial sugar concentration was within the range 18 - 36 g/L.

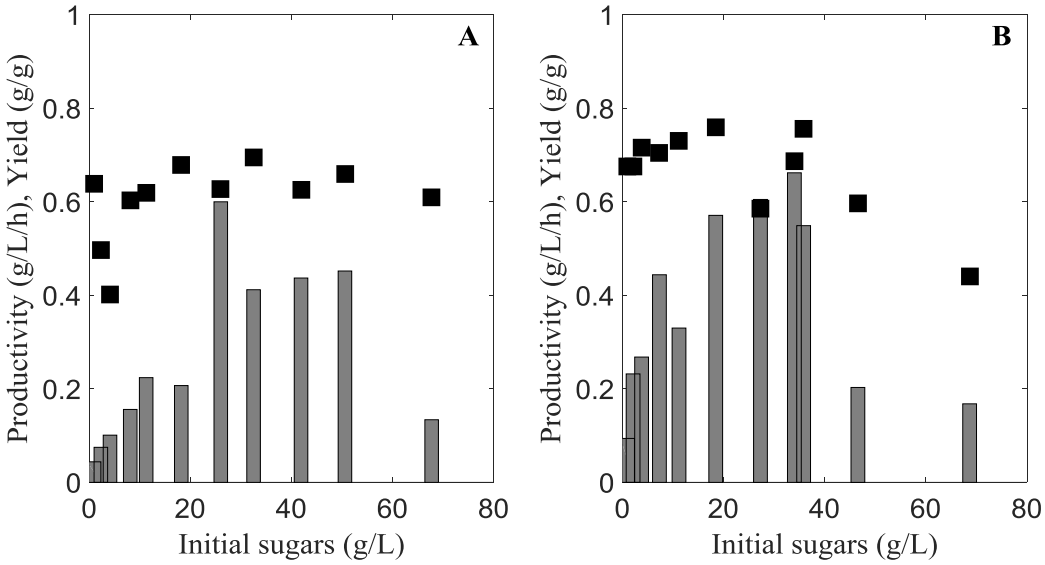

Figure 5.5 Productivity (grey bars) and final total consumed sugars to succinic acid conversion yield (square, ■) achieved by *A. succinogenes* (A) and *B. succiniciproducens* (B) during fermentations in Duran bottles (250 mL) on different initial mixed sugar concentrations

Lactic acid production that was observed only in the case of *B. succiniciproducens* followed a different trend than succinic acid production. Very low or zero concentrations of lactic acid were observed at low initial sugar concentrations (0 - 20 g/L), while above 20 g/L of total sugars an increasing trend in LA/SA molar ratio was observed with increasing initial total sugar concentration (Figure 5.6). In the case of *A. succinogenes*, the formic acid to succinic acid molar ratio (FA/SA) (Figure 5.7A) and the acetic acid to succinic acid molar ratio (AA/SA) (Figure 5.8A) followed a gradually increasing trend up to a total sugar concentration of 8 g/L followed by a decreasing trend at higher initial total sugar concentrations until a plateau was reached. The FA/SA molar ratio ranged from 0.793 to 1.663 mol/mol, while the AA/SA molar ratio ranged from 0.889 to 1.469 mol/mol in the case of *A. succinogenes*. In the case of *B. succiniciproducens*, both the FA/SA (0.323 - 1.152 mol/mol) and the AA/SA (0.627 - 1.255 mol/mol) molar ratios followed an increasing trend with increasing total sugar concentration. In each batch fermentation by-product formation stopped when the cells entered the stationary phase. Succinic acid production continued even during the stationary phase. That observation leads to the conclusion that acetic acid and formic acid are crucial for biomass production.

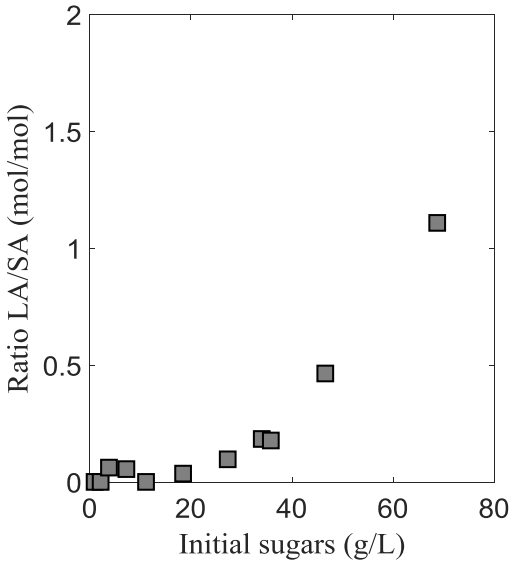

Figure 5.6 Final lactic acid to succinic acid molar ratios (LA/SA) observed in *B. succiniciproducens* fermentations at different initial mixed sugar concentrations. Standard deviation is less than 0.5% between six biological replicates.

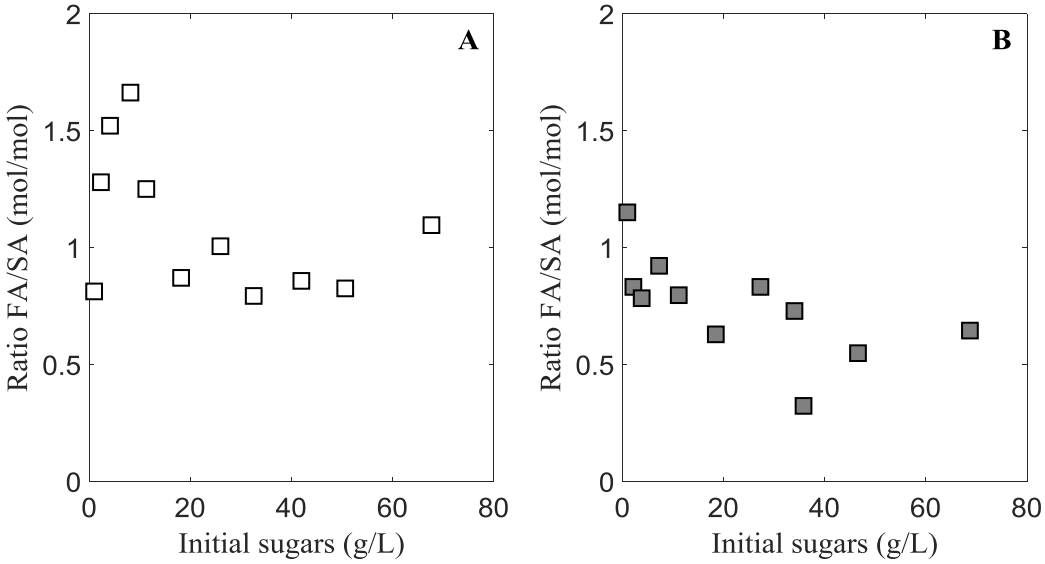

Figure 5.7 Final formic acid to succinic acid molar ratios (FA/SA) observed by *A. succinogenes* (A) and *B. succiniciproducens* (B) fermentations at different initial mixed sugar concentrations. Standard deviation is less than 0.5% between six biological replicates

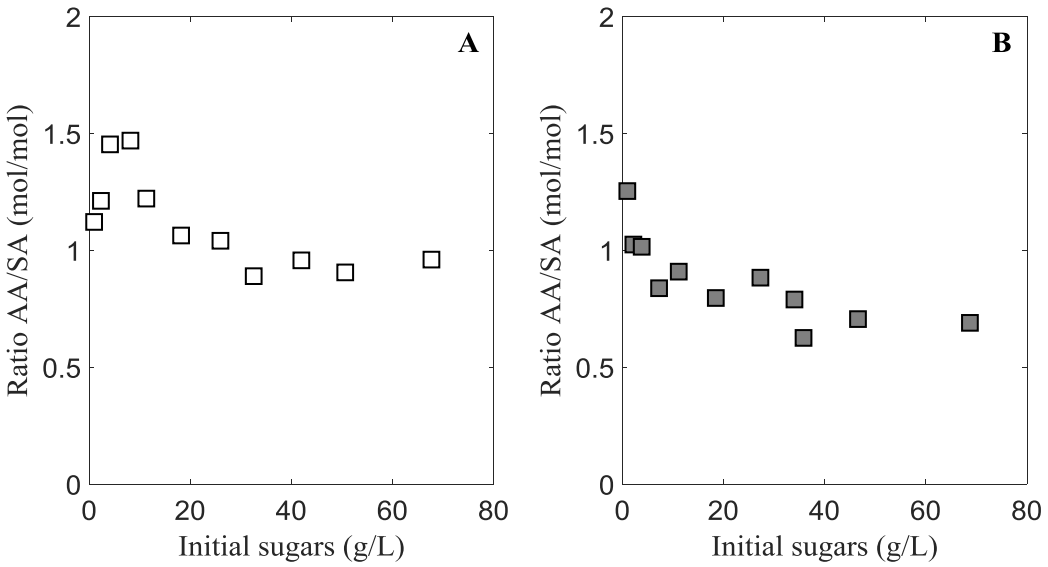

Figure 5.8 Final acetic acid to succinic acid molar ratios (AA/SA) observed by *A. succinogenes* (A) and *B. succiniciproducens* (B) fermentations at different initial mixed sugar concentrations. Standard deviation is less than 0.5% between six biological replicates

A few studies have included substrate and product inhibition in mathematical models for growth of *A. succinogenes* on glucose or glycerol as carbon sources (Lin et al., 2008; Corona-Gonzalez et al., 2008; Vlysidis et al., 2011). Substrate inhibition has been tested for glucose (Liu et al., 2008a) and sugar cane molasses (Liu et al., 2008b) and obtained results demonstrated that substrate inhibition starts at 50 g/L and 65 g/L, respectively. Zheng et al. (2009) evaluated the effect of initial sugar concertation on *A. succinogenes* CGMCC1593 using a mixture of glucose, xylose and arabinose, where the highest yield range (0.77 - 0.80 g/g) was observed at initial sugar concentrations in the range of 30 - 60 g/L. However, at concentrations higher than 80 g/L a decrease in yield was observed (Liu et al., 2008b).

The substrate and product inhibition on the growth of *A. succinogenes* have been previously expressed with modified Monod equations in cultures that utilized glucose as the major carbon source. The microorganism was capable of tolerating up to 143 g/L of glucose, while cell growth was completely inhibited at concentrations higher than 158 g/L (Lin et al., 2008). Furthermore, significant decrease in succinic acid yield and prolonged lag phase were observed at glucose concentrations higher than 100 g/L.

Xylose, one of the main sugar monomers generated from lignocellulose hydrolysis has been examined for succinic acid production by natural or genetically modified microorganisms. Table 5.3 summarises reported key fermentation data for succinic acid

Table 5.3 Compilation of reported fermentation data for xylose-based production of succinic acid

Microorganism	Carbon sources[1]	Nitrogen sources[1]	Fermentation mode	Succinic acid (g/L)	Yield (g-SA/g-TS[1])	Productivity (g/L/h)	Ref
Media prepared from purified xylose							
A. succinogenes CGMCC 2650 or BE-1	Xylose	YE 30 g/L	Batch	6.9	0.28	0.21	Li et al., 2010
B. succiniciproducens JF 4016	Xylose	YE 5 g/L; Peptone 10 g/L; NH_4SO_4 0.2 g/L	Batch	4.6	0.60	0.77	Scholten et al., 2008
E. coli AFP184	Xylose	$(NH_4)_2SO_4$ 3.3 g/L; CSL(50%) 15 g/L	Batch dual phase	28.5	0.36	1.09	Hodge et al., 2009
E. coli AFP184	Xylose	$(NH_4)_2SO_4$ 3.3 g/L; CSL(50%) 15 g/L	Batch dual phase	23.2	0.29	0.97	Andersson et al., 2007
E. coli AFP184	Xylose (50 %), Glucose (50%)	$(NH_4)_2SO_4$ 3.3 g/L; CSL(50%) 15 g/L	Batch dual phase	27.5	0.34	0.86	Andersson et al., 2007
E. coli SD 121	Xylose	YE 10 g/L; Tryptone 20 g/L; $(NH_4)_2SO_4$ 0.05 g/L	Batch (anaerobic)	11.16	0.56	0.27	Wang et al., 2011
E. coli BA 204	Xylose (84.4 %), Glucose	Tryptone, 3 g/L; YE 1.5 g/L	Batch dual phase	(9.18)[2]	(0.72)[2]	(0.57)[2]	Liu et al., 2012
A. succinogenes 130Z	Xylose (72.6 %), Gal, Glu, Man, Ara	YE 5 g/L	Batch	27.44	0.70	0.45	This study
B. succiniciproducens JF 4016				25.97	0.76	0.55	
Media prepared from xylose-rich crude hydrolysates from renewable resources							
A. succinogenes CICC 11014	Corncob hydrolysates, xylose (77.3%)	YE 11 g/L	Batch	23.64	0.58	0.49	Yu et al., 2010
E. coli recombinant BA 204	Pretreated corn stalk hydrolysate, xylose (74.7 %)	Tryptone 3 g/L; YE 1.5 g/L	Batch dual phase	(11.13)*	(1.03)*	(0.70)*	Liu et al., 2012
E. coli recombinant SD 121	Pretreated xylose mother liquor, xylose (51.7 %)	YE 17.84 g/L	Batch	52.09	0.63	0.62	Wang et al., 2011

[1] YE: Yeast Extract, CSL: Corn Steep Liquor, TS: Total Sugars, Gal: Galactose, Man: Mannose, Glu: Glucose, Ara: Arabinose, SA: Succinic acid; [2] Numbers in parenthesis indicate values of the anaerobic phase in dual-phase fermentations while numbers outside the parenthesis indicate overall results (both aerobic and anaerobic phases)

production from pure xylose or xylose-based renewable feedstocks. The xylose to succinic acid conversion yields obtained in this study for *A. succinogenes* (0.70 g/g) and *B. succiniciproducens* (0.76 g/g), when around 33-35 g/L of mixed sugars were employed using the same sugar ratio as the one present in SSL are in fact the highest reported values. However, most of the studies presented in Table 5.3 have used genetically modified *E. coli* strains. The highest productivities have been reported for the recombinant strain *E. coli* AFP 184 and were equal to 1.09 g/L/h (Hodge et al., 2009) and 0.97 g/L/h (Andersson et al., 2007) in dual phase batch fermentations using only commercial xylose as carbon source. In the same studies, the final succinic acid concentrations from pure xylose were 28.5 g/L (Hodge et al., 2009) and 23.2 g/L (Andersson et al., 2007) and the overall yields, including both the aerobic and anaerobic phase, were relatively low (0.36 g/g and 0.29 g/g, respectively). In this, the xylose content of the sugar mixture used was 72.6% and the obtained final succinic acid concentrations for *A. succinogenes* (27.44 g/L) and *B. succinicproducens* (25.97 g/L) were comparable to the reported titers for *E. coli* AFP 184, but at higher yields and lower productivities (Table 5.3).

5.3.3 Effect of product concentration on growth

The accumulation of the end-metabolites during fermentation can cause growth inhibition. Increased extracellular carboxylic acid concentration can damage the cell membrane and decrease intracellular pH. The level tolerance is strain specific (Kabara et al., 1972) and can be manifested by changes in composition, integrity, fluidity and hydrophobicity of the cell membrane (Jarboe et al., 2013). The inhibition effect on cell growth by different fermentation product concentrations individually as well as organic acid mixtures, which simulate the ratio of the acids that are produced by each microorganism, were evaluated for both *A. succinogenes* and *B. succiniciproducens*. Figures 5.9 - 5.13 present the normalised specific growth rates at various initial organic acid concentrations. Although *A. succinogenes* does not produce lactic acid, inhibition was evaluated for comparison purposes. All experiments were carried out in 96 well microplate reader.

Formic acid (Figure 5.11) caused the greatest inhibition effect for both microorganisms, followed by acetic acid (Figure 5.12), succinic acid (Figure 5.9) and lactic acid (Figure 5.10). The critical concentrations at which cells fail to grow were calculated to be 18.0, 38.0, 55.0 and 60.0 g/L for formic, acetic, succinic and lactic acid, respectively. Critical concentrations

of formic acid reported in the literature for *A. succinogenes* are 10.8 g/L (Lin et al., 2008) and 35.2 g/L (Li et al., 2010).

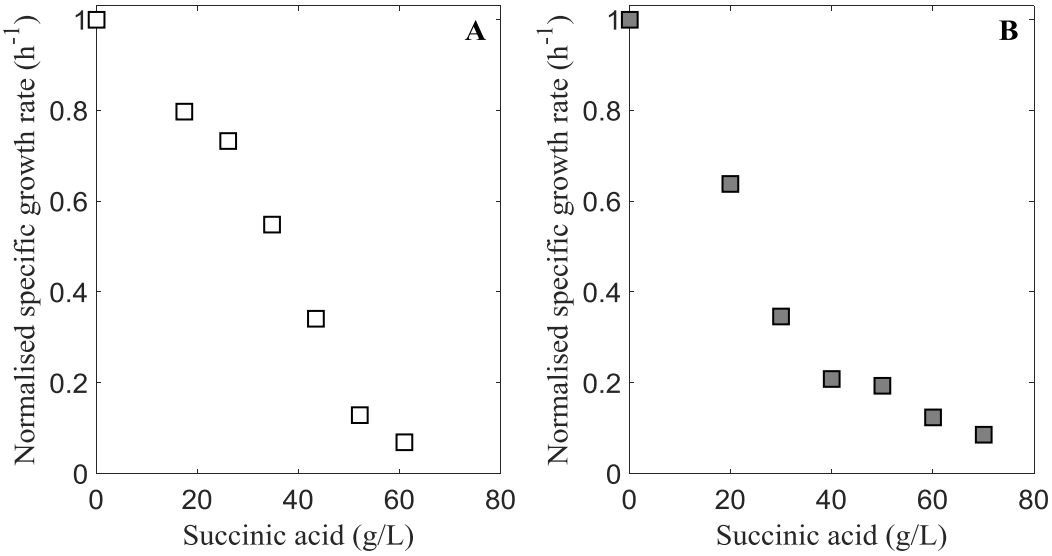

Figure 5.9 Effect of initial succinic acid concentration on specific growth rate of *A. succinogenes* (A) and *B. succiniciproducens* (B) in total culture volume of 200 µL

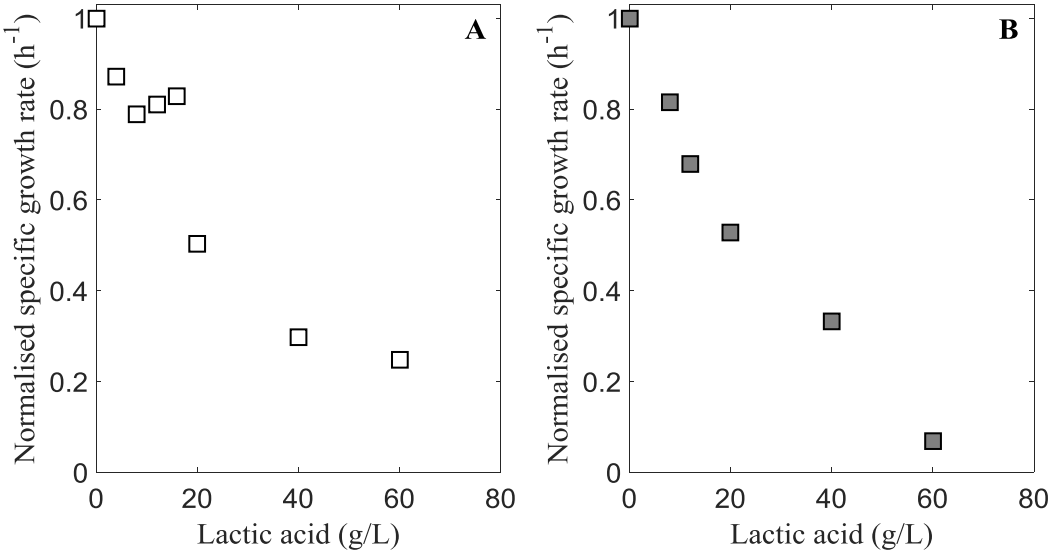

Figure 5.10 Effect of initial lactic acid concentration on specific growth rate of *A. succinogenes* (A) and *B. succiniciproducens* (B) in total culture volume of 200 µL

Regarding acetic acid, the reported critical inhibitory concentrations, 33.7 g/L (Lin et al., 2008) and 20 g/L (Li et al., 2010), are lower than the value obtained in this study. *B. succiniciproducens* shows similar product inhibition behavior with *A. succinogenes* as critical concentrations of the examined organic acids are 22 g/L for formic acid followed by 38 g/L

for acetic acid, 55 g/L for succinic acid and 62 g/L for lactic acid. Both microorganisms can tolerate higher concentrations of succinic acid than formic and acetic acids.

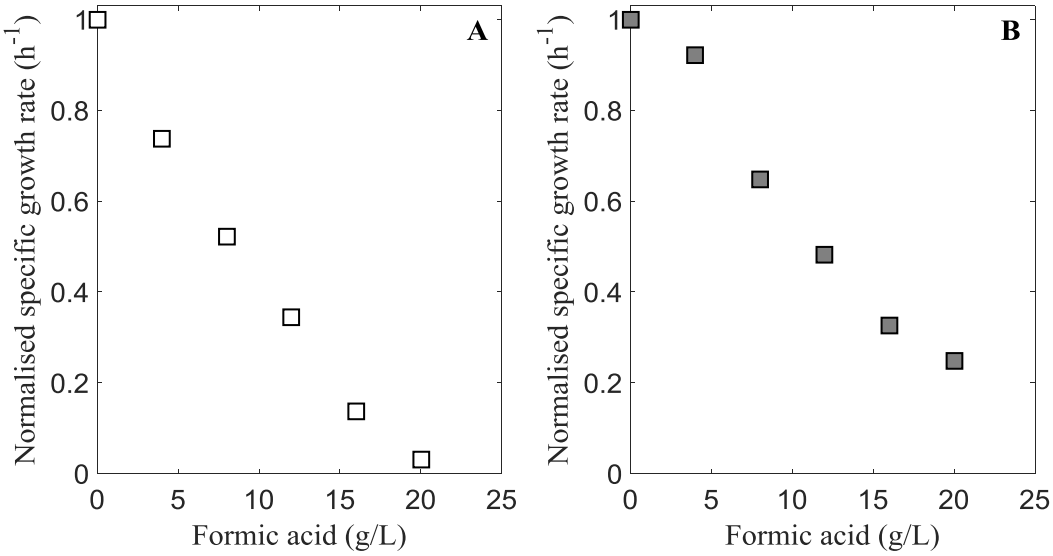

Figure 5.11 Effect of initial formic acid concentration on specific growth rate of *A. succinogenes* (A) and *B. succiniciproducens* (B) in total culture volume of 200 µL

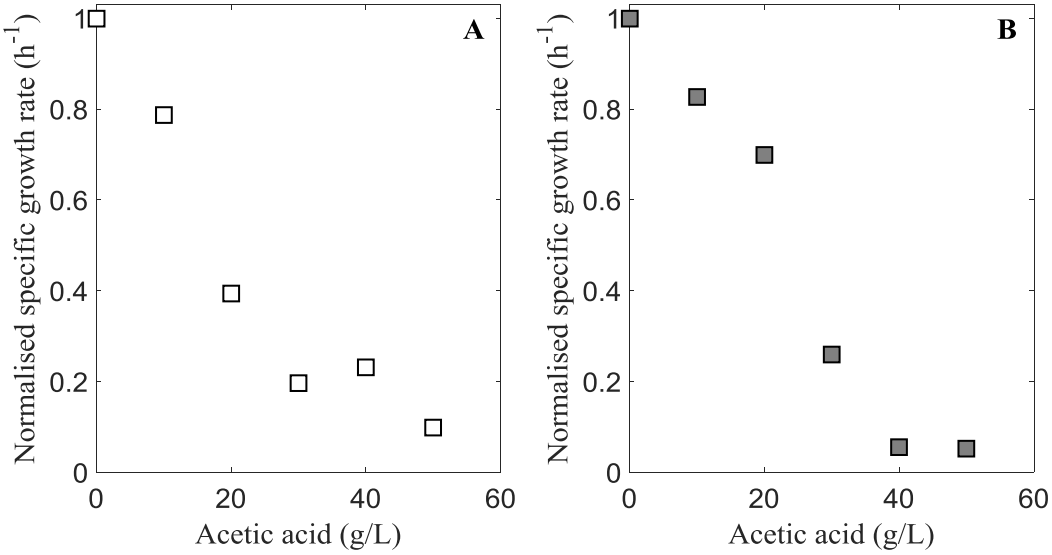

Figure 5.12 Effect of initial acetic acid concentration on specific growth rate of *A. succinogenes* (A) and *B. succiniciproducens* (B) in total culture volume of 200 µL

The synergistic inhibition effect was assessed by using a mixture of the organic acids in the ratio produced by each microorganism. The SA:FA:AA:LA (g/g) ratio was 1:0.45:0.59:0 for *A. succinogenes*, while for *B. succiniciproducens* it was 1:0.12:0.29:0.22. The critical concentrations found for these mixtures were 33 g/L for *A. succinogenes* and 35 g/L *B.*

succiniciproducens (Figure 5.13). We can thus conclude that both bacterial strains have similar tolerance to the examined fermentation products.

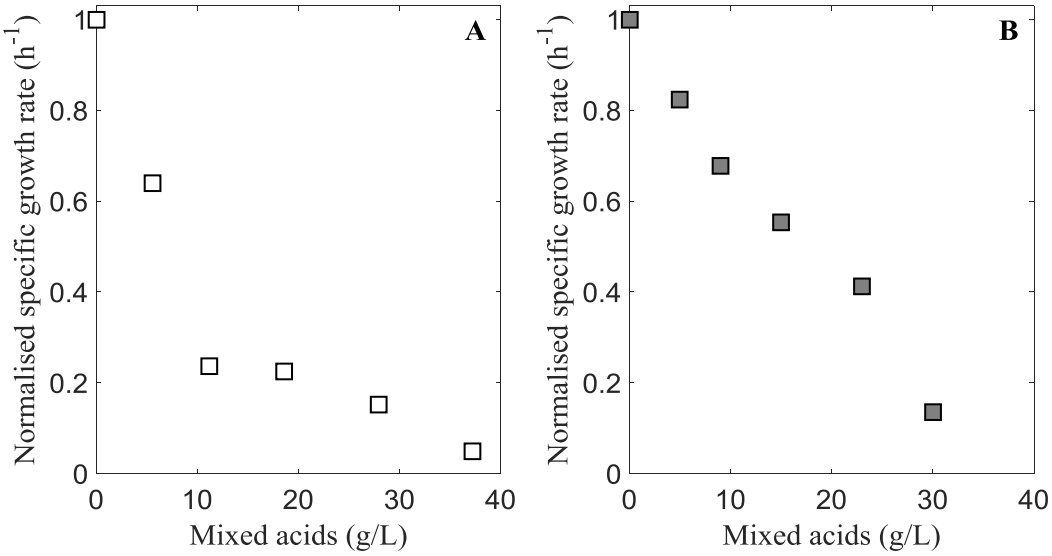

Figure 5.13 Effect of initial mixed acids concentration on specific growth rate of *A. succinogenes* (A) and *B. succiniciproducens* (B) in total culture volume of 200 μL

In another study, among the end-products investigated, formate was determined to impose the most inhibitory effect on succinic acid production, while the inhibitory concentrations of acetate, ethanol, formate, pyruvate and succinate were 46, 42, 16, 74 and 104 g L^{-1}, respectively (Lin et al., 2008) . Moreover, Corona-Gonzalez et al. (2008) cultivated *A. succinogenes* under different initial glucose and succinic acid concentrations in order to investigate their effect on the bioprocess using Jerusalimsky equations. It was demonstrated that the specific rates of biomass, succinate, formate and acetate production decreased when the initial succinic acid concentration increased, while the mathematical expressions used successfully predicted the substrate and product inhibitory effects. The product inhibition of *A. succinogenes* was compared to 3 mutant strains of *E. coli* (Qiang Li et al., 2010) exhibiting that *A. succinogenes* critical inhibitory concentrations of succinic, lactic, formic and acetic acids were 50, 18, 35.2 and 20 g/L, respectively. *E. coli* AFP111 showed similar tolerance with the examined studied microbial strains (*A. succinogenes* 130Z, *E. coli* NZN11, *E. coli* BL21) as the critical concentration of acetic acid was found to be 40 g/L. Qiang Li et al (2010) studied also the effect on growth and fermentation of by-products that are produced along with succinic acid and reported that three *E. coli* strains (NZ1111, AFP111, BL21) were more tolerant than *A. succinogenes* 130Z and that succinic acid had the milder inhibitory effect of the acids that were tested.

A. succinogenes and *B. succiniciproducens* are neutrophilic, meaning that high carboxylic acid concentration can cause inhibition. Cell growth inhibition has been proposed to be a result of the influx of the undissociated, lipophilic form (AH) of weak acids which can enter the cell by diffusion (Axe and Bailey, 1995). The inhibition degree of a weak acid depends on the relative acid strength, the carboxylic acid chain length and the extracellular pH, which are described below.

The relative strength of an acid depends on the dissociation constant (Ka) and the negative logarithm (pKa) (Atkins, 1990). At pH equal to pKa of an acid, the concentration of the undissociated and dissociated form of the acid is equal. The concentration of undissociated acid form increases with decreasing pH according to the Henderson–Hasselbalch equation:

$$pH = pKa + \log\frac{[A-]}{[HA]}$$

Weak acids have rather high pKa values, e.g. the values of pKa at zero ionic strength for acetic, formic, octanoic and levulinic acid are 4.75 (25°C), 3.75 (20°C), 4.89 (25°C) (Weast, 1975) and 4.66 (25°C) (Soni et al., 1982), respectively.

Carboxylic acid chain length affects the acid form that passes through the membrane by diffusion. According to Overton's Rule diffusion of a molecule through the membrane is a function of molecule hydrophobicity (Al-Awqati, 1999; Kamp and Hamilton, 2006). Acid transport is affected by the increase of hydrophobic chain length of the acid (Laura R. Jarboe, 2013). Experiments conducted across planar bilayer lipid membranes (Evtodienko et al., 1996) showed that the total transport of short chain (C2-C6) monocarboxylic acids is limited by diffusion of the anionic form [A-] of the acid at pH lower than the pKa, while at alkaline pH (>> pKa) the diffusion was limited by the neutral form of the acid. The transport of long-chain carboxylic acids (more than C8) was limited by diffusion of the anionic regardless the pH change (Evtodienko et al., 1996).

The pKa values, the concentrations of the produced acids, in combination with the pH and temperature of the medium, determine the concentration of the undissociated form of each acid in the fermentation broth. The pH of the cytosol is close to neutral, which triggers the dissociation of the acid resulting into the protons release and the counterion into the cytosol. The electrically charged acid form cannot pass through the lipophilic cell membrane

resulting in anion accumulation inside the cells cytosol, causing a change in the intracellular pH.

$$pH_{int} = \log\left\{\left(\frac{[A_{in}]}{[A_{out}]}\right)(10^{pKa} + 10^{pH_{out}}) - 10^{pKa}\right\}$$

Accumulation of the anions within the cell has been asserted as one of the main mechanisms of microbial inhibition by carboxylic acids. (Carpenter and Broadbent, 2009). Intracellular acidification causes disruption of the proton gradient across the cell membrane. When extracellular acid concentration is high, pH homeostasis is maintained through the synthesis of excessive ATP and the regulation of H^+ATPase, which expels protons from the cytoplasm (proton motive force) against the concentration gradient to maintain ΔpH (between the inner and outer surface of the cell) at low levels.

Actinobacillus succinogenes and *Basfia succiniciproducens* produce mainly succinic acid but formic acid acetic acids are also excreted from the cell and lactic acid in the case of *B. succiniciproducens*. The pKa value of the acids and the pH of the substrate play a critical role on the degree of tolerance as the intracellular pH is formed from the relation

The presence of more than one organic acids can cause synergistic inhibitory effects. Formic acid with a pKa of 3.75 has higher relative strength among succinic, lactic and acetic acids. Also the formic acid is a small molecule. These two properties can explain why formic acid caused the highest cell inhibition.

Table 5.4 Inhibitory product and carbon source concentrations on succinic acid production (initial inhibitory concentrations are enclosed in parenthesis). Missing data are either not mentioned or not investigated in the literature-cited references

Microorganism	Glucose (g/L)	Succinic acid (g/L)	Pyruvic acid (g/L)	Lactic acid (g/L)	Formic acid (g/L)	Acetic acid (g/L)	Ethanol (g/L)	Total products (g/L)	Reference
A. succinogenes CGMCC1593	> 100 (50-60)	-	-	-	-	-	-	-	Liu et al., 2008a
A. succinogenes CGMCC1593	> 100 (65)[1]	-	-	-	-	-	-	-	Liu et al., 2008b
A. succinogenes 130Z^T (ATCC 55618)	158 (100)	59.4	59.2	-	10.8 (1.8)	33.7	42	-	Lin et al., 2008
A. succinogenes 130Z^T (ATCC 55618)	> 100 (57.4)	-	-	-	-	-	-	-	Gonzalez et al., 2008
A. succinogenes 130Z^T (ATCC 55618)	-	40 (9.8)	-	-	-	-	-	- (20)	Gonzalez et al., 2010
A. succinogenes 130Z^T (ATCC 55618)	-	50 (<40)	-	18 (<9)	35.2 (<8.8)	20 (<10)	-	-	Qiang Li et al., 2010
E. coli NZN111	-	80 (<20)	-	18 (<9)	52.8 (<8.8)	60 (<20)	-	-	
E. coli AFP111	-	80 (<20)	-	18 (<9)	35.2 (<8.8)	40 (<20)	-	-	
E. coli BL21	-	>80 (<40)	-	18 (<9)	35.2 (<8.8)	60 (<20)	-	-	
A. succinogenes 130Z	>70 (50)[2]	65	-	75.14	22.56	50.6	-	33.15	This study
B. succinicciproducens JF4016	>70 (50)[2]	69.5	-	62.14	24.74	48.5	-	35.46	

[1] Total sugars from pretreated cane molasses; [2] Total sugars: 72.6% xylose, 12.2% galactose, 10.9% glucose, 4.2% mannose, and 0.1% arabinose

Chapter 6

Spent sulphite liquor fermentation

6.1 Introduction

Transformation of current industrial sectors into integrated and sustainable biorefineries is feasible through valorization of by-product and waste streams (Koutinas et al. 2014). In 2013, the worldwide sulphite pulping process produced around 2.6×10^6 t with the USA, Germany, Canada, Austria and Norway being the top five sulphite pulp producing countries (Anonymous 2015). The sulphite pulping process is conducted at temperatures in the range of 135 – 145 °C, at pH values of 1 – 2 for 6 – 12 h in batch digesters using aqueous solution of SO_2 and $MeHSO_3$, where Me stands for Ca, Mg, Na or NH_4 (Sjostrom, 1993). This process produces wood pulp and spent sulphite liquor (SSL) as aqueous waste stream that contains the solubilised lignin in the form of lignosulphonates (LS) and sugars resulting from hemicellulose degradation. The sugars contained in SSL depend on the type of wood used and the processing conditions employed in the plant. The SSL contains predominantly hexoses if softwood is employed, while pentoses are the main sugars when hardwoods are employed. The SSL is concentrated in multiple-effect evaporators and mainly employed for the recovery of LS that are currently used in various applications including concrete additives, cement dispersants, plasticisers, adhesives and feed additive (Restohlo et al., 2009). The current process (Howard process) used for LS precipitation involves $Ca(OH)_2$ or NaOH treatment that causes the destruction of C5 and C6 sugars. The sulphite pulping process generates around 8 – 9 m^3 of SSL per t of wood pulp produced (Lawford and Rousseau, 1993).

When hardwood is employed for wood pulp production, the SSL is rich mainly in xylose followed by galactose and glucose with lower amounts of mannose and arabinose (Table 6.1). SSL has been used as carbon source for fermentative production of various metabolic products, such as ethanol (Lawford and Rousseau, 1993), bacterial cellulose (Carreira et al., 2011) and xylitol (Heikkila et al., 1992). Besides sugars and LS, the SSL also contains phenolic compounds, furfural, 5-hydroxy-methyl-furfural (HMF), acetic acid and methanol. Various heavy metals and other substances originally present in wood can be also found in SSL. These compounds are inhibitory for microbial growth. Alexandri et al. (2016) reported that the extraction of phenolic compounds via solvent extraction could improve the production of succinic acid via fermentation. Overall, detoxification of SSL through the removal of LS and phenolic compounds will result in improved fermentation efficiency.

6.2 Spent sulphite liquor characterisation

SSL is the concentrated waste stream that is produced during pulp production using the hardwood Eucalyptus globulus as raw material. The thick SSL has been used in this study that was produced after concentration of the thin SSL via multi-effect evaporation. Table 6.1 presents the composition of SSL used in this study as well as five SSL streams reported in literature-cited publications. The pH value (2.7) of the SSL used in this study was the lowest among the streams reported in the literature. The high dry matter (816.5 ± 30 g/L) of the SSL used in this study as well as the SSL reported by Marques et al. (2009) indicates that these two streams were produced after concentration via evaporation. The SSL used in this study contains 410.1 ± 34.7 g/L of LS and 176.5 g/L of sugars, with xylose (72.6 %) being the predominant one.

Traces of cellobiose were also detected. Acetic acid, methanol, furfural and HMF are also contained in SSL at concentrations of 6.91 ± 0.49 g/L, 2.61 ± 0.29 g/L, 0.24 ± 0.03 g/L and 0.19 ± 0.05 g/L, respectively. The OH phenolic content (1.62 ± 0.08 g per 100 g dry matter) is produced via lignin degradation. The main phenolic compounds have been identified by Alexandri et al. (2016). One or more of the compounds present in SSL (e.g. acetic acid, methanol, furfural, HMF, LS and phenolic compounds) are inhibitory to microbial growth at varying critical concentrations depending on the microorganism (Palmqvist et al. 2000a,b; Salvachua et al. 2016). Therefore, the exploitation of the sugar content of SSL as carbon source for the production of bio-based chemicals and polymers necessitates the detoxification of SSL prior to fermentation. Furthermore, the recovery of LS constitutes current revenue for conventional sulphite pulp mills and the simultaneous exploitation of LS and sugars should be achieved.

6.3 Evaluation of SSL inhibitors on bacterial growth

Duran bottle fermentations were conducted with *A. succinogenes* and *B. succiniciproducens* with specific compounds that are contained in SSL (LS, acetic acid, furfural and methanol) at different initial concentrations. The tolerance of each microorganism was evaluated and the critical concentration of each inhibitor was estimated. Furthermore, Duran bottle fermentations with different initial SSL concentration were carried out in order to evaluate bacterial cell inhibition and succinic acid production.

Table 6.1 Characterization of the thick SSL used in this study (all results are average values of triplicate samples) and comparison with the respective compositions of thin and thick SSLs reported in literature-cited publications

Component	This study	Restolho et al. (2009) [1]	Marques et al. (2009) [2]	Chipeta et al. (2005) [3]	Xavier et al. (2010) [4]	Fernandez-Rodriguez et al. (2015) [5]
pH	2.7	3 - 3.7	3.7	3.3	3.4	3.3
Density (g/mL)	1.28 ± 0.007	-	1.48	-	-	1.029
Dry matter (g/L)	816.5 ± 30	161 - 173	840.6	753	148	7.23 ± 0.42
Lignosulfonates (g/L)	410.1 ± 34.7	79 - 87	486.9	-	78.2 ± 0.6	41.1 ± 3.05
OH phenolics (%, g/g-DM [6])	1.62 ± 0.08	-	-	-	-	-
Acetic acid (g/L)	6.91 ± 0.49	-	4.4	12.6	8.2 ± 0.3	0.25
Methanol (g/L)	2.61 ± 0.29	-	traces	-	-	-
HMF (g/L)	0.19 ± 0.05	-	-	-	-	-
Furfural (g/L)	0.24 ± 0.03	-	traces	-	< 0.1	0.004
Sugars (g/L)	176.5	40 - 57	134.7	145.2	46.7	23.31 ± 1.4
Xylose	128.1 ± 0.59	-	81.4	119	24.6 ± 0.5	-
Galactose	21.5 ± 5.5	-	31.1	11.1	4.5 ± 0.1	-
Glucose	19.3 ± 0.39	-	10.4	10.9	2.3 ± 0.1	-
Mannose	7.4 ± 1.3	-	4.4	-	8.5 ± 0.9	-
Arabinose	0.2 ± 0.05	-	4.4	4.2	7.8 ± 0.3	-

[1] Thin SSL from acidic magnesium based sulphite pulping of *E. globulus* (Portugal); [2] Thick SSL from magnesium-based acidic sulphite pulping of *E. globulus* (Portugal); [3] Thick SSL from acidic sulphite pulp mill (South Africa); [4] Thin SSL from magnesium based acidic sulphite pulping of *E. globules* (Portugal); [5] 10 times diluted thick SSL produced by acidic sulphite pulping of *E. globules*; [6] DM: Dry matter

6.3.1. Effect of acetic acid on bacterial growth and succinic acid production

The effect of acetic acid on bacterial growth was evaluated in Chapter 5 at a lower culture volume. Acetic acid inhibits bacterial growth by interfering in the difference between the intracellular pH and the pH of the environment (ΔpH). Acetic acid concentration in SSL ranges between 0.25 – 12.6 g/L in different industrial streams (Table 6.1). In the case of thick SSL used in this study, the acetic acid concentration was 6.9 g/L. Acetic acid concentrations of 10, 12 and 17 g/L were initially added to the medium that contained 25 g/L of mixed

sugars (at a ratio of 72.6% xylose, 12.2% galactose, 10.9% glucose, 4.2% mannose, and 0.1% arabinose), 5 g/L of yeast extract and minerals (as described in section 4.5). The specific growth rate was significantly affected by initial acetic acid concentrations higher than approximately 10 g/L (Figure 6.1).

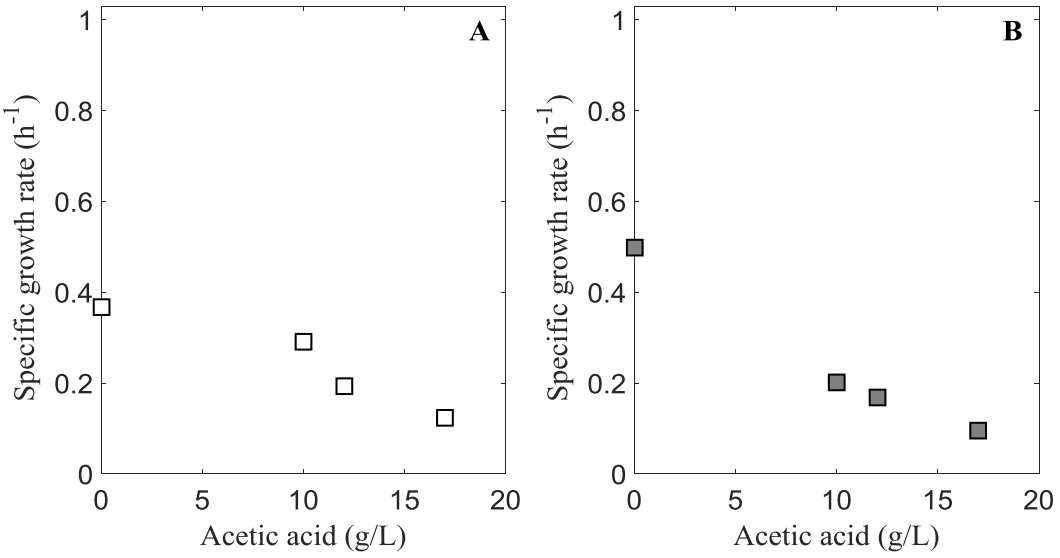

Figure 6.1 Effect of initial acetic acid concentration on specific growth rate of *A. succinogenes* (A) and *B. succiniciproducens* (B) during fermentations in Duran bottles (250 mL) using continuous supply of CO_2

Severe inhibition on succinic acid production was observed above 12 g/L of initial acetic acid concentration in both microorganisms (Figure 6.2). At 17 g/L of initial acetic acid concentration, the final succinic acid concentration was 9.8 g/L for *A. succinogenes* and 9.7 g/L for *B. succiniciproducens*, while around 10.5 g/L of total sugars remained unconsumed by both microorganisms (Figure 6.2). Up to 12 g/L initial acetic acid concentration, the final succinic acid concentration and unconsumed sugars remained almost stable.

In the case of *A. succinogenes*, the productivity was significantly reduced at acetic acid concentrations higher than 12 g/L, while in the case of *B. succiniciproducens* the productivity was significantly reduced even at acetic acid concentration of 12 g/L (Figure 6.3). The lowest productivity was 0.17 g/L/h for *A. succinogenes* and 0.13 g/L/h and *B. succiniciproducens*, which were observed at 17 g/L initial acetic acid concentration (Figure 6.3). It was observed that the total consumed sugars to succinic acid conversion yields were not affected by the initial acetic acid concentration for both *A. succinogenes* (0.73 - 0.78 g/g) and *B. succiniciproducens* (0.66 - 0.71 g/g). *A. succinogenes* seemed more tolerant than *B. succiniciproducens* to acetic acid concentrations up to 12 g/L.

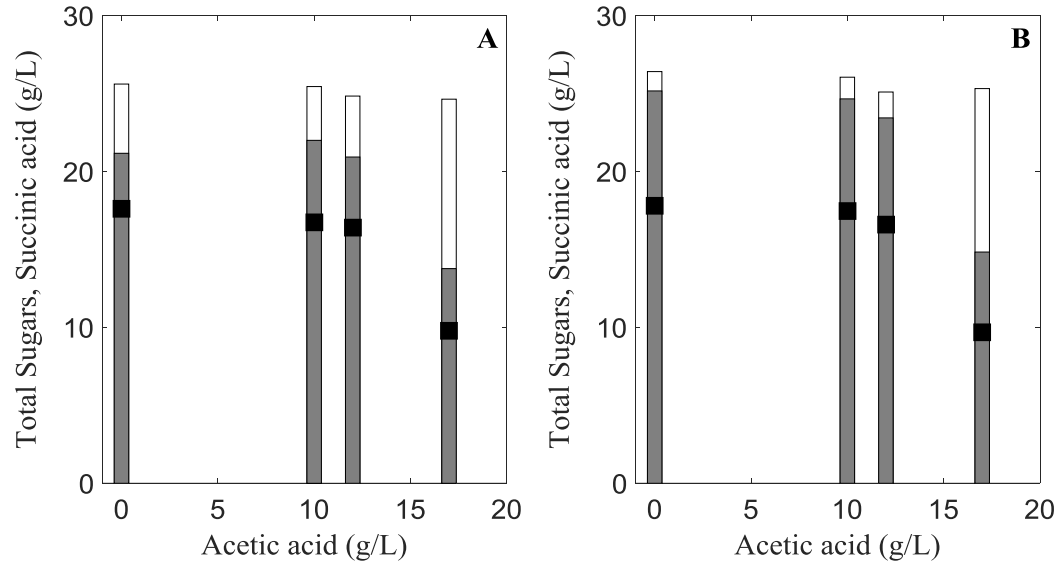

Figure 6.2 Total sugars (white bars), consumed sugars (grey bars) and final succinic acid concentration (square, ■) achieved by *A. succinogenes* (A) and *B. succiniciproducens* (B) during fermentations in Duran bottles (250 mL) on different initial acetic acid concentrations

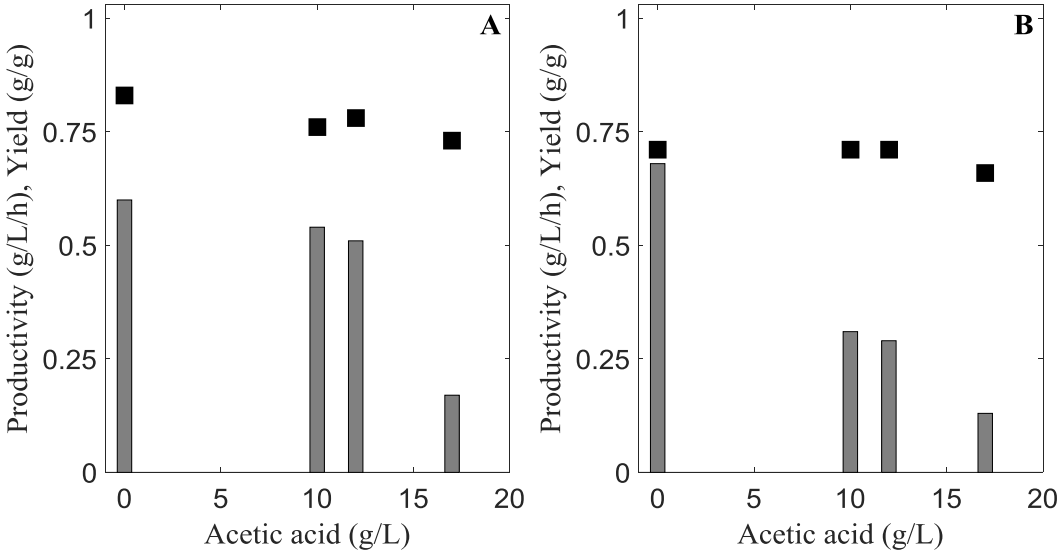

Figure 6.3 Productivity (grey bars) and final total consumed sugars to succinic acid conversion yield (square, ■) achieved by *A. succinogenes* (A) and *B. succiniciproducens* (B) in fermentations carried out at different initial acetic acid concentrations

Lactic acid production in the case of *B. succiniciproducens* followed an increasing trend at increasing initial acetic acid concentrations. Although lactic acid production is almost zero at fermentations with relatively low carbon source concentration (usually less than 25 g/L),

the stress caused by acetic acid resulted in higher final lactic acid concentrations (4.13 g/L at 17 g/L of initial acetic acid concentration) than the control experiment (0.62 g/L). The final LA/SA molar ratio followed an increasing trend with increasing acetic acid concentration (Figure 6.4).

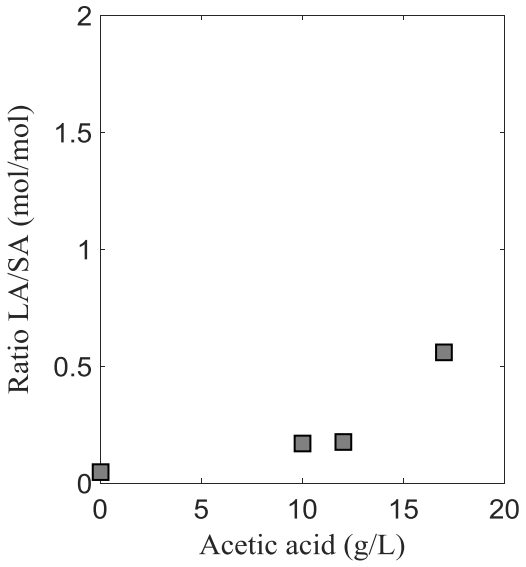

Figure 6.4 Final lactic acid to succinic acid molar ratios (AA/SA) observed in *B. succiniciproducens* fermentations at different initial acetic acid concentrations

The production of formic acid was reduced with increasing acetic acid concentration in both microorganisms. In the case of *A. succinogenes*, the FA/SA molar ratio remained almost constant (ca. 0.663-0.709 mol/mol) at all initial acetic acid concentrations (Figure 6.5A), while in the case of *B. succiniciproducens*, the FA/SA molar ratio followed a decreasing trend (Figure 6.5B). The production of acetic acid was reduced with increasing acetic acid concentrations in both microorganisms. The reduction of acetic acid production was higher in the case of *B. succiniciproducens* than *A. succinogenes*. The AA/SA molar ratio followed an increasing trend in the case of *A. succinogenes* and a decreasing trend in the case of *B. succiniciproducens* with increasing initial acetic acid concentrations (Figure 6.6).

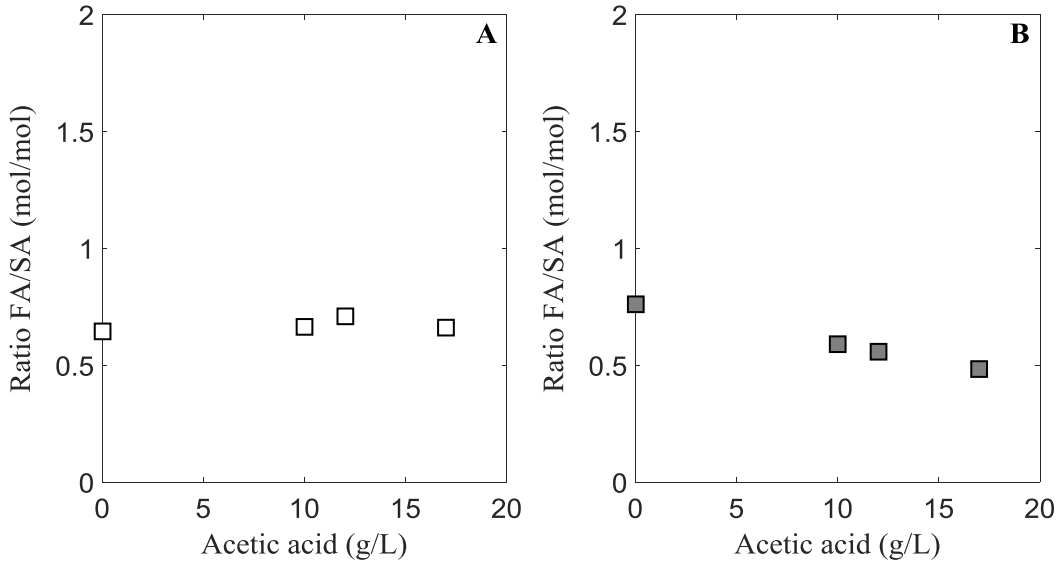

Figure 6.5 Final formic acid to succinic acid molar ratios (FA/SA) observed in *A. succinogenes* (A) and *B. succiniciproducens* (B) fermentations at different initial acetic acid concentrations

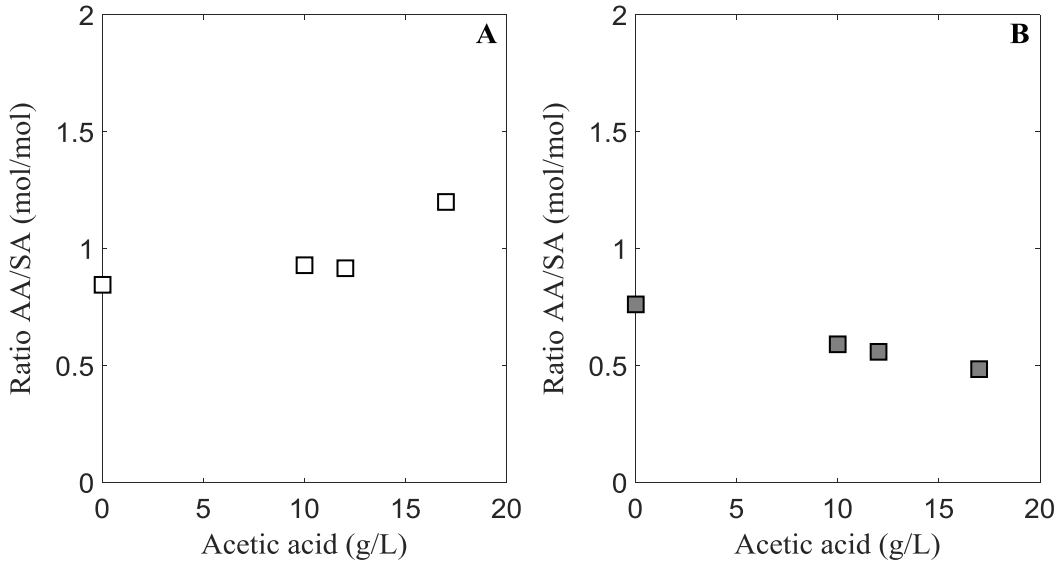

Figure 6.6 Final acetic acid to succinic acid molar ratios (AA/SA) observed in *A. succinogenes* (A) and *B. succiniciproducens* (B) fermentations at different initial acetic acid concentrations

6.3.2. Effect of furfural on bacterial growth and succinic acid production

Furfural concentration in the case of thick SSL was 0.24 g/L (Table 6.1). According to literature-cited publications, furfural is inhibitory even at very low concentrations (Wang et al., 2013; Sakai et al., 2007; Yong et al., 2015). The selected furfural concentrations of 0.5, 1,

2 and 3 g/L were initially added to the medium that consisted of 25 g/L of mixed sugars (at a ratio of 72.6% xylose, 12.2% galactose, 10.9% glucose, 4.2% mannose, and 0.1% arabinose), 5 g/L of yeast extract and minerals (as described in section 4.5). The specific growth rate was reduced at all initial furfural concentrations in both microorganisms. In the case of *A. succinogenes* growth, the inhibition trend was not as severe as in the case of *B. succiniciproducens* at initial furfural concentrations higher than 2 g/L (Figure 6.7).

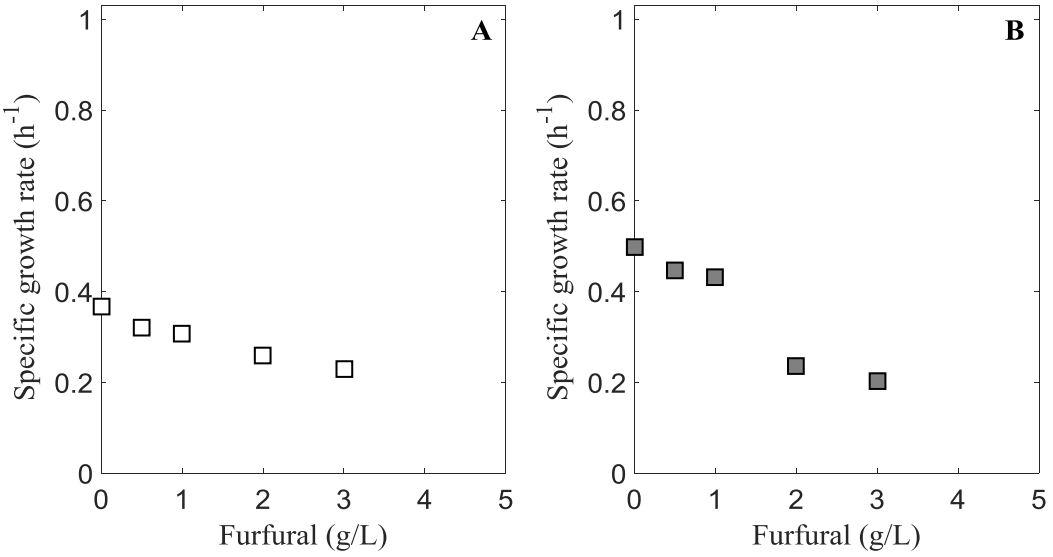

Figure 6.7 Effect of initial furfural concentration on specific growth rate of *A. succinogenes* (A) and *B. succiniciproducens* (B) during fermentations in Duran bottles (250 mL) using continuous supply of CO_2

The final succinic acid concentration and sugar consumption was not affected by the initial furfural concentrations that were used despite the fact that furfural is inhibitory to microbial cell growth (Figure 6.8). However, the productivity was severely affected even at initial furfural concentrations of 0.5 g/L, especially in the case of *A. succinogenes* (Figure 6.9). The total consumed sugars to succinic acid conversion yield was not significantly affected by furfural addition for either *A. succinogenes* or *B. succiniciproducens* (Figure 6.9).

Lactic acid production was almost zero in both microorganisms at all initial furfural concentrations. The production of formic acid in the case of *A. succinogenes* was increased with increasing furfural concentrations in the case of *A. succinogenes*, while in the case of *B. succiniciproducens* the final formic acid production remained almost constant. The FA/SA molar ratio in the case of *A. succinogenes* was reduced at initial furfural concentration of 0.5 g/L, following an increasing trend in higher initial furfural concentrations (Figure 6.10A). In the case of *B. succiniciproducens*, the FA/SA ratio remained stable (Figure 6.10B). The

production of acetic acid was reduced with increasing furfural concentration in both microorganisms. The AA/SA molar ratio in *A. succinogenes* followed an increasing trend at initial furfural concentrations higher than 1 g/L, while in *B. succiniciproducens* the AA/SA ratio remained stable (Figure 6.11).

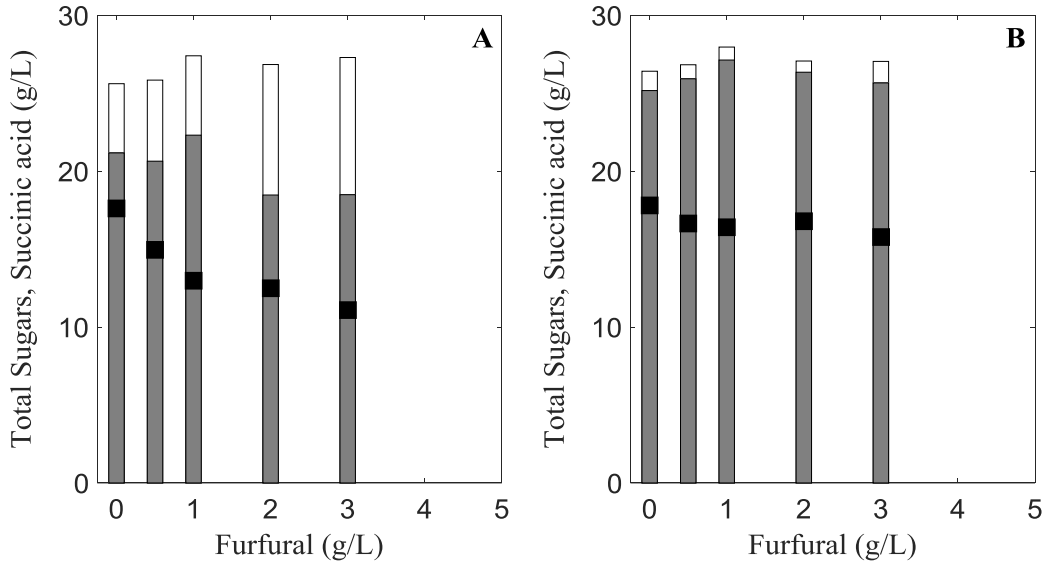

Figure 6.8 Total sugars (white bar), consumed sugars (grey bar) and final succinic acid concentration (square, ■) achieved by *A. succinogenes* (A) and *B. succiniciproducens* (B) during fermentation on different initial furfural concentrations

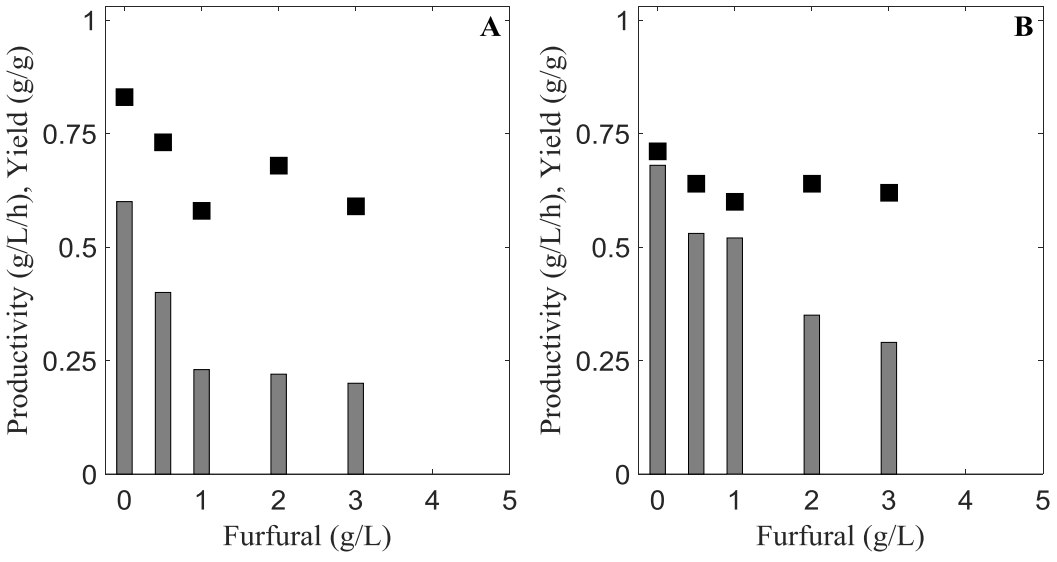

Figure 6.9 Productivity (grey bars) and final total consumed sugars to succinic acid conversion yield (square, ■) achieved by *A. succinogenes* (A) and *B. succiniciproducens* (B) in fermentations at different initial furfural concentrations

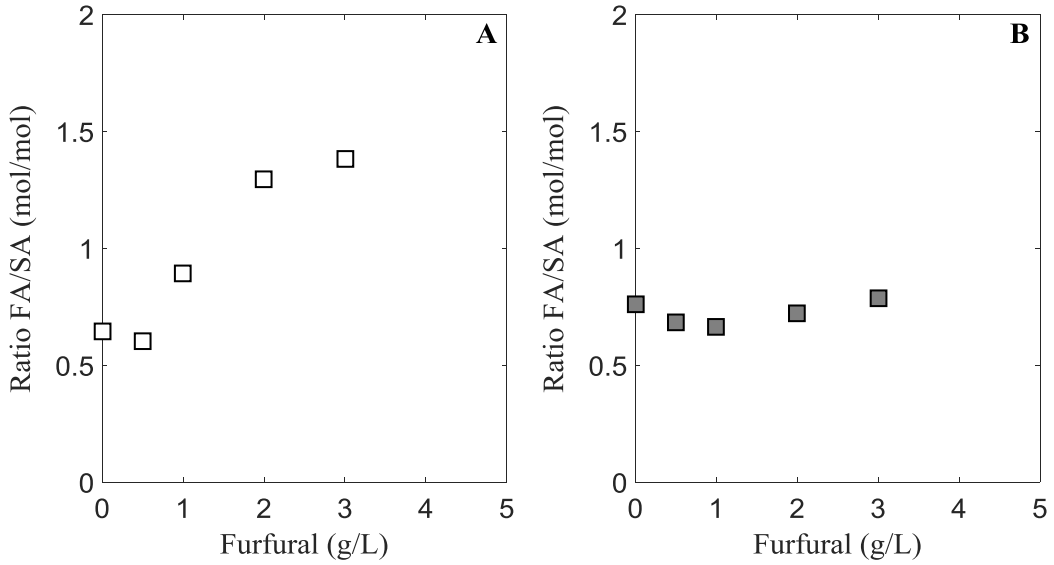

Figure 6.10 Final formic acid to succinic acid molar ratios (AA/SA) observed in *A. succinogenes* (A) and *B. succiniciproducens* (B) fermentations at different initial furfural concentrations

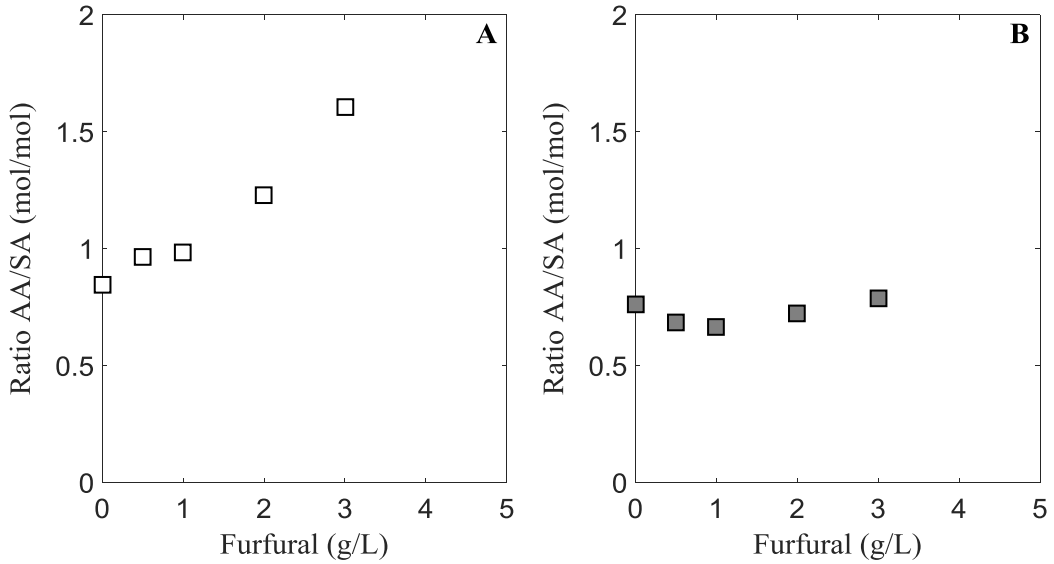

Figure 6.11 Final acetic acid to succinic acid molar ratios (AA/SA) observed in *A. succinogenes* (A) and *B. succiniciproducens* (B) fermentations at different initial furfural concentrations

An engineered *E. coli* strain could tolerate up to 3.2 g/L of furfural with decreased succinic acid fermentation efficiency (77.8% decrease in cell mass and 36.1% decrease in succinic acid concentration) (Wang et al., 2013). Critical inhibitory concentration, where growth was totally inhibited, was 6.4 g/L of furfural (12.8 g/L by HMF). The enzymes that were mostly affected, showing decreased enzyme activity by furfural addition, were phosphoenolpyruvate carboxylase, malate dehydrogenase and fumarate reductase (Wang et

al., 2013). The relative growth of *C. glutamicum* strain R-ldhA-pCRA723 with 21 mM furfural and 16 mM HMF decreased to 7% and 14% of the growth of the reference culture, respectively. *C. glutamicum* displayed sensitivities to furfural and HMF similar to those of yeasts such as *Saccharomyces cerevisiae* CBS 1200, *Candida shehatae* ATCC 22984, and *Pichia stipitis* NRRL Y 7124, but was more sensitive to furfural than bacteria such as *Z. mobilis* ATCC 10988, *E. coli* ATCC 1175, and *E. coli* LY01 (Sakai et al., 2007). The addition of 1 g/L of initial furfural concentration on *Rhodotorula glutinis* cultures resulted in 47% decrease of biomass and 50% of lipid accumulation, while the composition of fatty acids consisted mainly of C18 (Yong et al., 2015). When *Zymomonas mobilis* was cultivated in the presence of lignocellulosic biomass inhibitors, the aldehydes were more toxic than the organic acids (except for caproic acid). The most toxic compounds were vanillin, followed by 4-hydroxybenzaldehyde, syringaldehyde, furfural and HMF (Franden et al., 2013).

6.3.3. Effect of methanol on bacterial growth and succinic acid production

The concentration of methanol in the case of thick SSL was 2.61 g/L. Methanol concentrations of 0.5, 2, 4, 8, 10 and 15 g/L were initially added to the medium that consisted of 25 g/L of mixed sugars (at a ratio of 72.6% xylose, 12.2% galactose, 10.9% glucose, 4.2% mannose, and 0.1% arabinose), 5 g/L of yeast extract and minerals (as described in section 4.5). The specific growth rate was affected by initial methanol concentrations but severe inhibition was not observed even at 15 g/L of initial methanol concentration (Figure 6.12).

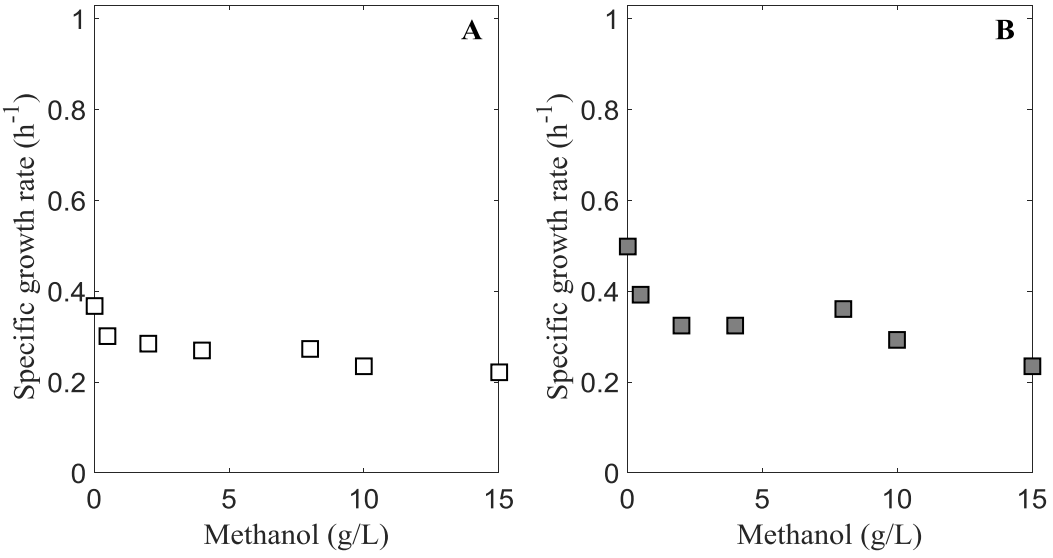

Figure 6.12 Effect of initial methanol concentration on specific growth rate of *A. succinogenes* (A) and *B. succiniciproducens* (B) during fermentations in Duran bottles (250 mL) using continuous supply of CO_2

The final succinic acid concentration in *A. succinogenes* (Figure 6.13A) and *B. succiniciproducens* (Figure 6.13B). was reduced at initial methanol concentrations higher than 8 g/L. The productivity was gradually reduced in the case of both *A. succinogenes* and *B. succiniciproducens* with increasing initial methanol concentration (Figure 6.14). Total consumed sugars to succinic acid conversion yield was not significantly affected at initial methanol concentrations lower than 4 g/L for both microorganisms.

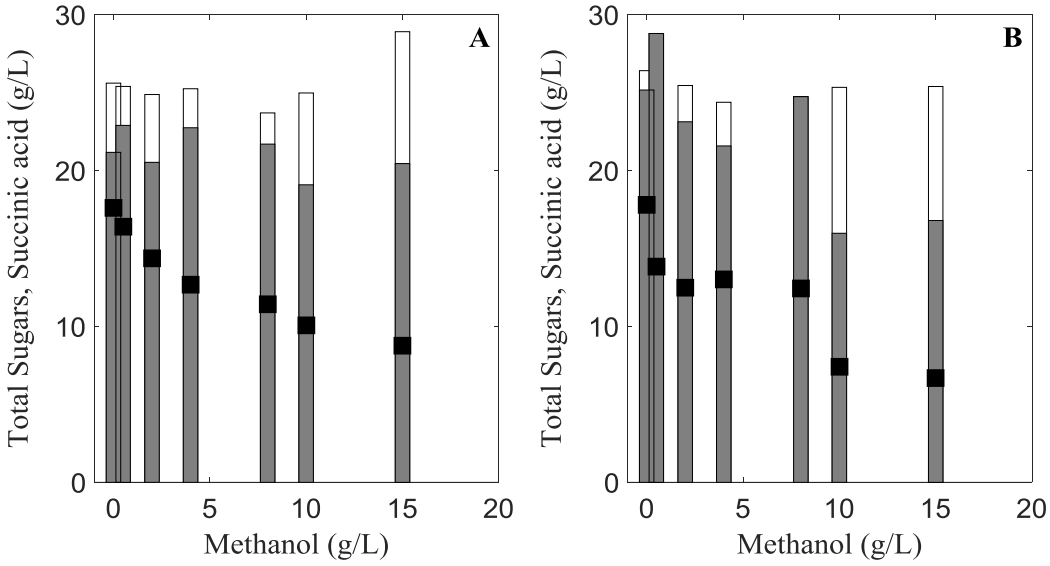

Figure 6.13 Total sugars (white bars), consumed sugars (grey bars) and final succinic acid concentration (square, ■) achieved by *A. succinogenes* (A) and *B. succiniciproducens* (B) during fermentations in Duran bottles (250 mL) using different initial methanol concentrations

Lactic acid production was observed only in the case of *B. succiniciproducens* at 10 g/L and 15 g/L initial methanol concentrations, while no lactic acid production was observed at lower methanol concentrations. Formic acid and acetic acid at the fermentations carried out with *A. succinogenes* followed a decreasing trend at increasing initial methanol concentrations, while the opposite response was observed in the case of *B. succiniciproducens*. The FA/SA ratio followed an increasing trend at increading methanol concentrations to both microorganisms. The AA/SA ratio was stable in A. succinogenes at all initial methanol concentrations while and followed an increasing trend. Overall, methanol caused the least inhibition on bacterial growth and succinic acid production compared to acetic acid and furfural.

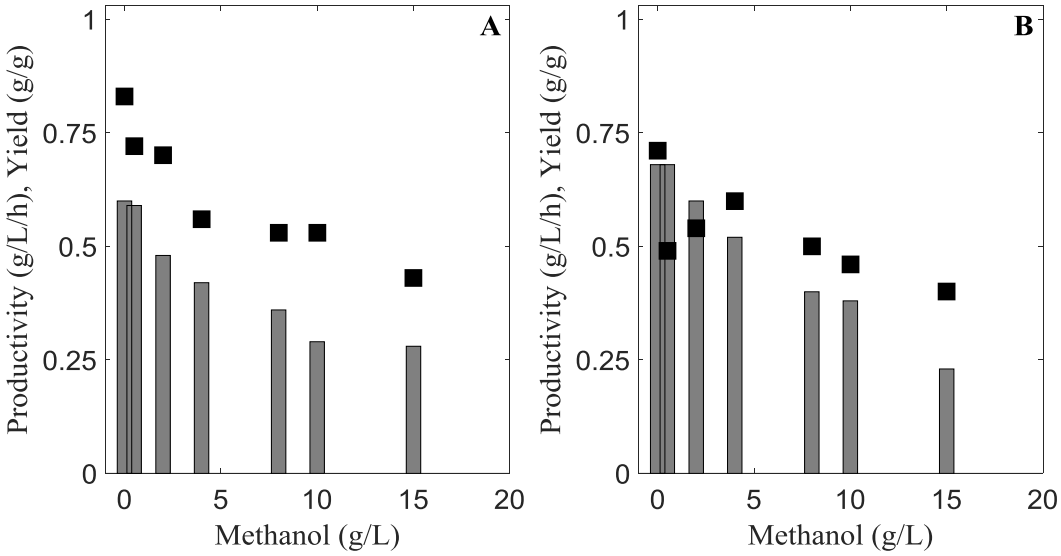

Figure 6.14 Productivity (grey bars) and final total consumed sugars to succinic acid conversion yield (square, ■) achieved by *A. succinogenes* (A) and *B. succiniciproducens* (B) in fermentations carried out at different initial methanol concentrations

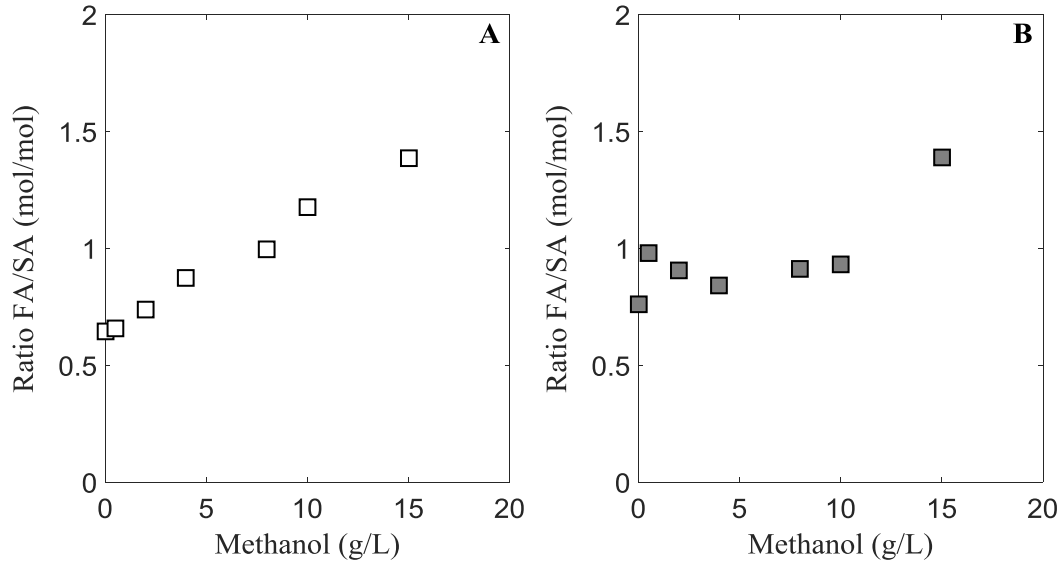

Figure 6.15 Final formic acid to succinic acid molar ratios (FA/SA) observed in *A. succinogenes* (A) and *B. succiniciproducens* (B) fermentations at different initial methanol concentrations in Duran bottle fermentations.

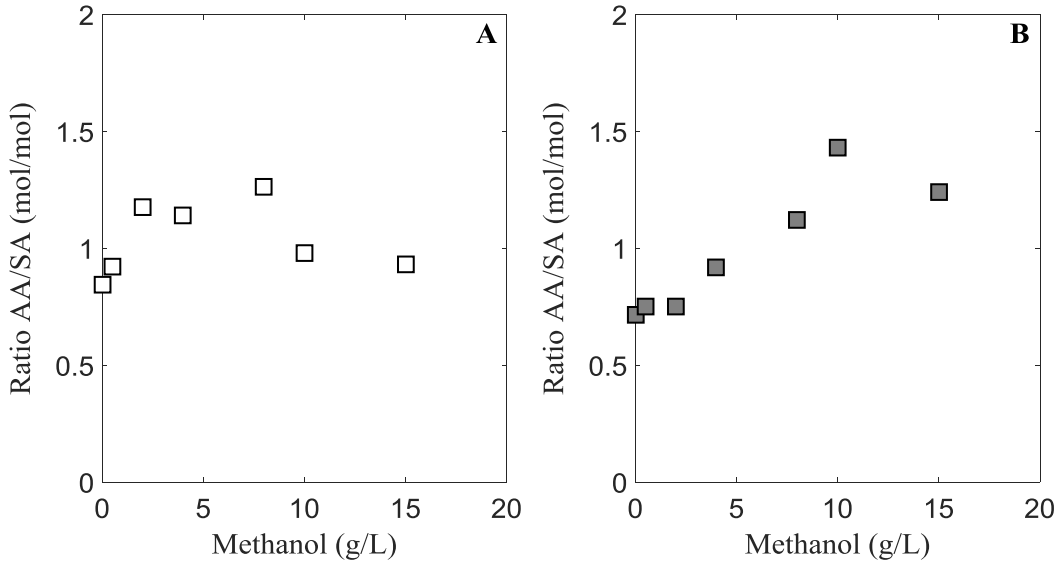

Figure 6.16 Final acetic acid to succinic acid molar ratios (AA/SA) observed in *A. succinogenes* (A) and *B. succiniciproducens* (B) fermentations at different initial methanol concentrations in Duran bottle fermentations.

In general, alcohols cause inhibition to the cells affecting the processes that are associated with the cell membrane. To overcome the stress, the cells alter the membrane lipid solubility (hydrophobic site of action). The effect on fatty acid composition depends on the properties of the molecule. Alcohol chain length is disanalogous to the severity of the membrane damage. Small molecules (up to four carbons in length) induce greater changes in fatty acid composition (synthesis of phospholipids containing two unsaturated fatty acids) than longer molecules that induce synthesis of phospholipids containing one unsaturated and one saturated fatty acid (i.e. hexanol) (Ingram and Vreeland, 1980). Alcohols interfere with the fluidity of the membrane causing microorganism to alter the fatty acid composition in order to tolerate stress. Furthermore, alcohols cause denaturation to the proteins that are found within the membrane and the cytosol (Huffer et al., 2011). Inhibition of methanol and ethanol was examined in the case of *E. coli*, *Bacillus cereus* and *Pichia pastoris* (Ganske and Bornscheuer, 2006). Ethanol caused a stronger inhibitory effect to microbial growth than methanol. Even at concentrations of 4% (v/v), ethanol caused higher inhibition to cell growth than methanol. In the case of methanol, *B. cereus* was more tolerant than *E. coli* and *P. pastoris* (Ganske and Bornscheuer, 2006). Table 6.2 presents final succinic acid concentration, yield and productivity as well as by-product to SA molar ratio at different initial inhibitor concentrations.

Table 6.2 Fermentation efficiency of *A. succinogenes* and *B. succiniciproducens* cultivated in Duran bottles at different initial inhibitor (acetic acid, furfural and methanol) concentrations

Inhibitor	Microorganism	Concentration (g/L)	Succinic acid (g/L)	Yield (g/g)	Productivity (g/L/h)	LA:FA:AA:SA (mol/mol)
Control	*A. succinogenes*	0	17.59 ± 0.03	0.83 ± 0.00	0.60 ± 0.00	0 : 0.645 : 0.846 : 1
	B. succiniciproducens	0	17.79 ± 0.37	0.71 ± 0.02	0.68 ± 0.01	0.046 : 0.763 : 0.718 : 1
Acetic acid	*A. succinogenes*	10	16.76 ± 0.69	0.76 ± 0.09	0.54 ± 0.02	0 : 0.664 : 0.929 : 1
		12	16.42 ± 0.08	0.78 ± 0.01	0.51 ± 0.00	0 : 0.709 : 0.915 : 1
		17	9.78 ± 0.77	0.73 ± 0.14	0.17 ± 0.01	0 : 0.663 : 1.210 : 1
	B. succiniciproducens	10	17.48 ± 0.12	0.71 ± 0.03	0.31 ± 0.00	0.169 : 0.590 : 0.611 : 1
		12	16.61 ± 0.07	0.71 ± 0.02	0.29 ± 0.00	0.175 : 0.558 : 0.568 : 1
		17	9.68 ± 0.69	0.66 ± 0.02	0.13 ± 0.01	0.559 : 0.484 : 0.626 : 1
Furfural	*A. succinogenes*	0.5	14.96 ± 0.20	0.73 ± 0.03	0.40 ± 0.03	0 : 0.605 : 0.965 : 1
		1	12.99 ± 1.20	0.58 ± 0.00	0.23 ± 0.02	0 : 0.894 : 0.984 : 1
		2	12.47 ± 0.50	0.68 ± 0.01	0.22 ± 0.01	0 : 1.297 : 1.230 : 1
		3	11.08 ± 3.32	0.59 ± 0.11	0.20 ± 0.06	0 : 1.384 : 1.606 : 1
	B. succiniciproducens	0.5	16.64 ± 0.97	0.64 ± 0.04	0.53 ± 0.03	0 : 0.684 : 0.906 : 1
		1	16.39 ± 0.21	0.60 ± 0.00	0.52 ± 0.00	0 : 0.664 : 0.940 : 1
		2	16.78 ± 0.66	0.64 ± 0.01	0.35 ± 0.01	0 : 0.722 : 0.774 : 1
		3	15.79 ± 0.57	0.62 ± 0.00	0.29 ± 0.01	0.058 : 0.788 : 0.782 : 1

Methanol	*A. succinogenes*	0.5	16.38 ± 0.54	0.72 ± 0.03	0.59 ± 0.02	0 : 0.658 : 0.924 : 1
		2	14.37 ± 1.48	0.70 ± 0.09	0.48 ± 0.04	0 : 0.738 : 1.177 : 1
		4	12.67 ± 0.41	0.56 ± 0.00	0.42 ± 0.01	0 : 0.876 : 1.141 : 1
		8	11.41 ± 0.31	0.53 ± 0.02	0.36 ± 0.01	0 : 0.997 : 1.263 : 1
		10	10.08 ± 0.19	0.53 ± 0.03	0.29 ± 0.00	0 : 1.177 : 0.981 : 1
		15	8.77 ± 0.00	0.43 ± 0.00	0.28 ± 0.00	0 : 1.388 : 0.993 : 1
	B. succiniciproducens	0.5	13.84 ± 0.41	0.48 ± 0.10	0.68 ± 0.01	0.083 : 0.981 : 0.753 : 1
		2	12.50 ± 0.00	0.54 ± 0.00	0.60 ± 0.00	0 : 0.908 : 0.751 : 1
		4	13.05 ± 0.06	0.60 ± 0.01	0.52 ± 0.00	0 : 0.842 : 0.919 : 1
		8	12.42 ± 0.69	0.50 ± 0.00	0.40 ± 0.00	0 : 0.913 : 1.122 : 1
		10	7.40 ± 0.21	0.46 ± 0.03	0.38 ± 0.01	0.610 : 0.932 : 1.234 : 1
		15	6.69 ± 1.49	0.40 ± 0.09	0.23 ± 0.00	0.695 : 1.389 : 1.240 : 1

6.3.4. Effect of lignosulphonates and spent sulphite liquor on bacterial growth and succinic acid production

The effect of SSL and LS on bacterial growth and succinic acid production was studied in order to elucidate the critical concentrations that lead to reduced fermentation efficiency. The LS are produced during the sulphite pulping process via biomass treatment with sulphurous acid at high temperatures and low pH, where lignin is separated from cellulose and hemicellulose through sulphonation and hydrolysis processes. The LS content in thick SSL is usually higher than 400 g/L (Table 6.1) in different industrial streams and therefore it is crucial to identify the dilution required at the beginning of fermentation. The first set of experiments was carried out using three different initial LS concentrations (36.1, 72.2 and 115.5 g/L) that were added into a fermentation medium that contained 25 g/L mixed sugars (at a ratio of 72.6% xylose, 12.2% galactose, 10.9% glucose, 4.2% mannose, and 0.1% arabinose), 5 g/L yeast extract and minerals. The second set of experiments was carried out with different SSL dilutions where the LS concentration varied.

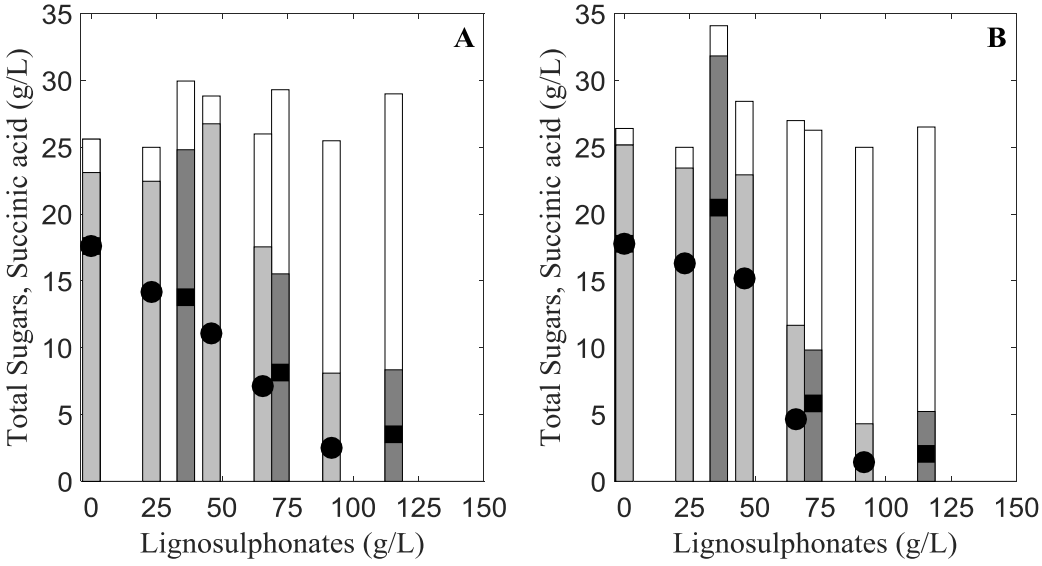

Figure 6.17 Total sugars (white bars), consumed sugars (grey bars) and final succinic acid concentration (■ and ●) achieved by *A. succinogenes* 130Z (A) and *B. succiniciproducens* (B) during fermentations in Duran bottles (250 mL) on different initial LS concentrations and various SSL dilutions. Fermentations carried out via SSL dilution are represented by light grey bars for sugar consumption and circles for succinic acid production.

The SSL was diluted 20, 10, 7 and 5 times corresponding to LS concentration of 22.94, 45.88, 65.54 and 91.76 g/L, respectively. Although other inhibitors are present in low concentrations, their synergistic effect could result in fermentation efficiency decrease. The

fermentation medium also contained the yeast extract mineral concentration used in the set of experiments carried out with LS addition. Mixed sugars were supplemented whenever needed, so as to reach a final total sugar concentration of 25 – 30 g/L. Fermentations on diluted SSL and LS were carried out with both *A. succininogenes* (Figure 6.17A) and *B. succiniciproducens* (Figure 6.17B). Specific growth rate could not be measured, since the brown color of LS and SSL disabled the measurement of optical density and the dry weight could not be measured due to the presence of LS. The initial inhibitory LS concentration for both succinic acid production and sugar consumption was around 45 g/L (Figure 6.17) for both microorganisms using either diluted SSL or added LS. Since there was no significant difference between cultures carried out with added LS and diluted SSL, it could be concluded that LS is the major inhibitory compound contained in SSL.

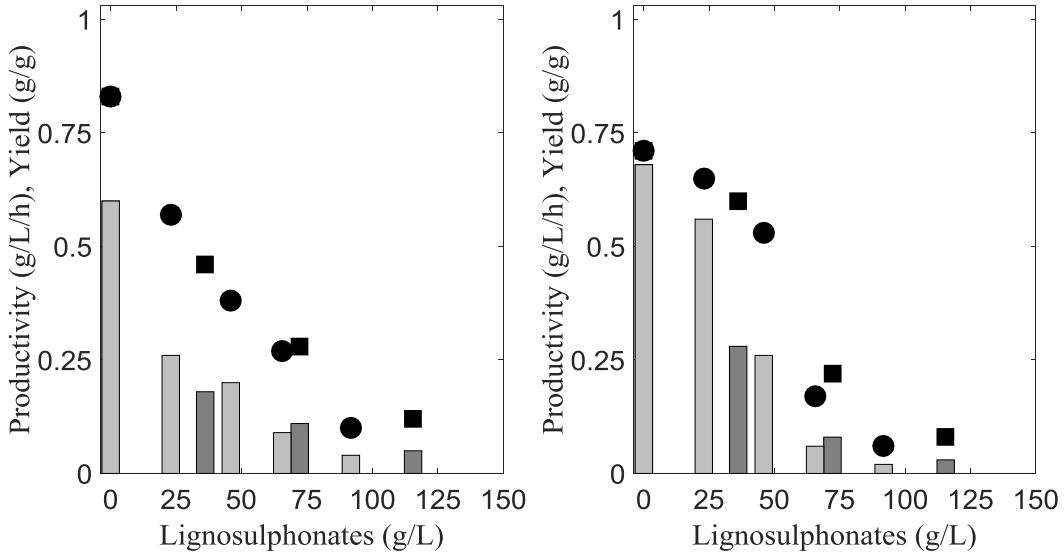

Figure 6.18 Productivity (grey bars) and final total consumed sugars to succinic acid conversion yield (■ and ●) achieved by *A. succinogenes* 130Z (A) and *B. succiniciproducens* (B) during fermentations in Duran bottles (250 mL) on different initial LS concentrations and various SSL dilutions. Fermentations carried out via SSL dilution are represented by light grey bars for sugar consumption and circles for succinic acid production.

Yield and productivity were gradually decreased with increasing initial LS concentration (Figure 6.18).When *A. succinogenes* was cultivated on the lowest LS concentration (22.9 g/L), the productivity was decreased by approximately 60% compared to the control culture carried out without SSL or LS. A less pronounced decrease in productivity was observed in the case of *B. succiniciproducens* cultures when cultivated at similar LS concentration (Figure 6.18B). The yield was higher in the case of *B. succiniciproducens*

cultures and was decreased at a slower than *A. succinogenes* cultures. Minimal sugar consumption and succinic acid production was observed at LS concentrations higher than 100 g/L for both microorganisms (Figures 6.17 and 6.18).

The by-product to succinic acid molar ratios observed in the previous cultures showed that the metabolism shifted towards the C3 pathway or the C3 pathway was not as much affected as the C4 pathway.

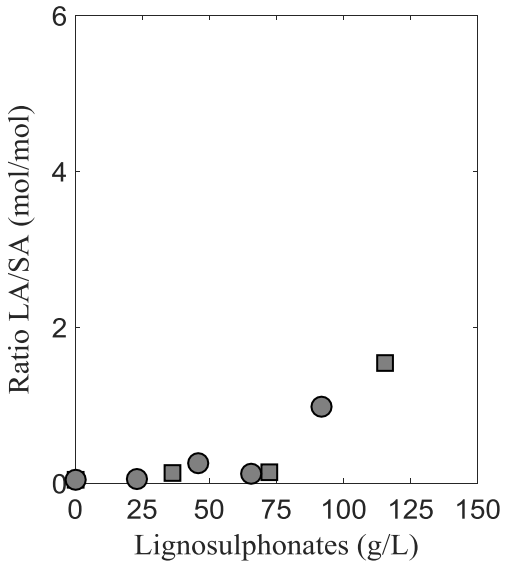

Figure 6.19 Final lactic acid to succinic acid molar ratios (LA/SA) observed in *B. succiniciproducens* cultures carried out in Duran bottles at different initial LS concentrations (■) and various SSL dilutions (●).

The final formic acid to succinic acid molar ratio followed an increasing trend for both microorganisms (Figure 6.20). The highest FA/SA molar ratio for *A. succinogenes* (2.275 mol/mol) and *B. succiniciproducens* (2.603 mol/mol) were observed at 115 g/L of initial LS concentration. The final acetic acid to succinic acid ratio was increased to higher values in the case of *B. succiniciproducens* cultures (Figure 6.21B) reaching values higher than 5 mol/mol. These results show that under extreme stress both microorganisms reduce the carbon flux through the TCA cycle but retain the regulation of the pyruvate metabolism, which leads to lactic acid, formic acid and acetic acid production. Lactic acid production was significantly increased in *B. succiniciproducens* cultures at initial LS concentrations higher than 75 g/L. The LA/SA molar ratio reached values close to 2 mol/mol at LS concentrations higher than 115 g/L (Figure 6.19).

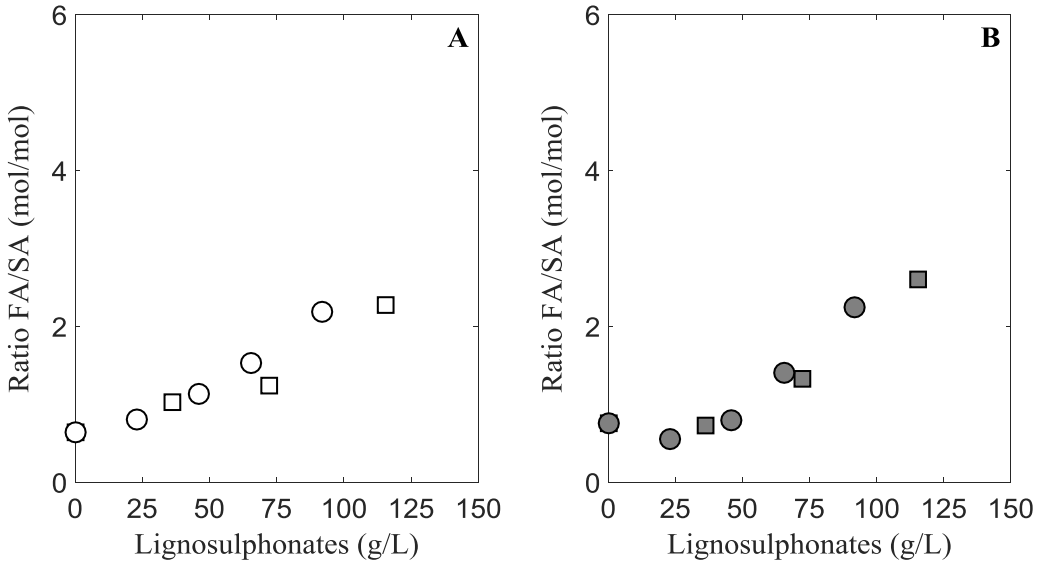

Figure 6.20 Final formic acid to succinic acid molar ratios (FA/SA) observed in *A. succinogenes* (A) and *B. succiniciproducens* (B) cultures carried out in Duran bottles at different initial LS concentrations (■) and various SSL dilutions (●).

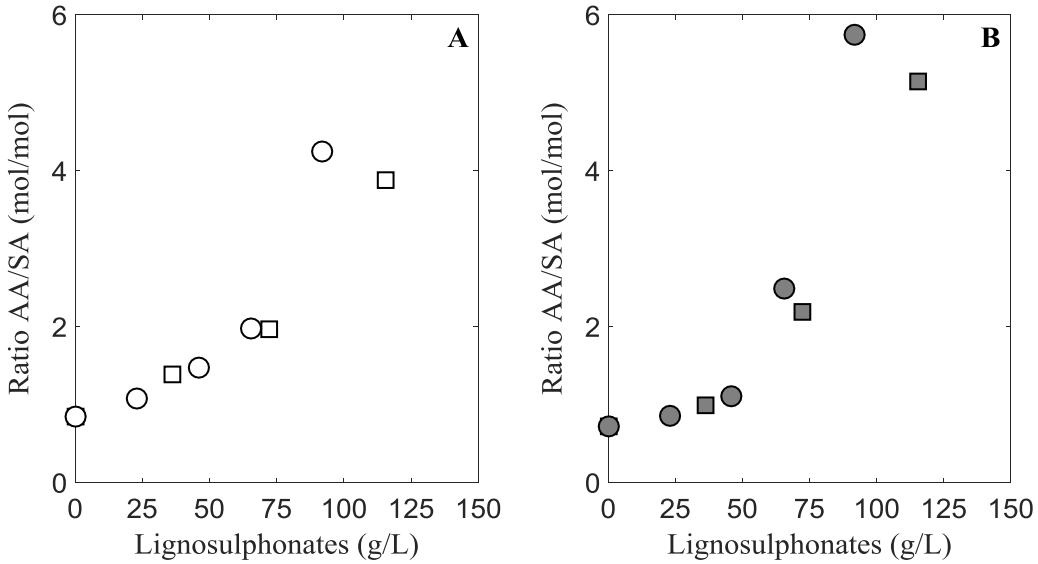

Figure 6.21 Final acetic acid to succinic acid molar ratios (AA/SA) observed in *A. succinogenes* (A) and *B. succiniciproducens* (B) cultures carried out in Duran bottles at different initial LS concentrations (■) and various SSL dilutions (●).

Table 6.3 Fermentation efficiency of *A. succinogenes* and *B. succiniciproducens* cultivated in Duran bottles at different initial LS concentrations

Inhibitor	Microorganism	LS concentration (g/L)	Succinic acid (g/L)	Yield (g/g)	Productivity (g/L/h)	LA:FA:AA:SA (mol/mol)
Control	*A. succinogenes*	0	17.59 ± 0.03	0.83 ± 0.00	0.60 ± 0.00	0 : 0.645 : 0.846 : 1
	B. succiniciproducens	0	17.79 ± 0.37	0.71 ± 0.02	0.68 ± 0.01	0.046 : 0.763 : 0.718 : 1
LS-based cultures	*A. succinogenes*	36.1	13.78 ± 0.8	0.46 ± 0.04	0.18 ± 0.03	0 : 1.032: 1.384: 1
		72.2	8.13 ± 2.3	0.28 ± 0.05	0.11 ± 0.01	0 : 1.243 : 1.964 : 1
		115.52	4.29 ± 0.87	0.17 ± 0.00	0.06 ± 0.00	0 : 2.275 : 3.875 : 1
	B. succiniciproducens	36.1	20.47 ± 1.36	0.6 ± 0.06	0.28 ± 0.04	0.134 : 0.725 : 0.992 : 1
		72.2	5.84 ± 0.24	0.22 ± 0.04	0.08 ± 0.04	0.140 : 1.327 : 2.187 : 1
		115.52	2.08 ± 0.74	0.08 ± 0.01	0.03 ± 0.00	1.538 : 2.603 : 5.141 : 1
SSL-based cultures	*A. succinogenes*	22.94	14.20 ± 0.31	0.57 ± 0.00	0.26 ± 0.03	0 : 0.809 : 1.079 : 1
		45.88	11.06 ± 0.26	0.38 ± 0.06	0.20 ± 0.02	0 : 1.138 : 1.478 : 1
		65.54	7.12 ± 0.54	0.27 ± 0.03	0.09 ± 0.01	0 : 1.532 : 1.973 : 1
		91.76	2.52 ± 0.12	0.10 ± 0.00	0.04 ± 0.00	0 : 2.193 : 4.246 : 1
	B. succiniciproducens	22.94	16.32 ± 0.41	0.65 ± 0.04	0.56 ± 0.01	0.050 : 0.551 : 0.853 : 1
		45.88	15.17 ± 0.67	0.53 ± 0.01	0.26 ± 0.01	0.258 : 0.797 : 1.104 : 1
		65.54	4.63 ± 0.23	0.17 ± 0.02	0.06 ± 0.00	0.116 : 1.410 : 2.485 : 1
		91.76	1.41 ± 0.12	0.06 ± 0.00	0.02 ± 0.00	0.984 : 2.246 : 5.746 : 1

Based on the results presented in Table 6.3 and Figures 6.17-6.21, both microorganisms can tolerate up to 50 g/L of initial LS concentration without severely affecting succinic acid final concentration. However, productivity and yield are reduced faster even at lower LS concentrations than 50 g/L. The bacterial strain *B. succiniciproducens* seems more efficient than *A. succinogenes* on succinic acid production when it is cultivated on SSL. Inhibition experiments that were carried out with *Rhodosporidium toruloides* for microbial oil production using initial concentrations in the range of 0.5 - 2 g/L of sodium lignosulphonate resulted in a 10% biomass increase, suggesting that it was a result of the surfactant properties (Zhao et al., 2012).

6.4 Pretreatment of SSL via ultrafiltration and nanofiltration

Several methods (e.g. chemical, mechanical) have been employed so far for the pretreatment of wood hydrolysates or residual liquors from the pulp and paper industry (Palmqvist et al., 2000a). Filtration has been employed for the separation of LS from residual liquors of the pulp and paper industry using hardwood or softwood with efficiencies higher than 90 % (Restolho et al., 2009). Membrane treatment of SSL generated from the acidic pulping process of hardwood could lead to the separation of LS in the retentate stream, while the permeate stream would be rich in C5 and C6 sugars that could be used as carbon sources in fermentation processes. In this way, the full potential of SSL could be exploited for the production of several value-added products.

The major objective of filtration was to separate the LS, while in the same time exploit the majority of sugars for bio-based succinic acid production. The removal of LS will also reduce their accumulation in the broth during fed-batch fermentation leading to inhibition of bacterial growth. Table 6.4 presents the composition of the diluted SSL used in each filtration experiment and the composition of total sugars, LS and OH phenolics of the retentate and permeate streams generated after each filtration experiment. The retentate stream was around 20% of the initial SSL volume employed. In the case that a membrane with 10 kDa MWCO was used, the LS concentration in the permeate stream was 24.7 g/L corresponding to around 50% of the total LS content in the initial SSL used. The membranes of 5 and 3 kDa MWCO were more effective in the retention of the LS with 69 % and 74 % of the initial LS content retained in the retentate fraction, respectively. The total loss of sugars was similar in all cases (17.6 - 20%). The membranes used in this study for ultrafiltration of SSL result in sugar losses in the retentate stream and LS losses in the permeate stream. SSL treated with 3 kDA

MWCO membrane was used as carbon source in the fermentations carried out by *A. succinogenes* and *B. succiniciproducens*.

Restohlo et al. (2009) reported that most ultrafiltration membranes used could not efficiently separate the lignosulphonates from the sugars during filtration of thin SSL produced from acidic magnesium-based sulphite pulping of *Eucalyptus globulus*. However, only the use of Microdyn-Nadir UP010 polyethersulphone membrane resulted in high retentions of LS (68%) and low retention of sugars (3%) during ultrafiltration of thin SSL (Restolho et al., 2009). The main problem of using only ultrafiltration membranes is the retention of high molecular weight lignosulphonates and the permeation of low molecular weight lignosulphonates. The complete separation of sugars from the permeate stream could be achieved via ion exchange treatment (Restolho et al., 2009). Bansail and Wiley (1974) reported 80-96% rejection of lignosulphonates using ultrafiltration with cellulose acetate membrane modules and softwood calcium-based SSL.

Table 6.4 Composition of total sugars, lignosulphonates and OH phenolics in initial, permeate and retentate streams produced via filtration using membranes with MWCO of 10, 5, 3 and 0.5 kDa

Membrane MWCO (kDa)	SSL streams	Volume (mL)	Total sugars (g/L)	Lignosulphonates (g/L)	OH phenolics (g/L)
10	Initial	400	18.1	39.8	1.3
	Permeate	324	18.4	24.7	1.3
	Retentate	76	18	104.3	1.3
5	Initial	400	18.8	35.6	1.3
	Permeate	317	18.9	13.6	1.3
	Retentate	83	18.1	119.2	1.3
3	Initial	400	18.6	37.7	1.3
	Permeate	329	18.7	12.1	1.2
	Retentate	71	18.4	157.1	1.7
0.5	Initial [1]	32	27	60	1.9
	Permeate [1]	20.95	27.2	4.1	1.4
	Retentate [1]	11.05	26.7	166.1	2.8

[1] Volume in L

In this study, sequential filtration was also carried out initially through the membrane of 10 kDa MWCO and subsequently through either 5 kDa or 3 kDa that resulted in 20 % retention of total sugars and 76 % retention of LS in the retentate stream. The permeate from sequential filtration through two membranes was not used as carbon source for succinic acid production as the separation of the LS was not improved. Fernandez-Rodriguez et al. (2015) used various filtration membrane systems for the separation of lignosulphonates and sugars from thin SSL produced from the same industrial plant as the SSL used in this study. Sequential filtration of SSL using 5 kDa and 1 kDa ceramic membranes led to the rejection of 72.8% LS and 26.5% of sugars, whereas sequential filtration of SSL using 15 kDa and 5 kDa ceramic membranes led to the rejection of 61.7% LS and 10.6% of sugars.

Pretreatment of 7 times diluted SSL via nanofiltration using the VSEP filter and 500 Da MWCO membrane led to almost complete retention of the LS in the retentate stream. The concentration of sugars in the permeate stream was 27.2 g/L, while the sugar content retained in the retentate fraction was around 34% of the initial sugars in the 7 times diluted SSL. The maximum volumetric recovery achieved via nanofiltration was 65.5 %. The total permeate (20.95 L) obtained contained 4.1 g/L LS. The retention of LS was higher than 95% in the retentate stream demonstrating that significant separation of LS could be achieved with this filtration approach.

The combination of ultrafiltration, nanofiltration and reverse osmosis could potentially lead to maximum lignosulphonate separation in the retentate stream and in the same time concentration of sugars in the permeate stream. However, Restolho et al. (2009) reported that many nanofiltration and reverse osmosis membranes demonstrated higher than 95% retention for the total sugars contained in the thin SSL. Ultrafiltration has been developed by Borregaard Industries (Norway) for the separation of lignosulphonates with purity around 95% and low sugar and salt content. The lignosulphonates derived via ultrafiltration could be employed for the extraction of high value products, such as vanillin (Lora, 2008).

6.5 Batch fermentations with ultrafiltrated SSL in Duran bottles

Batch fermentations were carried out in Duran bottles using the initial SSL as well as all permeate streams produced by ultrafiltration in order to evaluate their efficiency regarding succinic acid production. The initial total sugar concentration in these fermentations was around 18 g/L, while the initial LS concentration varied depending on the feedstock used (see Table 6.4). Table 6.5 shows that the final succinic acid concentration (6.15 – 6.5 g/L) was

similar when 10 times diluted SSL and permeates from ultrafiltration experiments were used as fermentation media by *A. succinogenes*. The highest succinic acid concentration (8.6 g/L) was produced when commercial mixed sugars were used. The highest succinic acid conversion yield (0.53 g per g consumed sugars) and productivity (0.57 g/L/h) were observed in the case that commercial mixed sugars were used indicating that both the untreated SSL and the permeates produced via ultrafiltration pose inhibitory effects to bacterial growth and product formation. In all fermentations carried out with *A. succinogenes* a residual sugar concentration of around 2 g/L was left in the medium because of its inability to catabolise galactose. SSL-based media led to higher by-product formation than in the case that commercial mixed sugars were used.

The cultivation of *B. succiniciproducens* on commercial mixed sugars (Table 6.5) led to the highest final succinic acid concentration (11.5 g/L), yield (0.63 g per g consumed sugars) and productivity (0.64 g/L/h). The cultivation of *B. succiniciproducens* on untreated 10 times diluted SSL led to the lowest final succinic acid concentration (7.6 g/L), yield (0.48 g/g) and productivity (0.5 g/L/h). All permeates resulted in similar fermentation efficiency by *B. succiniciproducens*. The highest by-product formation was observed when untreated SSL was used followed by all permeates.

The by-product formation in the fermentations carried out with SSL-based media was higher than the respective by-products produced in the fermentations carried out with commercial mixed sugars. The total by-product to succinic acid ratio for *A. succinogenes* and *B. succiniciproducens* was 0.79 g/g and 0.65 g/g in the fermentations where commercial mixed sugars were used, respectively. However, in the fermentations carried out with SSL-based media, the total by-product to succinic acid ratio was increased within the range of 1.22 - 1.35 g/g for *A. succinogenes* and 0.91 - 1.04 g/g for *B. succiniciproducens*.

In the case that untreated SSL was used, the ratio of total by-products to succinic acid was increased significantly in both cultures. Similar ratios of total by-products to succinic acid were observed by both strains when untreated and ultrafiltrated SSL were used. Succinic acid production from phosphoenolpyruvate requires 2 moles of NADH and leads to the production of 2/3 ATP. In the C3 metabolic pathway, the enzyme pyruvate formate lyase catalyses the conversion of pyruvate into formic acid and acetyl-CoA that is used as precursor for the production of structural compounds of the cells. Pyruvic acid could be also converted via pyruvate dehydrogenase into acetyl-CoA with generation of 1 mole NADH. The acetyl-CoA is converted via a two-step reaction into acetic acid with the production of 1 mole ATP.

Table 6.5 Results from batch cultures in Duran bottles using commercial mixed sugars, untreated SSL and pretreated SSL via ultrafiltration

Microorganism	Fermentation feedstock	Succinic acid (g/L)	Succinic acid yield (g per g consumed sugars)	Productivity (g/L/h)	LA/SA (g/g) [1]	FA/SA (g/g) [1]	AA/SA (g/g) [1]	Tot/SA (g/g) [1]
Actinobacillus succinogenes	Commercial mixed sugars	8.6	0.53	0.57	-	0.38	0.4	0.79
	Untreated (10 times diluted) SSL	6.15	0.38	0.34	-	0.66	0.56	1.22
	Ultrafiltrated SSL with 10 kDa membrane	6.3	0.40	0.42	-	0.60	0.74	1.35
	Ultrafiltrated SSL with 5 kDa membrane	6.5	0.41	0.43	-	0.52	0.68	1.21
	Ultrafiltrated SSL with 3 kDa membrane	6.5	0.41	0.43	-	0.53	0.63	1.16
Basfia succiniciproducens	Commercial mixed sugars	11.5	0.63	0.64	0.04	0.25	0.36	0.65
	Untreated (10 times diluted) SSL	7.6	0.48	0.50	-	0.41	0.62	1.03
	Ultrafiltrated SSL with 10 kDa membrane	7.9	0.50	0.53	-	0.37	0.60	0.97
	Ultrafiltrated SSL with 5 kDa membrane	7.8	0.49	0.52	-	0.42	0.62	1.04
	Ultrafiltrated SSL with 3 kDa membrane	8.2	0.52	0.55	-	0.36	0.54	0.91

[1] LA: lactic acid; SA: succinic acid; AA: acetic acid; FA: formic acid; Tot:SA: Total by-product to succinic acid production

When pyruvic acid is converted into lactic acid via lactate dehydrogenase then 1 mole of NADH is reduced. Therefore, increased acetic acid production is associated with increased ATP pool, whereas increased formic acid production is associated with increased acetyl-CoA production, the precursor of structural components. The results indicate that the inhibitors present in SSL-based media stimulate the production of acetic and formic acids in favour of succinic acid. It should be also noted that although lactic acid was produced by *B. succiniciproducens* in the fermentation carried out with commercial mixed sugars, in SSL-based media lactic acid was not produced.

6.6 Bioreactor fed-batch fermentations

Fed-batch bioreactor cultures for succinic acid production were carried out using both bacterial strains cultivated initially on commercial mixed sugars, untreated 7 times diluted SSL, ultrafiltrated SSL using 3 kDa MWCO membrane and nanofiltrated SSL treated with membrane of 500 Da MWCO (Table 6.4). In the fermentation that was carried out with commercial mixed sugars, the feeding solution used contained 400 g/L of commercial mixed sugars using the sugar ratio measured in SSL. The untreated SSL was used as feeding medium in the fermentation carried out with 7 times diluted SSL. In the other two fermentations, simulated feeding solutions were prepared containing 400 g/L of commercial mixed sugars supplemented with the appropriate lignosulphonate content depending on the permeate used at the beginning of fermentation. The feeding solution was added continuously, so as to maintain the total sugar concentration in the range of 5 - 20 g/L, with the flow rate adjusted manually according to the sugar consumption rate in each fermentation.

Figure 6.22 presents the fed-batch fermentations carried out by both strains using commercial mixed sugars. Galactose was not consumed only by *A. succinogenes* (Figure 6.22A), despite the fact that open reading frames encoding enzymes for galactose consumption have been identified (McKinlay et al., 2010). Salvachua et al. (2016) reported low galactose consumption rate (0.02 g/L/h) and a final conversion of around 60% when *A. succinogenes* was cultivated in hydrolysates containing mainly xylose as well as other C5 and C6 sugars. Succinic acid was produced during the growth, stationary and death phases for both strains (Figures 6.22C and 6.22D). The stationary phase begins when the total acid concentration reaches 35 g/L for *A. succinogenes* and 31.5 g/L for *B. succiniciproducens*. Growth inhibition occurs at 33 g/L and 35 g/L of total mixed organic acids for *A. succinogenes* for *B. succiniciproducens*, respectively (Chapter 5). During the death phase, succinic acid is still being produced but by-product formation stops (Figures 6.22C and

6.22D). Neither catabolite repression nor diauxic growth was observed in the fermentations carried out by both strains.

The strain *A. succinogenes* (Figures 6.22A and 6.22C) consumed all sugars except for galactose and resulted in the production of 26.9 g/L of succinic acid, a conversion yield of 0.55 g per g consumed sugars and a productivity of 0.58 g/L/h (Table 6.6). *B. succiniciproducens* resulted in the production of 34.2 g/L succinic acid (Figures 6.22D) with a conversion yield of 0.65 g/g and a productivity of 0.59 g/L/h (Table 6.6). *B. succiniciproducens* is able to produce succinic acid from both the reductive and the oxidative branch of the TCA cycle (Becker et al., 2013) contrary to *A. succinogenes*, where only the reductive pathway leads to succinic acid production.

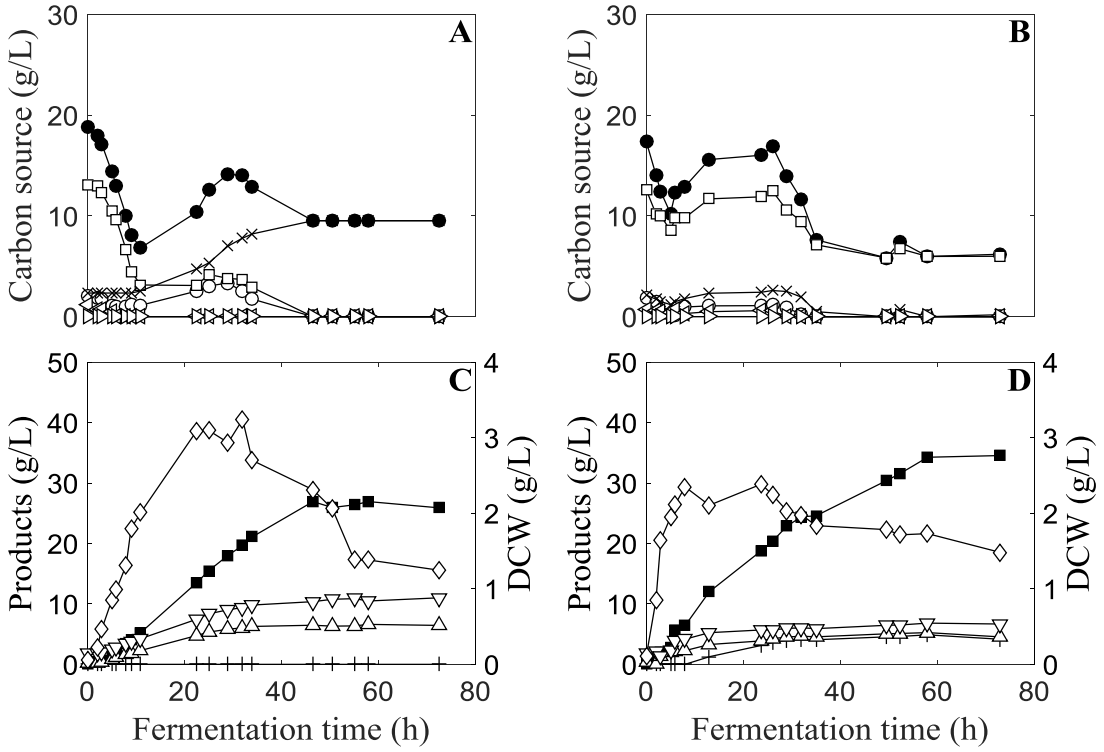

Figure 6.22 Consumption of C5 and C6 sugars, production of metabolic products and change of dry cell weight (DCW) during fed-batch bioreactor fermentations of *A. succinogenes* (A, C) and *B. succiniciproducens* (B, D) cultivated on mixed commercial sugars using the same sugar ratio as the one measured in SSL. Total sugars (●), xylose (□), glucose (○), galactose (×), arabinose (◁), mannose (▷), dry cell weight (◊), succinic acid (■),acetic acid (▽), formic acid (△), and lactic acid (+)

Table 6.6 Fed-batch bioreactor cultures using mixed commercial sugars, untreated SSL and pretreated SSL via ultrafiltration and nanofiltration

Microorganism	Fermentation feedstock	Succinic acid (g/L)	Succinic acid yield (g per g consumed sugars)[1]	Productivity (g/L/h)	LA/SA (g/g)[1,2]	FA/SA (g/g)[1,2]	AA/SA (g/g)[1,2]	Tot/SA (g/g)[1,2]
Actinobacillus succinogenes	Mixed sugars	26.9	0.55	0.58	-	0.23 (0.60)	0.33 (0.66)	0.57 (1.26)
	Untreated (7 times diluted) SSL	19.3	0.66	0.27	-	0.19 (0.48)	0.42 (0.82)	0.61 (1.3)
	Ultrafiltrated SSL with 3 kDa membrane	27.4	0.52	0.39	-	0.15 (0.38)	0.26 (0.51)	0.41 (0.89)
	Nanofiltrated SSL with 500 Da membrane	25.2	0.57	0.47	-	0.30 (0.77)	0.42 (0.83)	0.72 (1.60)
Basfia succiniciproducens	Mixed sugars	34.2	0.65	0.59	0.14 (0.19)	0.15 (0.37)	0.16 (0.31)	0.45 (0.87)
	Untreated (7 times diluted) SSL	8.6	0.45	0.11	0.63 (0.82)	0.33 (0.85)	0.34 (0.67)	1.30 (2.35)
	Ultrafiltrated SSL with 3 kDa membrane	27.8	0.63	0.41	0.14 (0.18)	0.08 (0.20)	0.21 (0.42)	0.43 (0.80)
	Nanofiltrated SSL with 500 Da membrane	33.8	0.58	0.48	0.35 (0.46)	0.12 (0.31)	0.26 (0.52)	0.74 (1.29)

[1] All metabolic product yields were calculated based on the quantities (g) of the organic acids produced during fermentation and removed during sampling and the quantities of individual sugars (g) consumed during fermentation, added via feeding and removed via sampling. The volumes considered included the volume of the fermentation broth, the volume of samples removed, the volume of feeding solution added and the volume of NaOH added.

[2] The ratio of mol/mol is presented in the parenthesis. LA: lactic acid; SA: succinic acid; AA: acetic acid; FA: formic acid; Tot:SA: Total by-product to succinic acid production

Table 6.7 Bio-based succinic acid production using lignocellulosic hydrolysates with varying sugar composition

Raw material	Pretreatment method	Major medium components (g/L)	Microorganism	Succinic acid (g/L)	Yield (g/g)	Productivity (g/L/h)	SA:FA:AA (mol basis)	Ref
Corn fiber	Dilute H_2SO_4 treatment followed by enzymatic hydrolysis with cellulase and β-glucosidase	Glucose (61.1), xylose (8.2), arabinose (9.6), yeast extract (10), biotin (10 μg/L)	*A. succinogenes* FZ6 (mutant)	70.6	0.88	0.7	1:0.01:0.08	Guettler et al., 1996
Corncob	Dilute H_2SO_4 treatment	Total sugars (50.6), xylose (38.1), yeast extract (11)	*A. succinogenes* CICC 11014	23.6	0.58	0.49	nk	Yu et al., 2010
Corn stover	Dilute alkaline treatment followed by enzymatic hydrolysis with cellulase and cellobiase	Simultaneous saccharification and fermentation with 70 g/L pretreated and dried corn stover, corn steep liquor (20)	*A. succinogenes* CGMCC 1593	47.4	0.72 [1]	0.99	1:0.06:0.44	Zheng et al., 2010
Corn straw	Dilute alkali treatment followed by enzymatic hydrolysis	Initial medium for batch culture: yeast extract (15), total sugars (40) with glucose (22.4) and xylose (13.7). Feeding medium with 200 g/L total sugars	*A. succinogenes* CGMCC1593	53.2	0.82	1.21	1:0:0.22	Zheng et al., 2009
Cotton stalks	Steam explosion followed by treatment with NaOH and H_2O_2 and enzymatic hydrolysis by cellulose and β-glucosidase	Simultaneous saccharification and fermentation with hydrolysate containing 65% glucose and 10% xylose, yeast extract (30)	*A. succinogenes* 130Z	63	0.64	1.17	nk	Li et al., 2013
Sugarcane bagasse	Treatment with ultrasonication followed by dilute H_2SO_4 treatment and detoxification with activated charcoal	Xylose (22.4), glucose (3.6), arabinose (3.9), yeast extract (4), corn steep liquor (5)	*A. succinogenes* NJ113	23.7	0.79	0.99	1:0:0.37	Xi et al., 2013
Sugarcane bagasse	Dilute H_2SO_4 treatment	Hemicellulose hydrolysate with xylose (52), yeast extract (2)	*A. succinogenes* CIP 106512	22.5	0.43	1.01	nk	Borges and Pereira, 2011
Corn stover	Mild alkaline wash (deacetylation) followed by dilute acid treatment	ca. xylose (58), glucose (8.7), arabinose (8.7), galactose (4.6), yeast extract (6), corn steep liquor (10)	*A. succinogenes* 130Z	42.8	0.74	0.30	1:0:0.47	Salvachua et al., 2016

[1] g succinic acid per g pretreated and dried corn stover (the main sugar was glucose)

In the fed-batch fermentations carried out by *B. succiniciproducens* with mixed commercial sugars, the production of 1 mole of succinic acid was combined with the production of 0.19 moles of lactic acid, 0.37 moles of formic acid and 0.31 moles of acetic acid. When *A. succinogenes* was used, the production of 1 mole of succinic acid was combined with the production of 0.6 moles of formic acid and 0.66 moles of acetic acid. The succinic acid to by-product ratio SA:FA:AA (mol/mol) observed for *A. succinogenes* (1:0.6:0.66) is higher than the ratios reported in literature-cited publications using xylose-based media (Table 6.7).

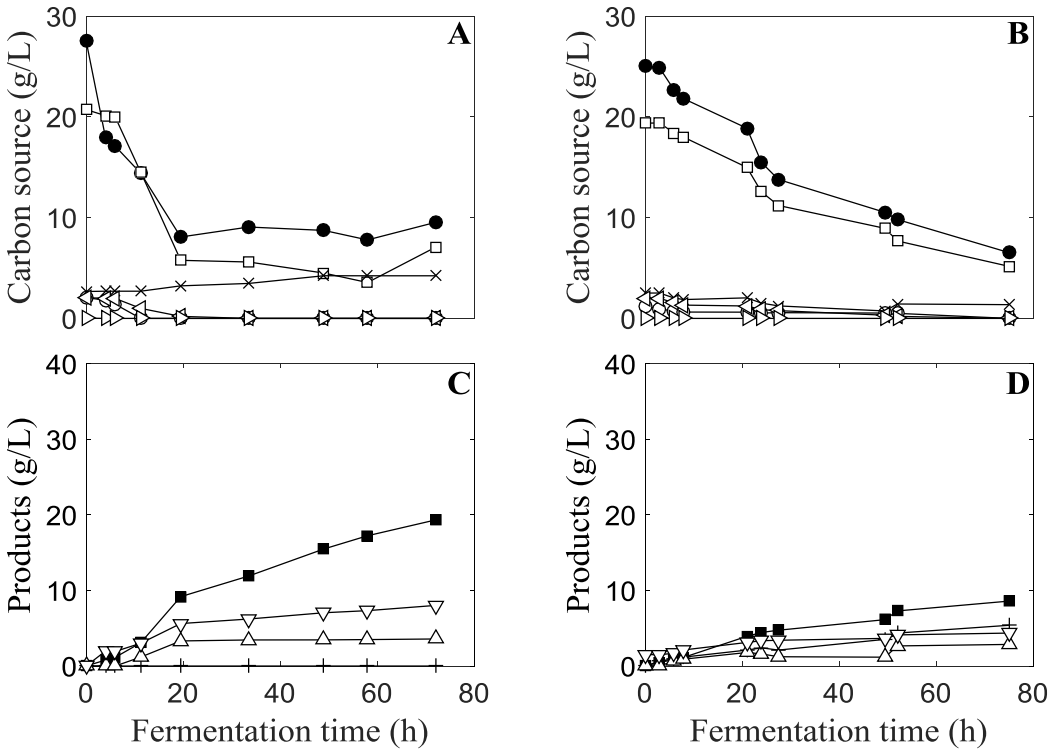

Figure 6.23 Consumption of C5 and C6 sugars and production of metabolic products during fed-batch bioreactor fermentations of *A. succinogenes* (A, C) and *B. succiniciproducens* (B, D) cultivated on untreated, seven times diluted, SSL. Total sugars (●), xylose (□), glucose (○), galactose (×), arabinose (◁), mannose (▷), dry cell weight (◊), succinic acid (■), acetic acid (▽), formic acid (△), and lactic acid (+)

Fed-batch fermentations were subsequently carried out using untreated, seven times diluted, SSL in order to evaluate the ability of both strains to grow and produce succinic acid in the untreated stream. The cultivation of *A. succinogenes* (Figures 6.23A and 6.23C) on untreated SSL led to the production of 19.3 g/L of succinic acid with a conversion yield of 0.66 g/g (Table 6.6). The remaining 11.3 g/L of total sugars at the end of fermentation corresponded to a higher sugar content than just galactose that is not consumed by *A.*

succinogenes. This means that the efficiency of *A. succinogenes* to grow and produce succinic acid is significantly impaired when cultivated in the crude SSL. The productivity (0.27 g/L/h) was significantly lower compared to the fermentation carried out with commercial mixed sugars. *B. succiniciproducens* was less tolerant than *A. succinogenes* when cultivated on untreated SSL (Figures 6.23B and 6.23D) as only 8.6 g/L of succinic acid concentration (0.45 g/g conversion yield) was produced (Table 6.6). The production of succinic acid was very slow and the concentration of total sugars dropped below 10 g/L after 50 h.

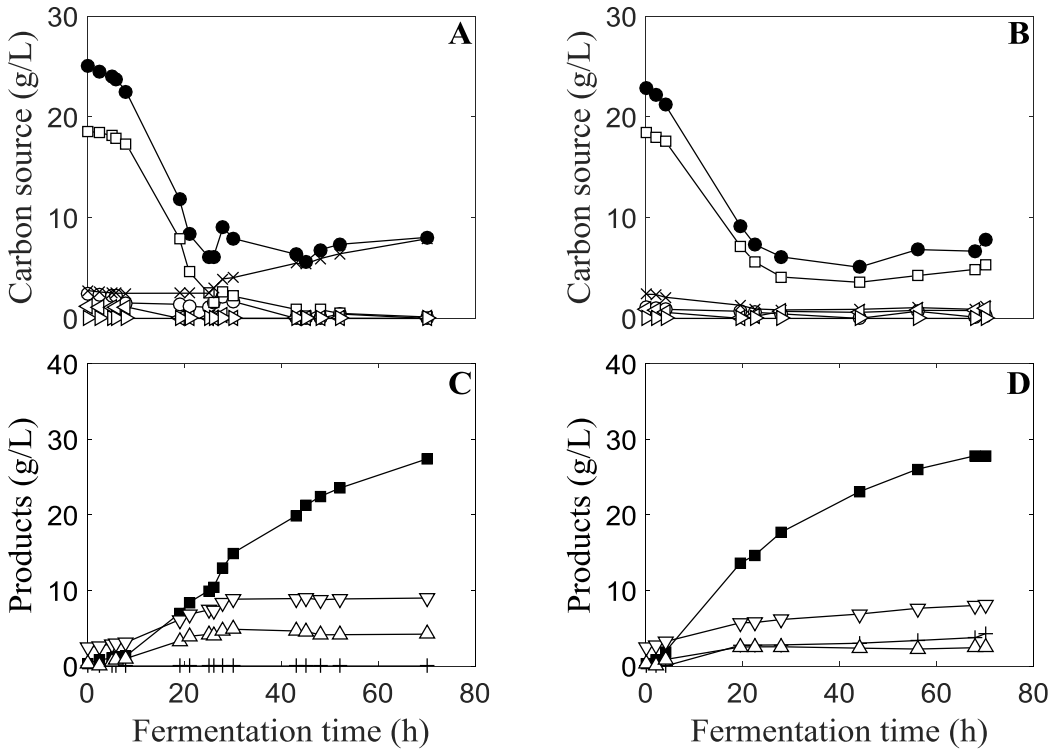

Figure 6.24 Consumption of C5 and C6 sugars and production of metabolic products during fed-batch bioreactor fermentations of *A. succinogenes* (A, C) and *B. succiniciproducens* (B, D) cultivated on SSL pretreated via ultrafiltration using a membrane with 3 kDa MWCO. Total sugars (●), xylose (□), glucose (○), galactose (×), arabinose (◁), mannose (▷), dry cell weight (◊), succinic acid (■),acetic acid (▽), formic acid (△), and lactic acid (+)

Figures 6.24 and 6.25 present the production of metabolic products and the consumption of sugars from fed-batch fermentations carried out with both strains using ultrafiltrated and nanofiltrated SSL, respectively. A membrane with 3 kDa MWCO was used for ultrafiltration. As shown in Table 6.6, both pretreatment methods led to similar succinic acid concentration (27.4 g/L and 25.2 g/L) when *A. succinogenes* was used. The total consumed sugar to succinic acid conversion yield is higher in the case of untreated SSL (0.66

g/g) and is reduced to 0.52 g/g and 0.57 g/g when ultrafiltrated and nanofiltrated SSL are used, respectively. As expected, the productivity increases gradually with increased level of SSL treatment to 0.47 g/L/h in the case of *A. succinogenes*. The productivity achieved with nanofiltrated SSL is still lower than the productivity achieved with commercial mixed sugars (0.58 g/L/h) when the strain *A. succinogenes* was used. This may have occurred because the permeate produced via nanofiltration of SSL still contains various inhibitors such as phenolic compounds.

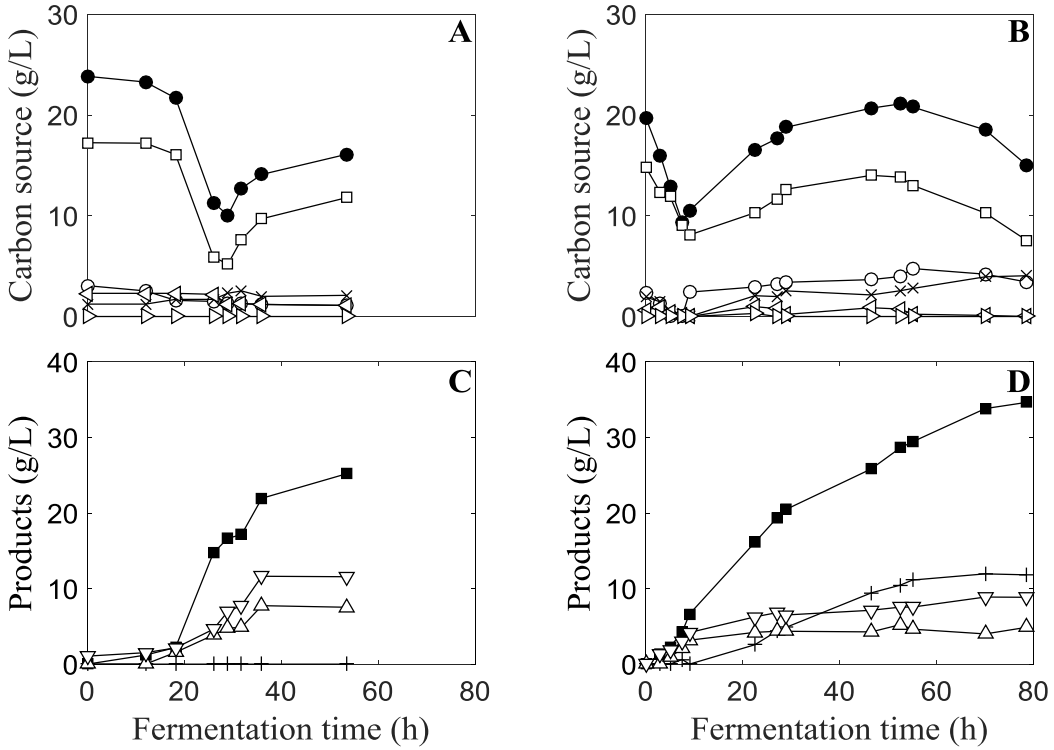

Figure 6.25 Consumption of C5 and C6 sugars and production of metabolic products during fed-batch bioreactor fermentations of *A. succinogenes* (A, C) and *B. succiniciproducens* (B, D) cultivated on SSL pretreated via nanofiltration. Total sugars (●), xylose (□), glucose (○), galactose (×), arabinose (◁), mannose (▷), dry cell weight (◊), succinic acid (■),acetic acid (▽), formic acid (△), and lactic acid (+).

In the case of *B. succiniciproducens*, the use of nanofiltrated SSL led to higher final succinic acid concentration (33.8 g/L) (Figures 6.25C and 6.25D) than the use of ultrafiltrated SSL (27.8 g/L) (Figures 6.24C and 6.24D). The productivity achieved by *B. succinciproducens* increases gradually to 0.48 g/L/h with increased level of pretreatment. This value is however lower than the one achieved when commercial mixed sugars were used (0.59 g/L/h). This means that inhibitory compounds (e.g. lignosulphonates, phenolic compounds) were still present in nanofiltrated SSL leading to reduced productivity by *B.*

succiniciproducens. Feeding of SSL-based media leads to the accumulation of inhibitory compounds during fermentation that result in lower final productivity. Alexandri et al. (2016) has shown that the removal of low molecular weight phenolic compounds led to improved fermentation efficiency by both strains.

The highest total by-product to succinic acid ratio was observed in the fermentations carried out with untreated SSL for *B. succiniciproducens* and with nanofiltrated SSL for *A. succinogenes* (Table 6.6). It is worth noting that the fermentations carried out with ultrafiltrated SSL resulted in the lowest total by-product to succinic acid ratio for both strains in all fermentations. Surprisingly, the total by-product to succinic acid ratio was increased significantly in fermentations carried out with nanofiltrated SSL as compared to the ratio observed with ultrafiltrated SSL. Lactic acid production was observed only in the case of *B. succiniciproducens* (Figure 6.22) as *A. succinogenes* cannot produce lactic acid.

Table 6.7 presents the fermentation efficiency reported by different literature-cited publications on succinic acid production achieved when *A. succinogenes* was cultivated in various lignocellulose hydrolysates. In general, hydrolysates that contain higher glucose content result in higher final succinic acid concentration and yield than hydrolysates rich in xylose. However, Salvachua et al. (2016) reported high succinic acid concentration (42.8 g/L) and yield (0.74 g/g) from a xylose-rich hydrolysate. The highest succinic acid production (70.6 g/L) with the highest conversion yield (0.88 g/g) was observed in corn fiber hydrolysates by mutant *A. succinogenes* FZ6 (Guettler et al., 1996). The by-products reported by Guettler et al. (1996) were lower than the other fermentations carried out in lignocellulosic hydrolysates. The highest productivity (1.21 g/L/h) was achieved by *A. succinogenes* CGMCC1593 when cultivated in glucose-based corn straw hydrolysates (Zheng et al., 2009).

In the case that nanofiltrated SSL is used (Table 6.6), the strain *B. succiniciproducens* results in higher succinic acid concentration with similar yield and productivity when compared to *A. succinogenes*. Furthermore, ultrafiltration leads to significant losses of LS, while nanofiltration results in the recovery of more than 95% of LS in the retentate stream. However, the sugar losses are 34% via nanofiltration and only 17.6% when ultrafiltration with 3 kDa MWCO membrane is used. The results obtained via fed-batch fermentation indicate that lower molecular weight compounds pose an inhibitory effect on succinic acid production. The separation of phenolic compounds could lead to the production of a value-added co-product and the improvement of succinic acid production via fermentation.

Chapter 7

Succinic acid production from pulp and paper industry waste - A transcriptomic approach

7.1 Introduction

The utilisation of renewable resources for the production of bio-based products is essential in order to develop sustainable bioprocesses and biorefineries. Xylose-rich hydrolysates produced from hemicellulose contained in lignocellulosic resources could be used for the production of succinic acid, one of the most important platform chemicals in the bio-economy era. Exploitation of pretreated SSL for succinic acid production by *A. succinogenes* was efficiently verified in Chapter 6. In this chapter, the metabolic potential of this strain is evaluated through RNA expression of the metabolic pathways involved in succinic acid production utilising glucose, xylose or SSL as carbon sources. A transcriptomic approach of the key enzymes of glucose and xylose catabolism, carboxylic acid production as well as oxidative phosphorylation led to a deep understanding on the energy consuming metabolic pathways. The transcriptomic analysis was carried out in batch cultures. cDNA libraries were synthesised at different time points of the fermentation, where major metabolic changes in extracellular metabolites or biomass production were observed. RT-qPCR was used to determine the expression levels of the genes of interest throughout fermentation. The bottlenecks of the fermentative production of succinic acid by *A. succinogenes* were addressed with particular focus on the effect of the inhibitory SSL on pathways that involve ATP consumption and NADH oxidation.

7.2. Succinic acid production via repeated fermentations

As reported in Chapter 6, the SSL contains various monosaccharides. The main sugar of SSL is xylose (72% of the total sugars) with glucose being also present at lower content (10% of total sugars). Therefore, xylose and glucose were selected as the control cases to compare with SSL as pure and crude carbon sources, respectively. Catabolic processes include glycolysis and pentose phosphate pathway (PPP) and involve the degradation of carbon source to produce phosphoenolpyruvate (PEP), the most important intermediate metabolite between catabolism and anabolism. *A. succinogenes* lucks glyoxylate and Entner-Doudoroff pathway fluxes (McKinlay et al., 2010). Two housekeeping genes (Christensen et al., 2004; Nielsen et al. 2005) were used for the quantification of the relative gene expression levels using RT-PCR, recF (encoding recombinase F) and rpoB (encoding β-subunit of RNA polymerase). Primers were designed using the Primer Express Software.

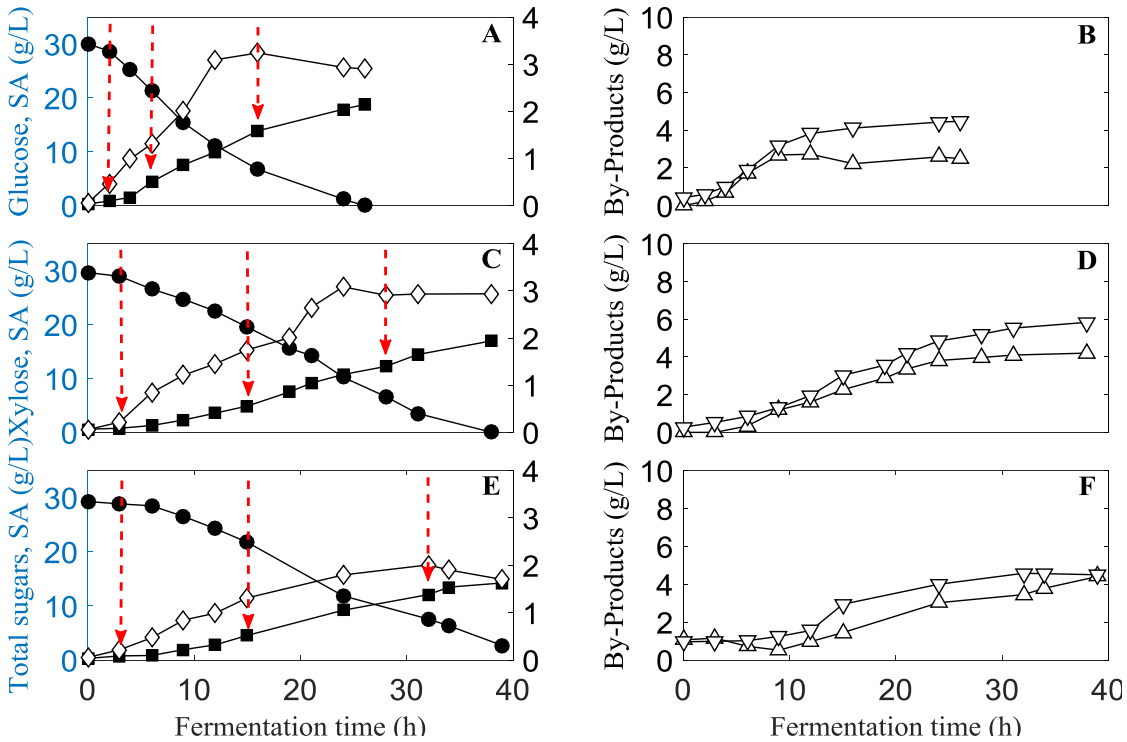

Figure 7.1. Metabolic profile and sampling during the fermentations carried out with glucose (A and B), xylose (C and D), and SSL (E and F) using *A. succinogenes* in bench top bioreactor. Carbon source (●), dry cell weight (◊), succinic acid (□), acetic acid (▽) and formic acid (△).

Figure 7.1 presents the profiles of sugar consumption, metabolic product synthesis and dry cell weight during three fermentations carried out on glucose, xylose and ultrafiltrated SSL in order to collect the samples required for transcriptome analysis. The sampling times are shown in Figure 7.1 with orange arrows. Initial sugar concentration was around 30 g/L of glucose, xylose or total sugars in the case of SSL. SSL was treated via ultrafiltration in order to remove the majority of the inhibitory compounds (mainly LS). The fermentation medium contained also 5 g/L yeast extract and minerals (per L): $NaH_2PO_4 \cdot H_2O$, 1.16 g; Na_2HPO_4, 0.31 g; NaCl, 1 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.2 g. $MgCO_3$ was not added in the medium to avoid interference during RNA extraction. The main criterion used for selecting the sampling times in the fermentations carried out with the three different substrates was the concentration of extracellular metabolic products. The samples were taken during the lag, exponential and stationery phases of bacterial growth. In the sample taken during lag phase, the succinic acid concentration in the fermentation broths was 0.88 g/L in glucose, 0.77 g/L in xylose and 0.78 g/L in SSL based cultures. The respective formic acid concentration was 0.23

g/L, 0.2 g/L and 1.16 g/L in glucose, xylose and SSL, respectively. The respective acetic acid concentration was 0.60 g/L, 0.53 g/L and 1 g/L in glucose, xylose and SSL, respectively.

In the sample taken during the exponential growth phase, the extracellular concentration of succinic acid was 4.42 g/L in glucose, 4.89 g/L in xylose and 4.56 g/L in SSL based cultures. The formic and acetic acid concentrations in the sample taken during the exponential growth phase were 1.17 g/L and 1.87 g/L in the glucose based culture, 2.26 g/L and 3.02 g/L in the xylose based culture and 1.45 g/L and 2.95 g/L in the SSL based culture, respectively.

In the sample taken during the stationary phase, the succinic acid concentration was 13.9 g/L in glucose, 12.23 g/L in xylose and 12.09 g/L in SSL based cultures. The formic acid acetic acid concentrations were 2.23 g/L and 4.12 g/L in glucose based cultures, 3.97 g/L and 5.2 g/L in xylose based cultures and 3.46 g/L and 4.58 g/L in SSL based cultures, respectively.

Table 7.1 Results from batch cultures in bench top bioreactors using glucose, xylose and pretreated SSL via ultrafiltration

Fermentation feedstock	Succinic acid (g/L)	Yield (g per g consumed sugars)	Productivity (g/L/h)	DCW (g/L)	FA/SA (mol/mol)	AA/SA (mol/mol)
Glucose	18.8	0.61	0.72	3.2	0.341	0.429
Xylose	17.0	0.55	0.45	3.1	0.634	0.648
Ultrafiltrated SSL	14.2	0.52	0.30	2.0	0.614	0.817

In the case of glucose, the final succinic acid concentration was 18.8 g/L with a yield of 0.61 g/g and a productivity of 0.72 g/L/h (Figure 7.1A). Formic and acetic acids were also produced with final concentrations of 2.5 and 4.1 g/L respectively, corresponding to 0.341 mol/mol FA/SA ratio and 0.429 mol/mol AA/SA ratio (Table 7.1B). The final concentration of succinic acid (17 g/L) in xylose was slightly lower than glucose with decreased yield (0.55 g/g) and productivity (0.45 g/L/h), respectively (Figure 7.1C). In the case of SSL, the consumption of sugars occurred simultaneously and catabolite repression was not observed. The use of ultrafiltrated SSL resulted in lower fermentation efficiency than glucose and xylose based cultures. The final succinic acid concentration and yield were 14.2 g/L and 0.52 g/g (Table 7.1E). Productivity was significantly decreased to 0.30 g/L/h. By-product formation was slightly higher in xylose than SSL based cultures (final formic acid concentration was 4.2 g/L and 3.4 g/L, respectively, while the final acetic acid concentration

was 5.6 g/L and 5.9 g/L, respectively) resulting in higher by-product to succinic acid molar ratios. In xylose, the molar ratios of FA/SA and AA/SA were 0.634 mol/mol and 0.648 mol/mol, respectively, while in SSL the respective molar ratios were 0.614 mol/mol and 0.817 mol/mol. Table 5.1 presents the fermentation results obtained in the fermentations presented in Figure 7.1.

7.3. Carbon source catabolism

Three microbial growth stages (lag, exponential and stationary phase) were selected for transcriptome analysis. The logarithmic fold change (log2) at the expression levels in SSL compared to the two control cases (glucose and xylose) are shown in Table 7.2. Gene overexpression related with catabolism of carbon sources at all growth phases in SSL compared to glucose were mainly observed in pathways that are involved in xylose transportation and the first steps of xylose degradation.

Table 7.2 Fold-change (log2) of relative expression levels of *A. succinogenes* genes involved in glycolysis and PPP in fermentations with SSL compared to glucose and xylose

Genes	EC Number	Lag phase			Exponential phase			Stationary phase		
		S/G	S/X	X/G	S/G	S/X	X/G	S/G	S/X	X/G
PTS Glucose transporter	2.7.1.199	0.78	-0.98	1.76	0.81	-0.35	1.17	-0.78	-1.50	0.72
PTS Mannose transporter	4.A.6.1.1	-4.94	-1.61	-3.33	2.37	1.89	0.48	2.93	2.97	-0.05
ABC transporter		1.92	3.62	-1.70	2.38	3.09	-0.71	1.00	5.06	-4.06
Low aff Xylose symporter	1.3.1.74	6.52	1.07	5.45	6.22	2.44	3.78	5.07	3.65	1.42
Xylose transporter (HGF)	3.A.1.2.4	4.61	-1.02	5.63	4.86	-0.71	5.57	3.02	0.07	2.95
Galactokinase	2.7.1.6	3.25	1.77	1.48	0.68	0.85	-0.17	1.56	2.46	-0.90
1-P Fructokinase	2.7.1.56	-0.36	0.15	-0.51	1.31	0.68	0.63	1.90	1.15	0.75
Glucose 6-P isomerase	5.3.1.9	1.31	1.64	-0.33	1.13	1.49	-0.37	1.45	0.70	0.75
6-P fructokinase	2.7.1.11	-0.09	-0.96	0.87	1.32	1.22	0.10	0.42	0.28	0.14
Fructose bis P aldolase	4.1.2.13	1.03	0.12	0.90	1.16	1.52	-0.35	0.71	-0.37	1.08
Glyceraldehyde 3 P DH	1.2.1.12	-2.02	-1.65	-0.37	0.15	0.49	-0.34	-0.80	0.31	-1.12
Phosphoglycerate kinase	2.7.2.3	-2.40	-2.30	-0.11	-0.55	-0.06	-0.49	0.58	-0.10	0.68
Phosphogluconate DH	1.1.1.44 / 1.1.1.343	-2.36	-2.63	0.28	-1.67	-2.60	0.92	-1.34	-1.68	0.34
Ribulose P Epimerase	5.1.3.1	1.68	1.12	0.56	0.48	0.83	-0.35	0.05	0.52	-0.47
Transaldolase	2.2.1.2	3.10	2.09	1.01	-0.12	2.08	-2.20	-0.10	-0.08	-0.02
Xylulokinase	2.7.1.17	6.51	1.50	5.01	6.13	0.81	5.32	3.27	0.39	2.88

Principal component analysis of the relative expression levels, revealed (Figure 7.2) a separate group, indicated with brown color, when the *A. succinogenes* cells were grown in SSL. Glucose and xylose, on the other hand, formed two distinguished groups that overlapped

to some extent. The genes responsible for SSL group isolation mainly participate in the pentose phosphate pathway. The major differences compared to xylose were observed at the expression levels of the following enzymes: transaldolase, phosphogluconate dehydrogenase, ribokinase, and low affinity xylose transporter. Also the genes that regulate the function of glycolysis enzymes and participated in the group isolation were: glucose 6-phosphate isomerase and 6-phosphate fructokinase.

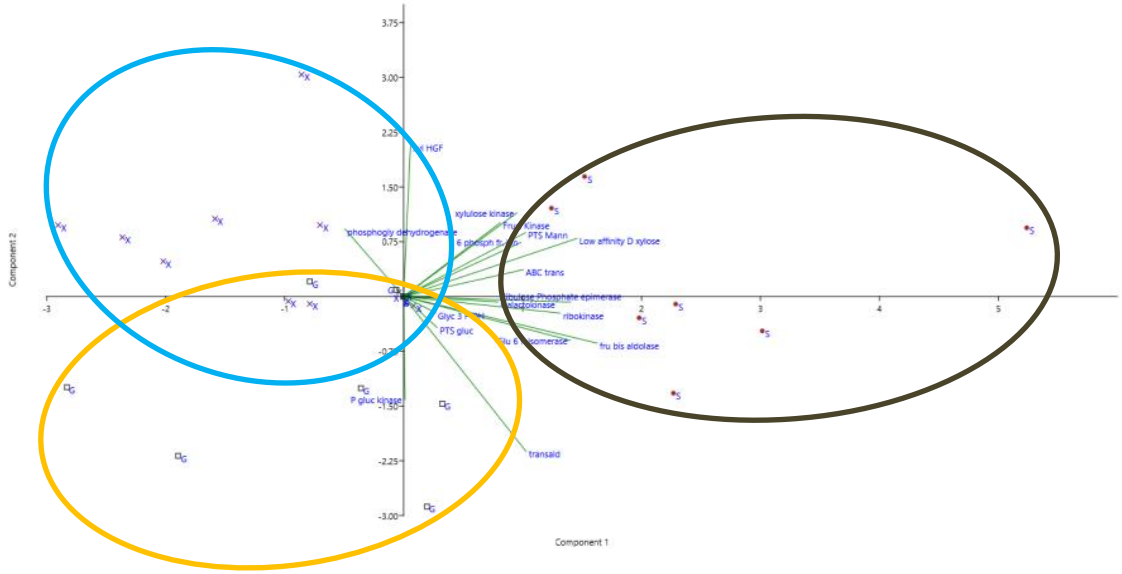

Figure 7.2. Principal component analysis of relative expression levels during exponential phase of genes involved in glycolysis and pentose phosphate pathways. Glucose (yellow circle), Xylose (blue circle), SSL (brown circle).

Starting from PTS glucose transporter (EC 2.7.1.199), its relative expression levels did not result in statistical significant differences between the substrates. The fold change between the substrates indicates that PTS glucose might not be glucose specific as it was upregulated in xylose compared to glucose in both lag and exponential phases. RNA expression of PTS Mannose (EC 4.A.6.1.1) was significantly upregulated during exponential and stationary phases in SSL compared to both glucose and xylose. The initial concentration of mannose in SSL based fermentations was around 1.5 g/L. Also the expression levels of ABC transporter were examined. ABC transporters in bacteria could act as importers and exporters and they are used to pump transport substrates against the concentration gradient using ATP as the driving force. In *A. succinogenes* the relative expression levels of ABC transporter in SSL were statistically significant compared to xylose. Three fold upregulation of ABC transporter occurred in SSL compared to xylose in both lag and exponential phases, leading to a 5-fold upregulation in the stationary phase. Also fold change of expression levels of ABC

transporters were above 2 in SSL compared to glucose, but were not statistically significant. RNA expression of putative low affinity D xylose proton symporter (EC 1.3.1.74) that was observed during lag ($1.19 < r > 1.41$), exponential ($4 < r > 10$) and stationary phase ($1.41 < r > 2$) in SSL was significantly higher compared to both xylose and glucose (Figure 7.3). Expression levels of low affinity D-xylose symporter were significantly higher when xylose was the carbon source ($0.84 < r > 1.19$, in lag and exponential phase and $0.5 < r > 0.71$ in stationary phase) compared to glucose ($r < 0.1$ in all growth phases). The fact that low affinity D xylose symporter is highly expressed in SSL compared to xylose leads to the conclusion that other monosaccharides except for xylose are imported into the cell by this transporter. On the other hand, high affinity ATP-dependent xylose transporter (HGF) (EC 3.A.1.2.4) is xylose specific and fold change between SSL and xylose was very low with no statistical significant difference. Compared to glucose, the relative expression levels of HGF were 5-fold higher in SSL and were statistically significant. Galactokinase (EC 2.7.1.6) is a galactose transporter in *A. succinogenes*. Although galactose is not consumed, galactokinase was active in all substrates. Specifically in SSL, fold change in lag phase and stationary phase was higher compared to both glucose and xylose. Probably 1-phosphofructokinase (EC 2.7.1.56) relative expression levels were higher in SSL compared to glucose and xylose.

Gene expression studies of the bacterium *Gluconobacter oxydans* NL71, that is able to produce D-xylonic acid from D-xylose, under the stress of the major lignocellulosic biomass inhibitors (formic acid, furfural, and 4-hydroxybenzaldehyde) has shown that the majority of the expressed genes of *Gluconobacter oxydans* NL71 encoding enzymes involved in transportation were upregulated in the presence of inhibitors (Miao et al., 2017).

Statistically significant difference in SSL compared to glucose and xylose during exponential growth was observed at relative expression levels of the genes, glucose 6-phosphate isomerase (EC 5.3.1.9), 6-phosphate fructokinase (EC 2.7.1.11) and fructose bisphosphate aldolase (EC 4.1.2.13). Fold change ranged between 1.1 - 1.8 during exponential phase. Allosteric regulation occurs in 6-phosphate fructokinase as it is suppressed when ATP levels are high. This indicates that available ATP during exponential phase when the cells grew in SSL might be lower that the synthetic substrates with commercial sugars. Glyceraldehyde 3 phosphate dehydrogenase (EC 1.2.1.12) was not significantly affected by the presence of inhibitors in SSL. Phosphoglycerate kinase (EC 2.7.2.3) was significantly downregulated in SSL during lag phase compared to both glucose and xylose (Figure 7.3).

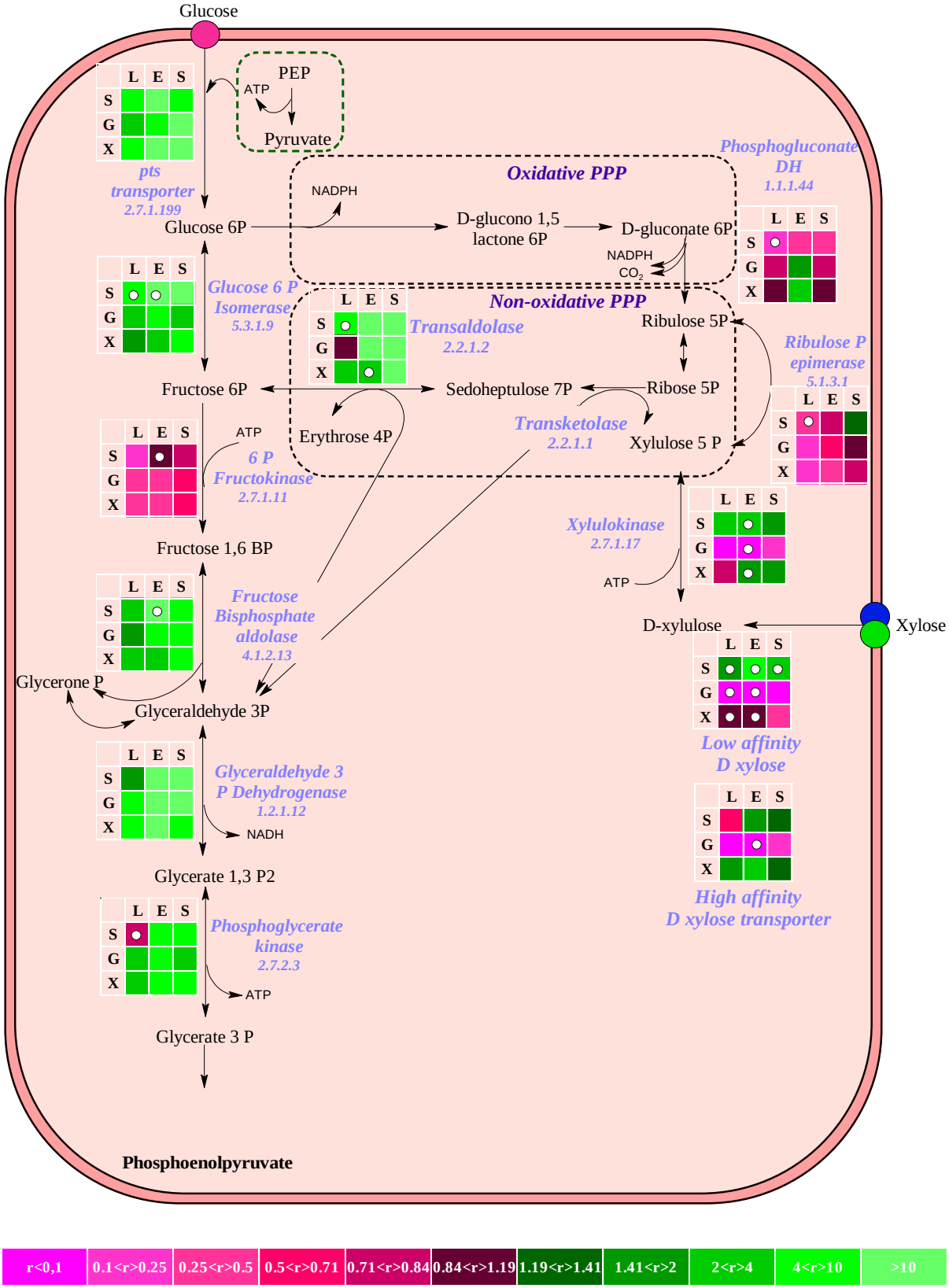

Figure 7.3. Heat map of transcriptional changes in *A. succinogenes* genes involved in glycolysis and pentose phosphate pathway metabolic pathways during fermentations in benchtop bioreactors in SSL (S), Glucose (G) and Xylose (X) during lag phase (L), exponential phase (E) and stationary phase (S).

The role of PPP to the cell's metabolism is very important. The PPP acts as an alternative mechanism to glycolysis, providing maintenance of carbon homoeostasis, depending on the cell requirements. The oxidative branch of pentose phosphate pathway is unidirectional and consists of three sequential enzymes that lead to the production of ribulose-5-phosphate, yielding two moles of NADPH per one mol of glucose-6-phosphate. The production of reducing molecules (NADPH) is crucial to maintain redox balance and defeat stress. The first enzyme that contributes to glucose oxidation is glucose-6-phosphate dehydrogenase, (EC 1.1.1.49/1.1.1.363), followed by 6-phosphoglucolactonase (EC 3.1.1.31) and finally phosphogluconate dehydrogenase (EC 1.1.1.44/1.1.1.343) leading to the production of ribulose-5-phosphate. Glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase are associated with NADPH production. Since oxidative PPP is unidirectional, the relative expression levels of phosphogluconate dehydrogenase (EC 1.1.1.44/1.1.1.343) were specified as an indication of the oxidative PPP regulation. Phosphogluconate dehydrogenase was significantly downregulated in SSL compared to xylose and glucose in both lag and exponential phases (Figure 7.3).

The non-oxidative branch provides to glycolysis intermediate metabolites from the oxidative branch and *vice versa*, contributing also to nucleotide and amino acid biosynthesis. The most important molecules that are produced through the non-oxidative PPP are ribose-5-phosphate, a precursor of DNA and RNA biosynthesis and other sugar phosphate precursors of amino acid biosynthesis (e.g. erythrose 4P, a precursor of histidine). Ribulose phosphate epimerase (EC 5.1.3.1) was significantly increased in SSL (0.83 fold relative expression levels). Transaldolase (EC 2.2.1.2), which is very important for the balance of metabolites between glycolysis and PPP, was upregulated in SSL compared to xylose in both lag and exponential phases and during lag phase compared to glucose. Differences in relative expression levels of xylulokinase (EC 2.7.1.17) were statistically significant in all substrates, during all growth phases. During exponential phase, a 6.13 fold increase was observed in SSL compared to glucose and a 0.81 fold increase was observed in SSL compared to xylose. Also in xylose a 5.32 fold increase was observed compared to glucose.

RNA expression levels of *Gluconobacter oxydans* NL71 for the production of D-xylonic acid have been investigated in the presence of lignocellulosic hydrolysate inhibitors. In the presence of inhibitors, upregulation of ribose-5-phosphate isomerase B and of the glucose-6-phosphate 1-dehydrogenase, involved in PPP, was observed (Miao et al., 2017). *Corynebacterium glutamicum* is a microorganism with high biotechnological interest that has been widely used for the production of succinic acid. Under the stress of succinic acid (0.0625

M), glucose consumption and succinic acid production is significantly decrased. Transcriptome analysis showed significant downregulation of a few genes that were involved in glycolysis, specifically whiB4, a gene that encodes a regulatory protein, was downregulated by the addition of succinic acid. Overexpression of whiB4 increased glucose consumption rate and succinic acid production overcoming the stressed previously caused by succinic acid (Chung et al., 2017). Moreover an increase in the metabolic flux from PEP to OAA, improved succinate production up to 152.2 g/L with a further improved yield and productivity.

7.3. TCA cycle and pyruvate metabolism

The tricarboxylic acid cycle and pyruvate metabolism as well as the metabolite balance between these two cycles are responsible for the distribution of the carbon flux among catabolism, anabolism and energy supply of the cell. In *A. succinogenes*, these pathways are very important for the production of succinic acid and the concentration of other extracellular products. Principal component analysis of the relative expression levels (Figure 7.4), revealed a separate group, indicated with brown color, when the *A. succinogenes* cells were grown in SSL. Glucose and xylose formed two distinguished groups that overlapped to some extent. The genes that contribute to the separation were mainly genes that participate in TCA cycle (*PEPCK*, *MDH*, fumarate reductases, fumarase) and the formation of acetyl-CoA (pyruvate kinase, pyruvate dehydrogenase complex and phosphogly-acetyl-transferase). Glucose and xylose relative expression levels formed two groups that are somewhat overlapped.

Phosphoenolpyruvate carboxykinase (*PEPCK*), in most bacteria catalyses the decarboxylation of oxaloacetate to phosphoenolpyruvate (Sauer and Eikmanns, 2005). PEP carboxykinase in *A. succinogenes* and other bacteria (*R. flavefaciens*, *A. succiniciproducens* and *B. succiniciproducens*) is responsible for CO_2 fixation that fulfills anaplerotic function under glycolytic conditions. *PEPCK* and malate dehydrogenase (*MDH*), which are the first steps in the TCA cycle towards succinic acid production, were overexpressed during the exponential phase in SSL compared to glucose and xylose (Figure 7.5). The fold change of *PEPCK* (EC 4.1.1.49) during exponential phase was above 2.14 in SSL compared to xylose and around 1.56 compared to glucose (Table 7.2). The fold change of *MDH* (EC 1.1.1.37) during exponential phase was above 2.33 in SSL compared to xylose and around 1.4 compared to glucose. Both *PEPCK* and *MDH* showed statistical significant difference in the gene expression of SSL compared to both glucose and xylose. Fumarate hydratase (EC 4.2.1.2) was under-expressed in SSL during lag phase. Statistical significant difference was

observed during exponential phase between SSL and xylose. Fold change or relative expression levels was 1.18 in SSL compared to xylose. Fumarate reductase (EC 1.3.5.4) subunits were all significantly affected and will be discussed in great detail in Section 7.4 related to oxidative phosphorylation. Succinyl-CoA synthetase (EC 6.2.1.5) was not significantly affected by the presence of inhibitors in SSL.

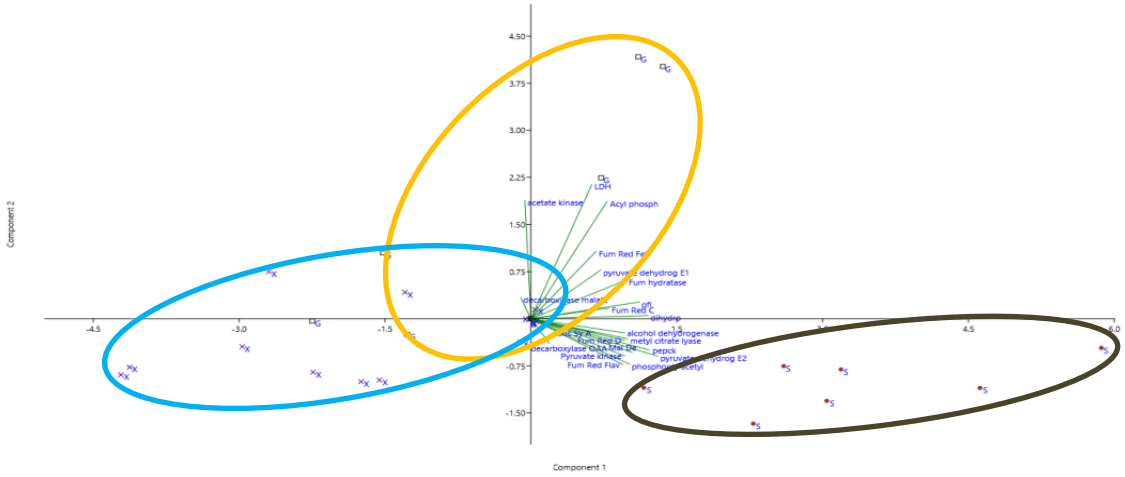

Figure 7.4. Principal component analysis of relative expression levels during exponential phase of genes involved in TCA cycle and pyruvate metabolism pathways. Glucose (yellow circle), Xylose (blue circle), SSL (brown circle).

Oxaloacetate decarboxylase (*OAAdec*) (EC 4.1.1.3) and malate decarboxylase (*Maldec*) (EC 1.1.1.37), which are very important enzymes for the carbon shift between C3 and C4 pathways, were not significantly affected by the presence of inhibitors in SSL, indicating that the co-factors that regulate these enzymes were not found in SSL. CO_2 (McKinlay et al., 2005) and H_2 gas (Van der Werf et al., 1997) enhanced the flow of carbon through these enzymes towards the C4 pathway. Neither of these enzymes was significantly affected by the presence of inhibitors during exponential phase. Citrate lyase (isocitrate) hydro-lyase (EC 2.3.3.1) showed low levels of RNA expression while no statisticall deffierence was observed between the substrates. Upregulation of aconitate hydratase was 1.68 fold in SSL compared to glucose and 3.15 compared to xylose. Pyruvate kinase (EC 2.7.1.40) catalyses the conversion of PEP to pyruvate. Pyruvate kinase (*PK*) relative expression levels were upregulated in SSL compared to both glucose (0.66 fold) and xylose (1.11 fold) during exponential phase.

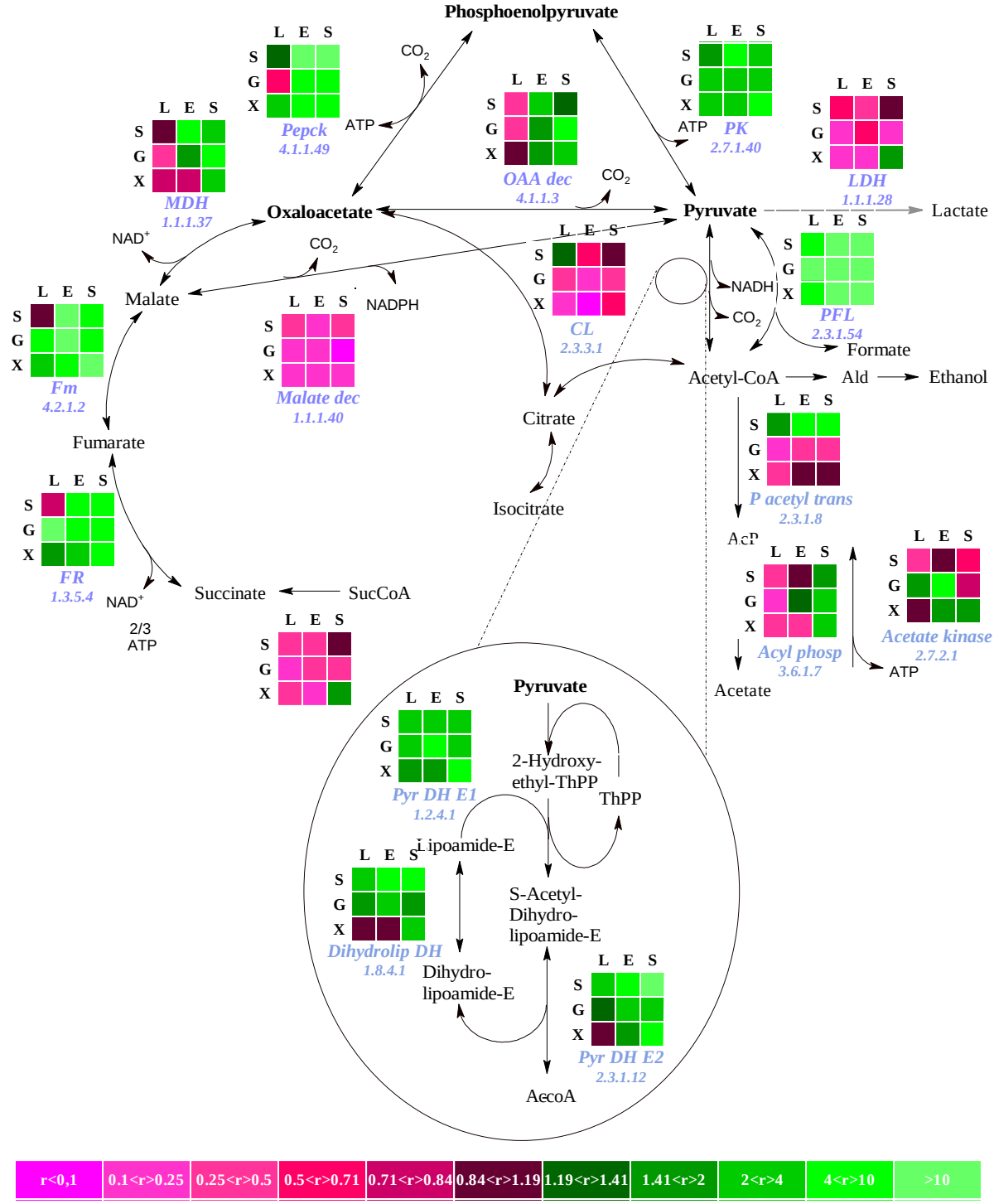

Figure 7.5. Heat map of transcriptional changes in *A. succinogenes* genes involved in TCA cycle and pyruvate metabolism pathways during fermentations in benchtop bioreactor in SSL (S), Glucose (G) and Xylose (X) during lag phase (L), exponential phase (E) and stationary phase (S).

Pyruvate dehydrogenase complex consists of three enzymes, pyruvate dehydrogenase E1 (*Pyr DH E1*), dihydrolipoamide dehydrogenase (*Dihydrolip DH*) and pyruvate dehydrogenase E2 (*Pyr DH E2*), which catalyse the oxidative decarboxylation of pyruvate

with parallel formation of acetyl-CoA, CO_2 and NADH. The first enzyme *Pyr DH E1* (EC 1.2.4.1) was not significantly affected by the presence of inhibitors in SSL. The second enzyme *Dihydrolip DH* was significantly affected in all the substrates and the third enzyme, *Pyr DH E2* (EC 2.3.1.12), was significantly overexpressed during exponential phase in SSL compared to both glucose and xylose (Figure 7.5). Fold change of *Dihydrolip DH* during exponential phase was 0.91 in SSL compared to glucose, 2.51 in SSL compared to xylose and -1.60 in xylose compared to glucose (Table 7.3). *Pyr DH E2* relative expression levels in SSL were 1.67 fold over-expressed compared to glucose and 2.39 fold over-expressed compared to xylose.

Table 7.3 Fold-change (log2) of relative expression levels of *A. succinogenes* genes involved in TCA cycle and pyruvate metabolism in fermentations with SSL compared to glucose and xylose.

Genes	EC Number	Lag phase			Exponential phase			Stationary phase		
		S/G	S/X	X/G	S/G	S/X	X/G	S/G	S/X	X/G
PEPCK	4.1.1.49	1.04	-0.72	1.76	1.56	2.14	-0.58	1.31	0.53	0.78
MDH	1.1.1.37	1.08	0.34	0.74	1.40	2.33	-0.93	-1.22	-0.12	-1.10
Fumarate hydratase	4.2.1.2	-2.34	-2.03	-0.31	0.23	1.18	-0.96	0.09	-1.85	1.94
Fumarate Reductase A	1.3.5.4	-4.69	-1.21	-3.47	1.16	1.99	-0.82	0.41	0.24	0.17
Fumarate Reductase B	1.3.5.4	-0.95	-1.45	0.50	-0.34	0.81	-1.15	-0.38	-1.17	0.79
Fumarate Reductase C	1.3.5.4	-3.81	-1.13	-2.68	0.20	0.90	-0.70	0.82	-0.01	0.83
Fumarate Reductase D	1.3.5.4	1.92	1.44	0.48	1.36	1.53	-0.17	-0.11	0.11	-0.23
Succinyl CoA synth	6.2.1.5	1.31	0.96	0.35	0.79	1.30	-0.51	2.01	-0.71	2.72
OAA decarboxylase	4.1.1.3	0.03	-1.08	1.11	0.53	0.37	0.15	-1.85	-1.44	-0.41
Malate decarboxylase	1.1.1.37	1.18	0.51	0.67	0.02	-0.35	0.37	2.02	0.88	1.13
Citrate lyase	2.3.3.1	1.78	2.95	-1.17	1.68	3.15	-1.47	1.22	0.86	0.36
Pyruvate kinase	2.7.1.40	-0.71	-1.11	0.40	0.66	1.11	-0.44	0.21	-0.56	0.77
Pyruvate DH E1	1.2.4.1	0.22	0.75	-0.53	-0.13	1.11	-1.24	0.29	-0.21	0.50
Dihydrolip DH		0.73	1.22	-0.49	0.91	2.51	-1.60	1.88	1.12	0.76
Pyruvate DH E2	2.3.1.12	1.07	1.28	-0.22	1.67	2.39	-0.72	1.67	1.41	0.26
PFL	2.3.1.54	-1.81	-0.98	-0.83	0.60	1.22	-0.62	0.86	-0.12	0.98
P acetyl transferase	2.3.1.8	3.23	1.74	1.50	4.92	3.43	1.49	3.34	2.26	1.08
Acetate kinase	2.7.2.1	-2.21	-1.78	-0.43	-2.48	-0.44	-2.04	-0.18	-1.21	1.03
Acyl phosphatase	3.6.1.7	0.99	0.47	0.51	-0.45	1.23	-1.69	-0.31	-0.31	0.00
LDH	1.1.1.28	2.29	1.54	0.75	-0.80	0.69	-1.49	1.97	-0.74	2.71

Pyruvate formate lyase (*PFL*), which catalyses the reaction of pyruvate to formate and acetyl-CoA was highly active in all substrates and relative expression levels were higher than

10 in all substrates. Nevertheless, significant difference was observed with SPSS statistical analysis in SSL, where *PFL* (EC 2.3.1.54) was downregulated compared to glucose with -1.81 fold change and -0.98 compared to xylose. During exponential phase, significant statistical difference was also observed in SSL. Upregulation of PFL with a 0.6 fold change was observed compared to glucose and 1.22 fold change compared to xylose (Figure 7.5). Upregulation of *PFL* in SSL compared to glucose resulted in higher final formic acid concentration, leading to the conclusion that under stress conditions the pathway that leads to formic acid production is crucial.

Phosphate acetyltransferase (EC 2.3.1.8) converts acetyl-CoA and inorganic phosphate to acetyl phosphate and CoA, while acetate kinase (EC 2.7.2.1) and acyl phosphatase (EC 3.6.1.7) catalyse the reverse reaction of acetyl phosphate to acetate. Phosphate acetyltransferase was overexpressed in SSL compared to both glucose and xylose, in all growth phases. The enzymes that are responsible for acetate production were highly expressed in stationary phase, despite the fact that acetate production stopped at late exponential phase. Low relative expression levels of lactic acid dehydrogenase (*LDH*) (EC 1.1.1.28) were detected despite the fact that lactic acid was not produced as an extracellular product.

In *E. coli* that has the ability to produce succinic acid, the enzymes that were mostly affected, showing decreased enzyme activity, by furfural addition were phosphoenolpyruvate carboxylase, malate dehydrogenase and fumarate reductase (Wang et al., 2013).

7.4. Oxidative phosphorylation

A. succinogenes utilizes fumarate instead of O_2 as a terminal electron acceptor during oxidative phosphorylation. Formate and H_2 are electron donors. Fumarate respiration results in the production of succinic acid, when fumarate is reduced through the elctron transport chain in the bacterial membrane. The electron transport chain consists of fumarate reductase, menaquinone (MK) and either hydrogenase or formate dehydrogenase. These reactions result in the generation of an electrochemical potential which drives ATP synthesis.

The logarithmic fold change (log2) of the expression levels of oxidative phosphorylation and fumarate respiration in SSL compared to the two control cases (glucose and xylose) are shown in Table 7.4. The relative expression levels of these genes during exponential phase are shown in principal component analysis (Figure 7.6). A separate group of relative expression levels is clearly formed (indicated with brown color) when the *A. succinogenes* cells were grown in SSL. Glucose and xylose on the other hand form two

groups that are overlapped in some extent. The genes responsible for this isolation were mainly those that participate in the fumarate respiration coupled with oxidative phosphorylation.

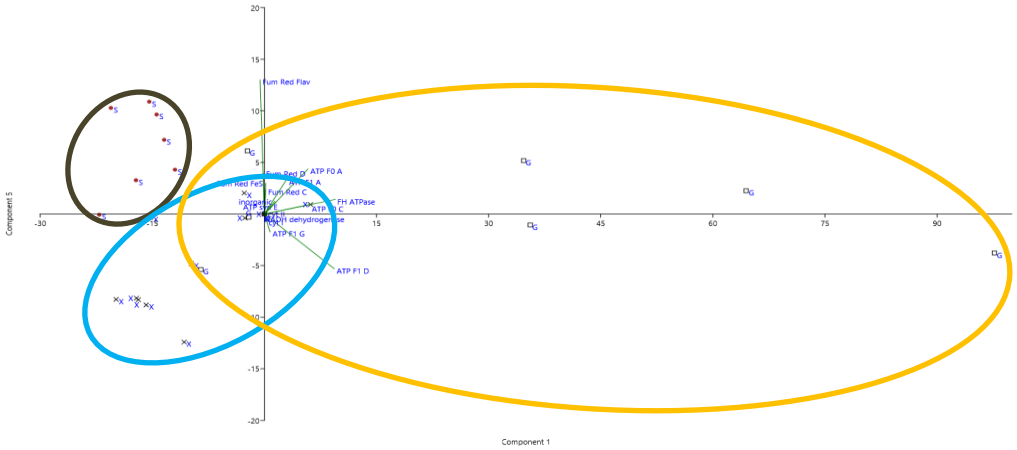

Figure 7.6. Principal component analysis of relative expression levels during exponential phase of genes involved in TCA cycle and pyruvate metabolism pathways. Glucose (yellow circle), Xylose (blue circle), SSL (brown circle).

NADH dehydrogenase (EC 1.6.99.3) relative expression did not show statistical significant differences using SPSS. Also fold change of SSL compared to the control cases did not show any significant difference in regulation (Table 7.4). All fumarate reductase (EC 1.3.5.4) subunits had statistically significant differences in the relative expression levels among the substrates. Specifically, relative expression of fumarate reductase subunit A (which corresponds to flavoprotein subunit) was significantly increased in SSL compared to both glucose and xylose during exponential phase. Fold change in expression levels were 1.16 and 1.99 compared to glucose and xylose, respectively. In lag phase, the genes were under-expressed especially in SSL and xylose. Fumarate reductase subunit B (iron-sulphur subunit) was significantly affected in xylose compared to SSL and glucose during exponential phase. Fold change of SSL compared to xylose was 0.81, while fold change of xylose compared to glucose was -1.15. The same was observed for subumit D, during exponential phase. Fold change of SSL compared to xylose was 1.53, while fold change of xylose compared to glucose was -0.17. Finally, subumit C relative expression levels were only significantly incrased in SSl compared to xylose with a fold change of 0.9 (Table 7.4). Cytochrome d oxidases (EC 1.10.3.14) in oxidative phosphorylation hold a major role in the electron transfer.

Table 7.4 Fold-change (log2) of relative expression levels of *A. succinogenes* genes involved in TCA cycle and oxidative phosphorylation pathways in fermentations with SSL compared to glucose and xylose.

Genes	EC Number	Lag phase			Exponential phase			Stationary phase		
		S/G	S/X	X/G	S/G	S/X	X/G	S/G	S/X	X/G
NADH dehydrogenase	1.6.99.3	-1.62	-1.17	-0.44	0.08	-0.50	0.58	0.43	-0.95	1.37
Fumarate Reductase A	1.3.5.4	-4.69	-1.21	-3.47	1.16	1.99	-0.82	0.41	0.24	0.17
Fumarate Reductase B	1.3.5.4	-0.95	-1.45	0.50	-0.34	0.81	-1.15	-0.38	-1.17	0.79
Fumarate Reductase C	1.3.5.4	-3.81	-1.13	-2.68	0.20	0.90	-0.70	0.82	-0.01	0.83
Fumarate Reductase D	1.3.5.4	1.92	1.44	0.48	1.36	1.53	-0.17	-0.11	0.11	-0.23
Cytochrome oxidase I	1.10.3.14	-0.40	0.11	-0.51	-1.25	-0.56	-0.68	0.11	-0.35	0.46
Cytochrome oxidase II	1.10.3.14	0.92	0.61	0.31	-0.86	-0.03	-0.83	-0.51	-0.78	0.28
ATP synthase F0 sub a	3.6.3.14	-0.91	-0.39	-0.52	-1.68	0.76	-2.44	0.25	0.01	0.25
ATP synthase F0 sub c	3.6.3.14	-0.37	-0.28	-0.09	-1.45	0.23	-1.68	-0.02	-1.33	1.32
ATP synthase F1 sub a	3.6.3.14	-1.01	-0.36	-0.64	-1.89	-0.01	-1.88	-0.62	-7.40	6.77
ATP synthase F1 sub d	3.6.3.14	-1.01	-0.62	-0.39	-2.11	-0.34	-1.76	-0.58	-1.45	0.87
ATP synthase F1 sub e	3.6.3.14	-0.27	0.01	-0.28	-0.89	-1.25	0.36	-0.15	0.05	-0.20
ATP synthase F1 sub g	3.6.3.14	-1.23	-1.30	0.07	-1.18	-0.24	-0.94	1.04	-0.06	1.10
ATP synthase F1 sub a	3.6.3.14	-1.01	-0.36	-0.64	-1.89	-0.01	-1.88	-0.62	-7.40	6.77
ATP synthase F1 sub b	3.6.3.14	-2.24	-1.78	-0.45	-3.12	-1.23	-1.89	-1.54	-2.25	0.71
Inorganic pyrophosphatase	3.6.1.1	-0.19	0.08	-0.27	-2.97	0.81	-3.78	-0.90	-2.09	1.19
DCU antiporter		3.15	0.43	2.71	1.61	1.86	-0.25	0.18	1.24	-1.06
0671 antiporter		0.46	0.37	0.09	-0.35	0.30	-0.66	1.22	0.38	0.84
1999 antiporter		-1.23	-1.21	-0.02	-3.13	-1.72	-1.41	-0.53	-0.89	0.35
0079 antiporter		1.65	2.57	-0.92	4.12	3.83	0.29	3.63	2.62	1.01

ATP synthase (EC 3.6.3.14) is a protein molecule with many subunits, which is the last protein complex of the oxidative phosphorylation. ATP synthase is activated by the electron transfer through the electron transport chain and produces ATP. All ATP synthase subunits have been selected for RT-PCR. The F0 ATP synthase unit consists of subunit α and c and F1 ATP synthase unit consists of subunits α, b, d, e and g. Relative expression levels of all subunits were high in exponential and stationary phase. Statistical significant differences during exponential phase in RNA expression levels of the genes that regulate ATP synthase subunits (except subunit e) were observed in SSL compared to glucose and in xylose compared to glucose. Statistically significant differences between SSL and xylose were not observed. All subunits were downregulated in SSL compared to glucose during exponential phase, reaching up to -3 fold change in relative expression levels. Similar behavior was observed in xylose compared to glucose.

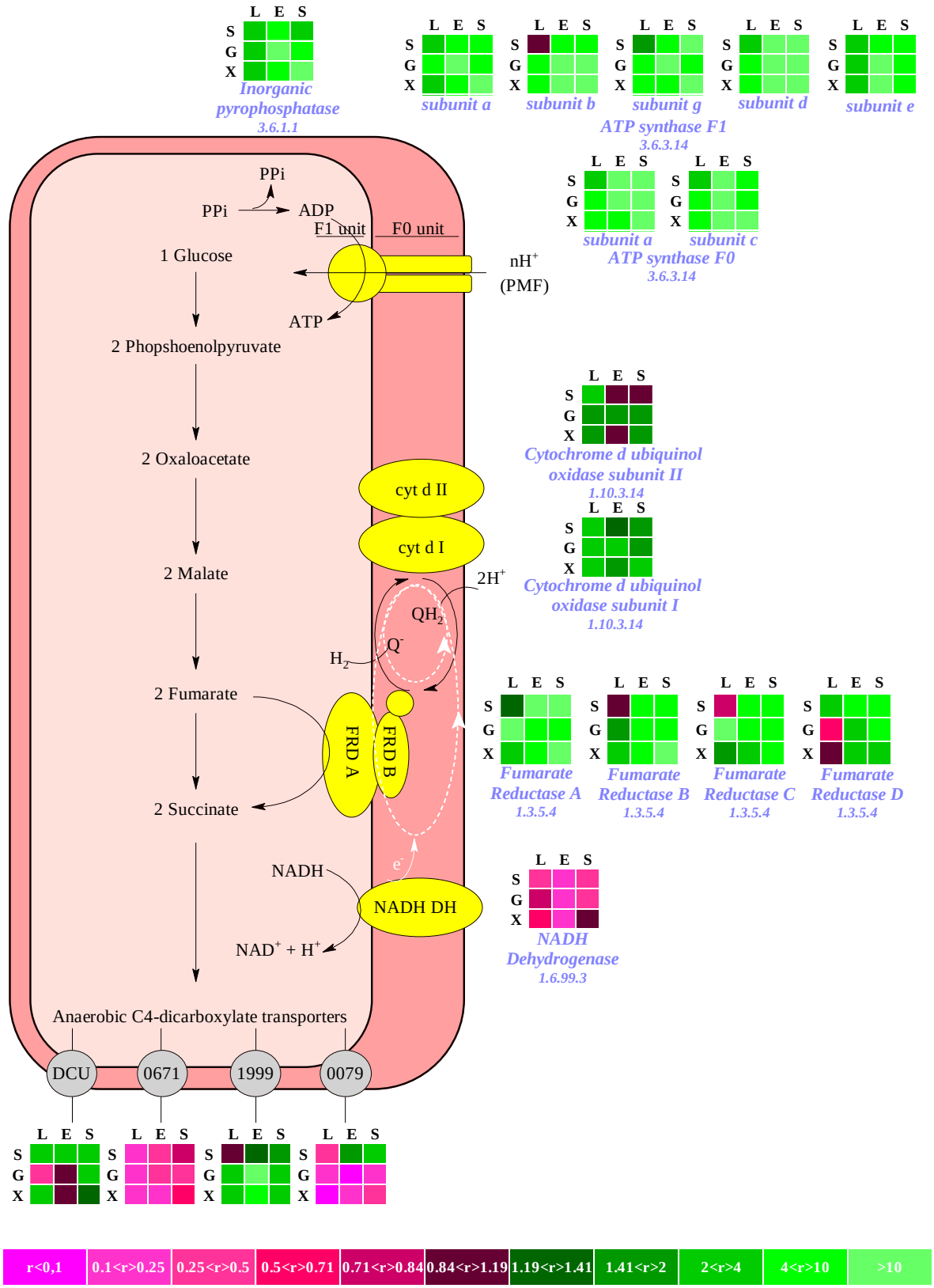

Figure 7.7 Heat map of transcriptional changes in *A. succinogenes* genes involved in TCA cycle and oxidative phosphorylation pathways during fermentations in benchtop bioreactor in SSL (S), Glucose (G) and Xylose (X) during lag phase (L), exponential phase (E) and stationary phase (S).

These results indicate that pentose phosphate pathway that is activated for xylose degradation might fulfill energy requirements that generally result from oxidative phosphorylation. Another enzyme that participates in oxidative phosphorylation pathway is inorganic pyrophosphatase (EC 3.6.1.1) that catalyses the conversion of pyrophosphate to two phosphate ions. Although relative expression levels were high in all growth phases, statistical difference between the substrates was not observed.

The relative expression levels of proteins that regulate C4 carboxylic acid transportation out of the cell were also analysed. DCU transporter plays an important role on C4 carboxylic acid transportation. Some other proteins encoded by genes Asuc_0671, Asuc_1999 and Asuc_0079, were also determined as C4 carboxylic acid transporters (McKinlay et al., 2010). During exponential phase, only DCU antiporter was statistically significant overexpressed in SSL, compared to both glucose and xylose. Fold change of DCU was 1.61 in SSL compared to glucose and 1.86 compared to xylose.

A transcriptome and metabolome study in the presence of furfural and HMF of *Saccharomyces cerevisiae* strain VTT C-10883 revealed the decrease of intracellular redox-cofactors concentrations, the decrease of intracellular ATP concentration that affects the redox balance. The most important metabolic groups that were influenced by furfural addition were "Cell rescue, defense and virulence", with ATP efflux pumps upregulated, leading to lower intracellular ATP concentration. Also genes involved in pseudohyphal growth were up-regulated, which often results from nitrogen starvation. Down regulation of genes involved in amino acid metabolism, glyoxylate cycle, electron transport and amino acid transport was observed (Ask et al., 2013). Transcriptomic analysis was also performed in *S. cerevisiae* T2, an industrial important strain for the production of ethanol using hardwood SSL. The transcriptome was analysed in yeast cells grown on the main inhibitors contained in SSL (acetic acid, furfural and HMF) and in hardwood SSL (HWSSL) and the profiles were compared to the profile of cells grown in media without inhibitors. When HWSSL was used 242 genes had significant expression levels. The majority of the different expression levels (206) were observed only in SSL, while only 14.9% of the gene responses were common between SSL and each individual inhibitor, compared to the control fermentation. Specifically, 27 gene responses were common between SSL and acetic acid, 6 were common between SSL and furfural and 10 between SSL and HMF, compared to the control fermentation. The genes that were mostly affected by acetic acid were related to uptake and energy metabolic pathways, while furfural and HMF had an impact on the redox balance of the cell. Gene responses in the presence of HWSSL were much more diverse than the

individual inhibitory compounds and affected many pathways, mainly those related with cellular structure and function. Specifically, a decrease in the expression of ribosomal proteins, synthesis and transport of proteins as well as metabolism of carbohydrates, lipids, vitamins and vacuolar proteins was observed. Also, increase in the expression of genes that are involved in multidrug resistance, iron transport and pheromone response was observed (Paramjit et al., 2013). In *Corynebacterium glutamicum* the stress by succinic acid resulted in downregulation of ABC type transport system, transcription regulation, amino acid biosynthetic pathway, DNA repair system, and electron carriers (Chung et al., 2017).

Chapter 8

Integrated succinic acid production and separation using an electrochemical bioreactor

8.1 Introduction

Membrane electrolysis is an electrochemical extraction technique in which electrodes are present in the fermentation broth. Typically water reduction (cathode) occurs in the fermentation broth, producing hydrogen gas and hydroxide ions, whereas water oxidation (anode) occurs in an extracting solution, producing oxygen gas and protons (Figure 8.1). The current drives the charged carboxylic acids across an anion exchange membrane by electromigration from the high-volume fermenter into a low-volume acid concentrate extract. These membranes are permeable to many carboxylic acid anions (e.g. acetic acid, butyric acid, succinic acid), but impermeable to cells and solids, resulting in a combined extraction, clarification, acidification and concentration step in a single unit. One of the main disadvantages on bacterial succinic acid production is the pH control close to neutral levels that results in the formation of succinate salts that later have to be recovered into their acid form. In this case, the applied current drives the anion of the succinate from the fermentation broth into an extract solution with pH lower than the pKa of succinic acid (Andersen et al 2014).

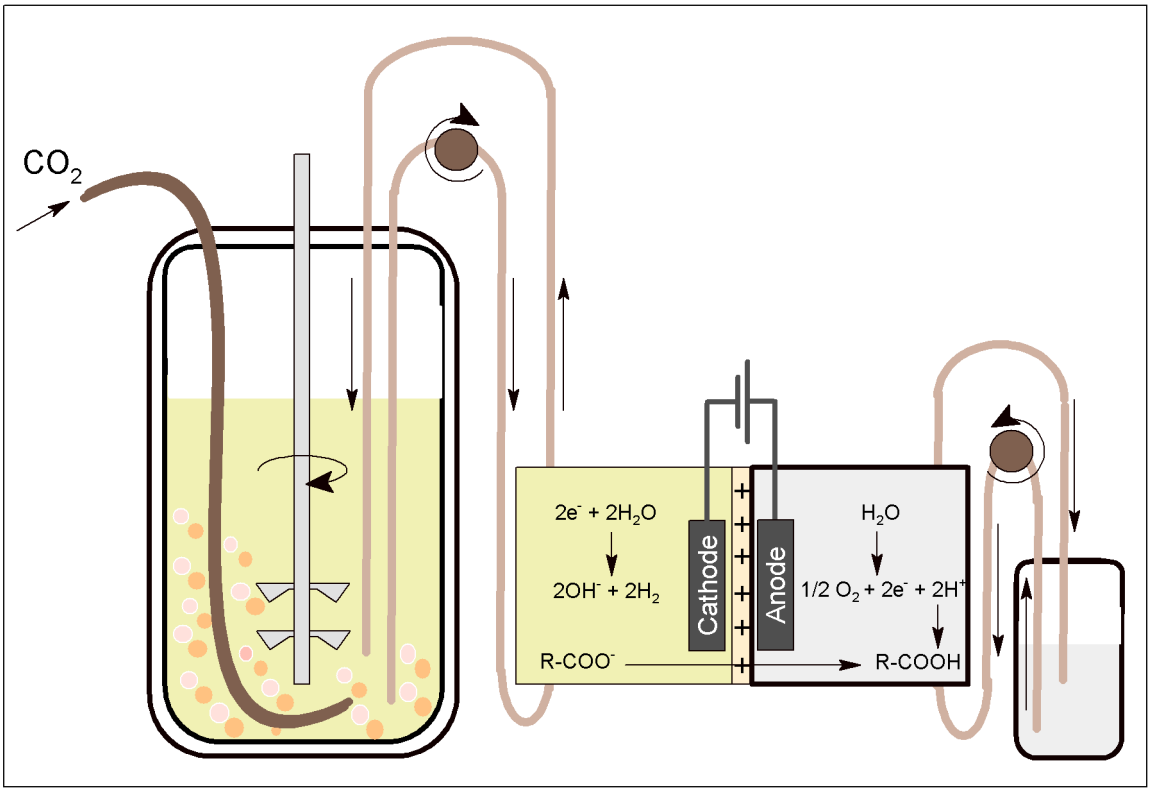

Figure 8.1 Succinic acid production coupled with *in situ* electrochemical extraction - Bioreactor set-up

The hydroxide ions generated in electrolysis replace pH control, thus dosing the fermentation with costly sodium or potassium hydroxide is no longer necessary (Andersen et al. 2014; Andersen et al. 2015). Electrochemical cell fermentation could be ideal for microbial cultures that are favored by reduced environments. Andersen et al. (2015) monitored the bacterial communities with and without current supply and observed a shift in the bacterial community that favored volatile fatty acid elongation probably due to the efficient utilisation of the produced hydrogen. An electrochemical bioreactor can supply reducing power that could enhance the production of succinic acid. Hydrogen or electricity can be used as additional reducing power and offer electron donors for fumarate respiration resulting in increased succinate yield and ATP synthesis via electron transport-mediated phosphorylation (Park et al. 1999).

8.2. Succinic acid production coupled with *in situ* electrochemical extraction using xylose as carbon source

Fermentations coupled with *in situ* electrolytic membrane extraction were carried out with *B. succiniciproducens*. Two cases, utilising glucose and xylose, were selected as carbon sources to prove that electrolysis could enhance succinic acid production. Fermentations with xylose as carbon source were initially applied to prove that electrolytic membrane extraction coupled with fermentation is feasible in pure cultures. A fed-batch fermentation using xylose as carbon source was carried out as the control case (Figure 8.2).

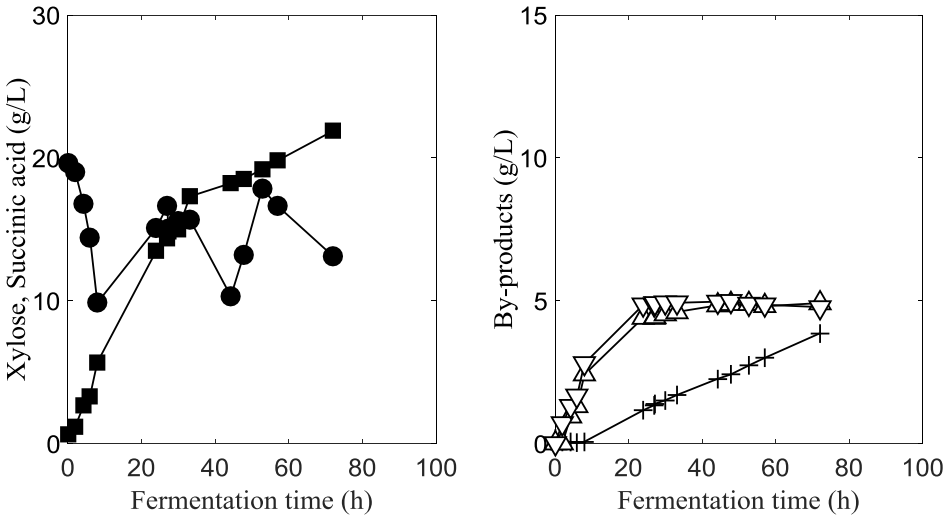

Figure 8.2 Consumption of xylose and production of metabolic products during fed-batch bioreactor fermentation of *B. succiniciproducens* cultivated on xylose and 5 g/L yeast extract. Xylose (●), succinic acid (■), lactic acid (+), formic acid (△), acetic acid (▽).

The feeding medium used was a concentrated xylose solution without any nutrient supplements. The final succinic acid concentration was 21.9 g/L with yield and productivity of 0.56 g/g and 0.30 g/L/h, respectively. The production of formic and acetic acids started soon after inoculation with final concentrations of around 4.9 g/L, while lactic acid production began at 8 h and its accumulation continued until the end of fermentation (3.8 g/L). The by-product to succinic acid molar ratios were 0.23 mol/mol, 0.59 mol/mol, 0.44 mol/mol for LA:SA, FA:SA and AA:SA, respectively.

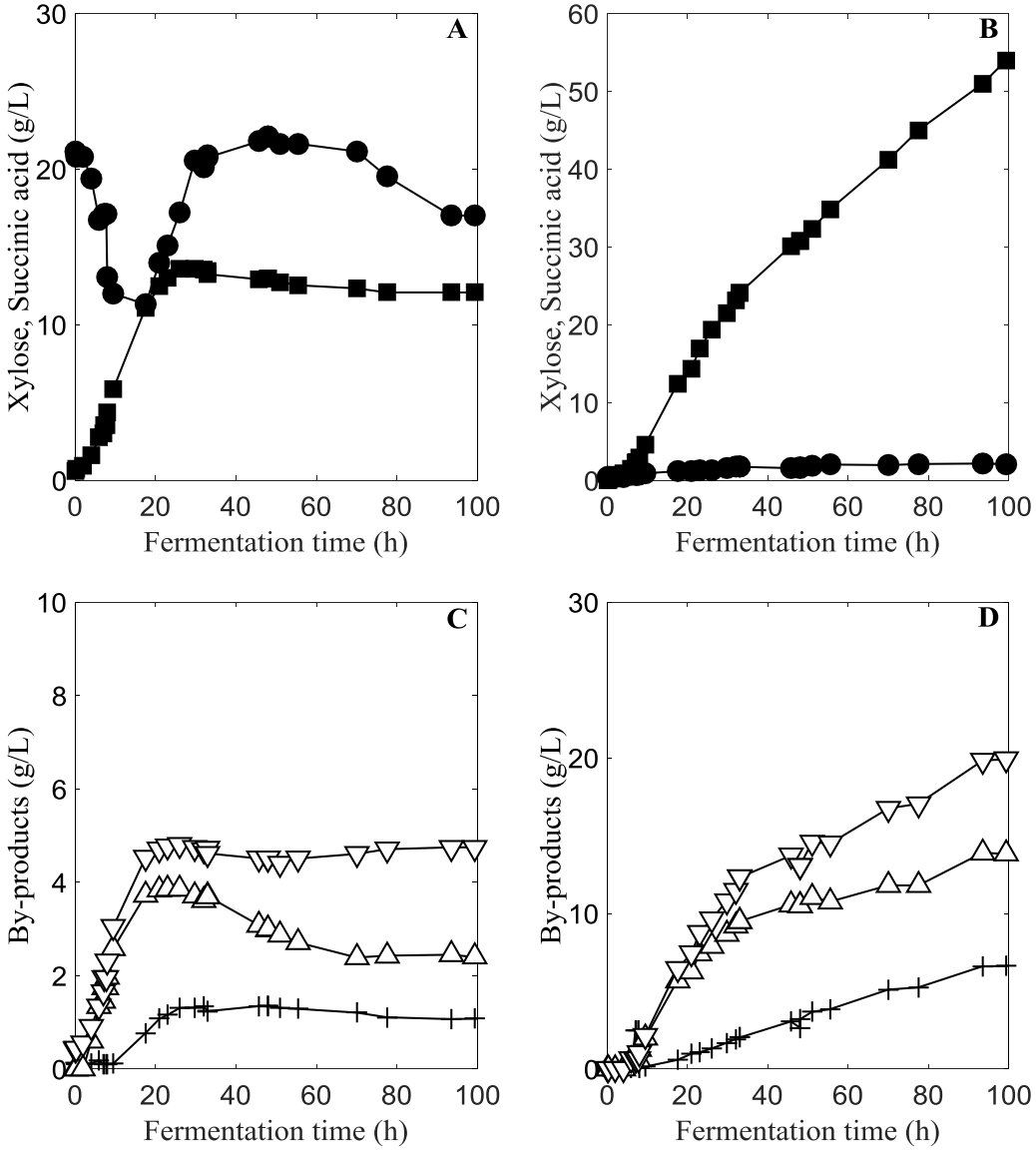

Figure 8.3 Xylose and metabolic products observed during fed-batch bioreactor fermentations of *B. succiniciproducens* cultivated on xylose and 5 g/L yeast extract integrated with *in-situ* extraction of metabolic products using membrane electrolysis. (A and C: bioreactor; B and D: extraction cell) Xylose (●), succinic acid (■), lactic acid (+), formic acid (△), acetic acid (▽). The feeding solution used was a concentrated xylose solution.

The first experiment of fed-batch fermentations coupled with an electrochemical cell that consisted of an anion selective membrane, was carried out at the same total fermentation volume (3 L) as the control culture. The feeding medium used was a concentrated xylose solution. The fermentation broth was recycled through the cathode compartment of the electrochemical cell (250 mL) with a flow rate of 3 L/h. Figure 8.3 presents the experimental results obtained during fermentation coupled with the electrochemical cell. The fermentation stopped suddenly at 30 h contrary to the control culture where the fermentation lasted for more than 70 h. The succinic acid concentration at 30 h was 13.6 g/L with a yield and productivity of 0.56 g/g and 0.44 g/L/h, respectively. The succinic acid concentration in the extract solution continuously increased at a rate of 0.5 g/L/h even after the fermentation was over, resulting in a succinic acid concentration of 61 g/L at 120 h. A slightly decreased succinic acid concentration (12 g/L) was observed in the bioreactor at 120 h. The final by-product to SA molar ratio was 0.12 mol/mol, 0.76 mol/mol, 0.69 mol/mol for LA:SA, FA:SA and AA:SA, respectively. The acetic acid concentration (4.75 g/L) at 30 h was similar to the control culture, while the formic acid (3.7 g/L) and lactic acid (1.3 g/L) concentrations were lower. The molar ratios of FA:SA and AA:SA were higher than the control experiment, while in the case of the LA:SA molar ratio a similar value was observed.

The cause of the sudden end of the fermentation could be the depletion of essential minerals like phosphate and chloride salts that were initially added into the medium. The membrane that was used is also permeable by other negatively charged ions e.g. phosphate (PO_4^{-3}) and chloride (Cl^-) that are contained in the fermentation medium. Depletion of these minerals from the fermentation medium resulted in the premature end of the fermentation at around 30 h. Inorganic phosphorus (IP) consumption in the control fermentation was 5.63 mg/L/h for the first 24 h until maximum growth was reached and 1.88 mg/L/h until the end of the fermentation. In the case of the fermentation coupled with the electrochemical cell, the concentration of inorganic phosphorus was decreased at a rate of 15.7 mg/L/h during the first 8 h of the fermentation, indicating that extraction of phosphate anions (PO_4^{-3}) from the fermentation medium occurred. IP reduction followed a 5.4 mg/L/h rate until the end of the fermentation.

The subsequent control fed-batch fermentation was carried out using exactly the same conditions as the cultures presented in Figures 8.2 and 8.3, but with a feeding solution containing also 1% (w/v) yeast extract besides xylose. The same fermentation was carried out using the integrated system, with the only difference being that the feeding solution also contained minerals. This approach was used in order to evaluate whether the depletion of

nutrients led to the premature end of the fermentation when the electrochemical cell was integrated with the bioreactor. The control fed-batch fermentation was carried out using xylose and feeding consisted of a concentrated xylose solution supplemented with 1% (w/v) yeast extract (Figure 8.4). This fermentation resulted in 20.5 g/L of succinic acid concentration with 0.56 g/g yield and 0.42 g/L/h productivity. The final by-product molar ratios were 0.20 mol/mol, 0.72 mol/mol, 0.55 mol/mol for LA:SA, FA:SA and AA:SA, respectively.

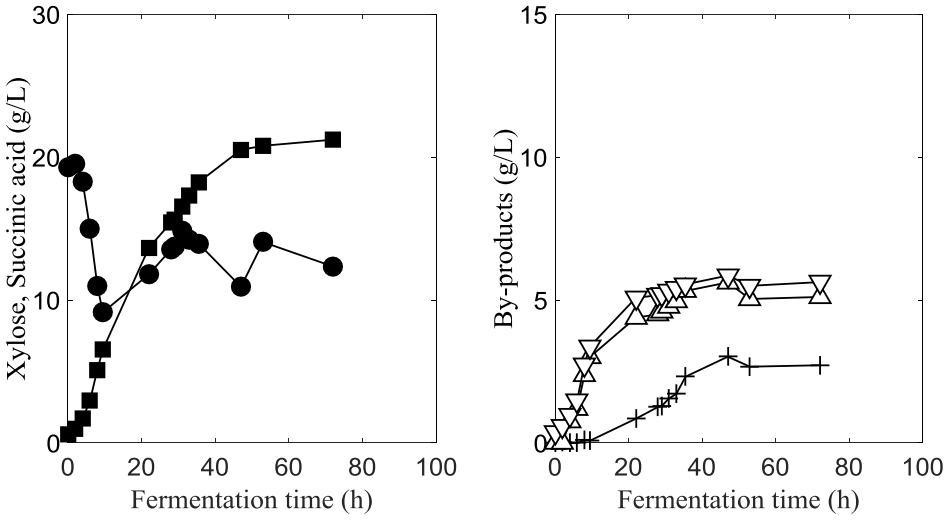

Figure 8.4 Consumption of xylose and production of metabolic products during fed-batch bioreactor fermentations of *B. succiniciproducens* cultivated on xylose and 5 g/L yeast extract. Glucose (●), succinic acid (■), lactic acid (+), formic acid (△), acetic acid (▽). The feeding medium used was a concentrated xylose solution supplemented with 1% (w/v) yeast extract.

Figure 8.5 presents the results obtained during the fed-batch fermentation where the integrated system was used and the feeding solution was supplemented with 1% (w/v) yeast extract and minerals with the following content: 58% (w/v) $NaH_2PO_4 \cdot H_2O$, 15.5% (w/v) Na_2HPO_4, 50% (w/v) NaCl, 10% (w/v) $MgCl_2 \cdot 6H_2O$,and 10% (w/v) $CaCl_2 \cdot 2H_2O$. The final succinic acid concentration was higher (21.9 g/L) than the one (13.6 g/L) achieved in the integrated system presented in Figure 8.3. The overall succinic acid to substrate conversion yield was 0.63 g/g, which is higher than the control fed-batch fermentation. The productivity achieved was 0.46 g/L/h, which is also higher than the control fed-batch fermentation. The final by-product molar ratios were 0.12 mol/mol, 0.50 mol/mol, 0.46 mol/mol for LA:SA, FA:SA and AA:SA, which are lower than the control fermentation presented in Figure 8.4.

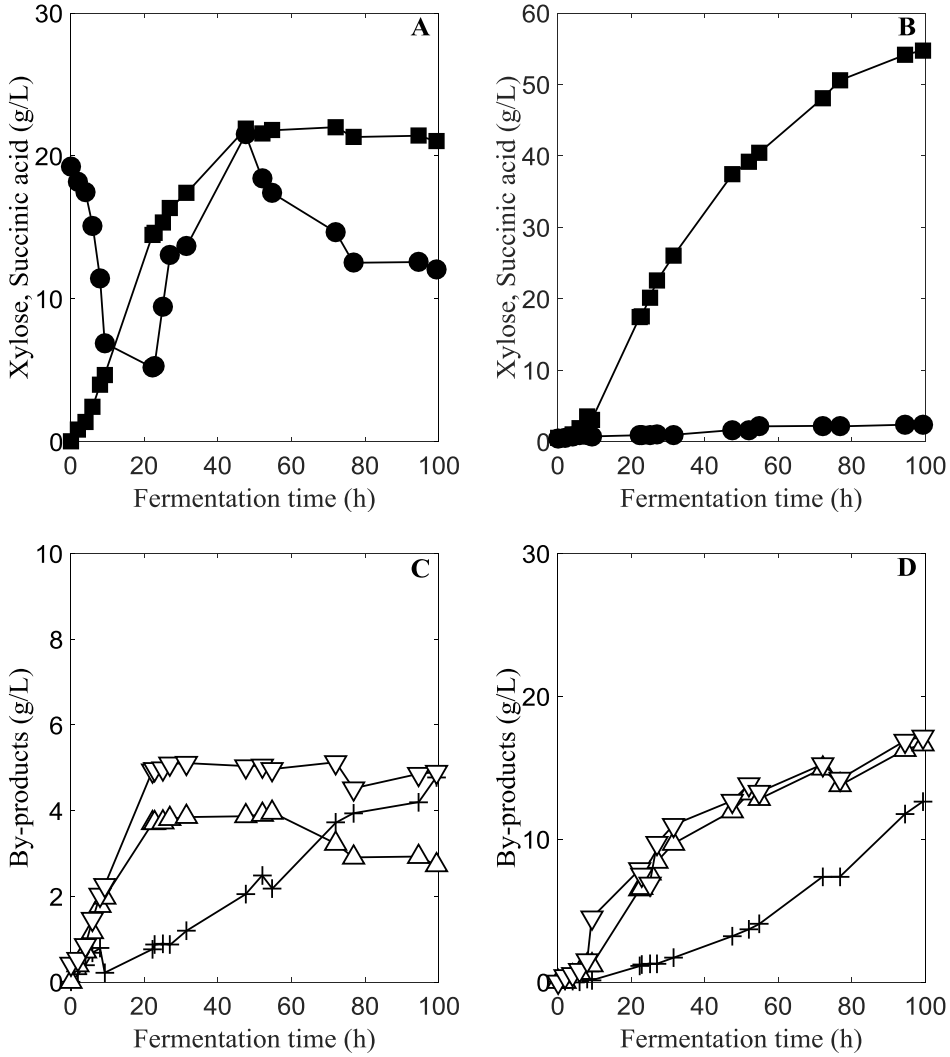

Figure 8.5 Xylose and metabolic products observed during fed-batch bioreactor fermentations of *B. succiniciproducens* cultivated on xylose and 5 g/L yeast extract integrated with *in-situ* extraction of metabolic products using membrane electrolysis. (A and C: bioreactor; B and D: extraction cell). Xylose (●), succinic acid (■), lactic acid (+), formic acid (△), acetic acid (▽). The feeding solution used was a concentrated xylose solution supplemented with 1% (w/v) yeast extract and minerals.

The molar ratios of FA:SA and AA:SA were lower than the previous fed-batch fermentation using the integrated system (Figure 8.3) when the feeding solution was supplemented with yeast extract and minerals. This could be explained by the fact that hydrogen gas that is generated in the cathode compartment of the electrochemical cell, where the bacterial cells and the fermentation medium are recycled, favor the carbon flux towards the C4 pathway instead of the C3 pathway. Reversible shunts through the C4 and C3 pathways are possible through malate decarboxylase and OAA decarboxylase [McKinlay et

al., 2008, Becker et al., 2013]. McKinlay and Vieille (2008) have investigated hydrogen gas supply along with $NaHCO_3$ on the metabolic flux of *A. succinogenes* using C^{13} glucose, concluding that hydrogen enhances carbon flux in C4 pathway versus C3 pathway.

In order to prove that excess mineral supplementation was beneficial only in the integrated system where depletion of nutrients occurred due to electrolytic extraction, a fed-batch fermentation was carried out without simultaneous succinic acid extraction using the feeding solution that was supplemented with yeast extract and minerals (Figure 8.6). It is clearly shown that *B. succiniciproducens* was inhibited when this feeding addition was employed. The final succinic acid concentration was 4.9 g/L with a yield and productivity of 0.45 g/g and 0.09 g/L/h, respectively. These results show that

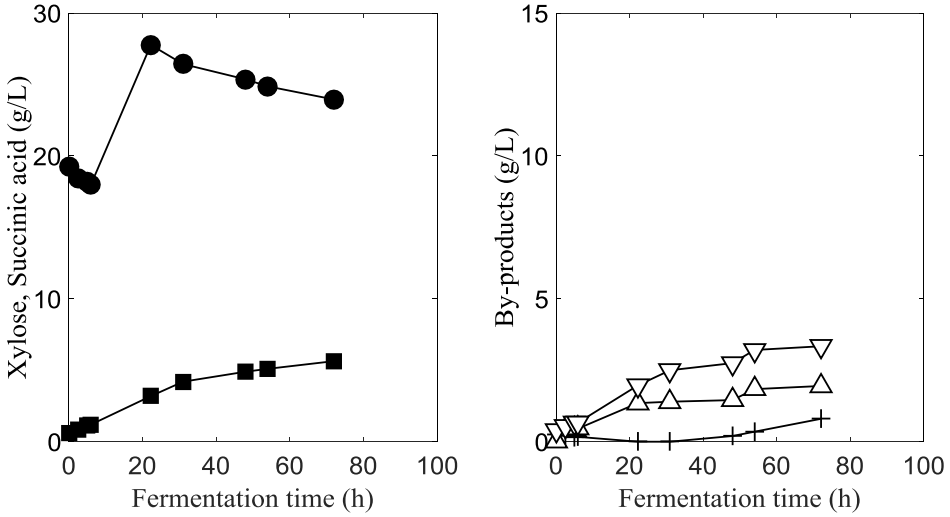

Figure 8.6 Consumption of xylose and production of metabolic products during fed-batch bioreactor fermentations of *B. succiniciproducens* cultivated on xylose and 5 g/L yeast extract. Glucose (●), succinic acid (■), lactic acid (+), formic acid (△), acetic acid (▽). The feeding solution was a concentrated xylose solution supplemented with 1% (w/v) yeast extract and minerals.

8.3. Succinic acid production coupled with *in situ* electrochemical extraction using glucose as carbon source

Commercial glucose was also investigated as carbon source in fed-batch fermentations in control cultures or cultures integrated with *in situ* electrochemical extraction. These fermentations were carried out using a feeding medium supplemented either with only yeast extract in the case of the control culture or with yeast extract and minerals in the case of the

integrated system. The control fed-batch fermentation with glucose (Figure 8.7) resulted in 20 g/L of succinic acid with 0.59 g/g yield and 0.37 g/L/h productivity. Although total by-product formation was similar to the xylose control fermentation presented in Figure 8.4, a significant difference was observed in the distribution of by-products. Glucose consumption led to higher lactic acid production. The lactic acid to succinic acid ratio (LA:SA) in glucose was much higher than xylose, while the formic to succinic acid ratio (FA:SA) and the acetic acid to succinic acid ratio (AA:SA) in glucose were lower than in the culture carried out on xylose. Specifically, the LA:SA ratio was 0.41 mol/mol in glucose compared to 0.20 mol/mol in xylose, while FA:SA and AA:SA were 0.35 mol/mol and 0.30 mol/mol, respectively, in glucose and 0.72 mol/mol and 0.55 mol/mol in xylose.

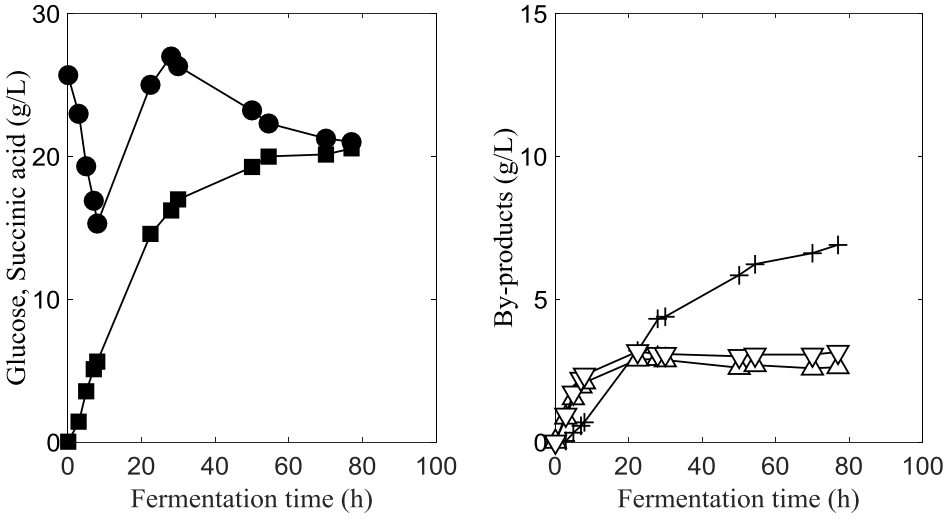

Figure 8.7 Consumption of glucose and production of metabolic products during fed-batch bioreactor fermentations of *B. succiniciproducens* cultivated on glucose and 5 g/L yeast extract. Glucose (●), succinic acid (■), lactic acid (+), formic acid (△), acetic acid (▽). The feeding solution contained glucose supplemented with 1% (w/v) yeast extract.

An overall increase in succinic acid fermentation efficiency was observed when electrolytic membrane extraction was applied (Figure 8.8) as compared to the control culture (Figure 8.7). The highest succinic acid concentration (22.1 g/L) in the fermentation broth was observed at 48.5 h (Figure 8.8A). Succinic acid was simultaneously extracted during fermentation (Figure 8.8B). The yield (0.68 g/g) and productivity (0.46 g/L/h) achieved in the integrated system were higher by 15% and 24%, respectively, than the control culture. The succinic acid to substrate conversion yield was higher in glucose than xylose. Lactic acid

production was observed throughout fermentation as when xylose was used as carbon source (Figure 8.5C).

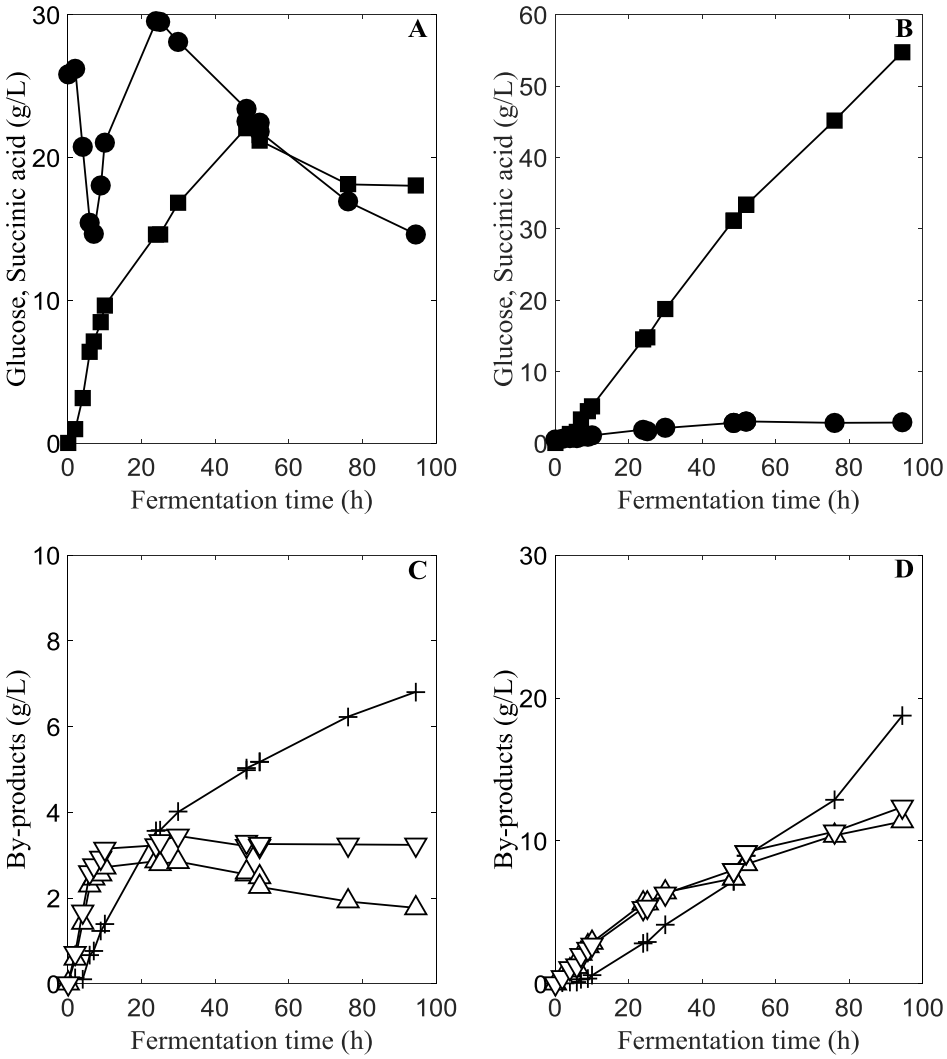

Figure 8.8 Glucose and Metabolic products during fed-batch bioreactor fermentations of *B. succiniciproducens* cultivated on glucose and 5 g/L yeast extract integrated with *in-situ* extraction of metabolic products using membrane electrolysis. (A and C: bioreactor; B and D: extraction cell). Glucose (●), succinic acid (■), lactic acid (+), formic acid (△), acetic acid (▽). The feeding solution contained glucose supplemented with 1% (w/v) yeast extract and minerals.

This microorganism forms a biofilm that is usually attached on the baffles and the impellers of the bioreactor. In the fermentation coupled with the electrochemical cell, a biofilm was formed on the membrane and the spacer between the membrane and the stainless steel, anode electrode (Figure 8.9A). In this case, the biofilm could interfere with the

resistance of electrolysis reaction resulting in voltage increment. The cells become self-immobilised into a polysaccharide-based structure that consists mainly of glucose. Scanning electron microscopy has been used to visualize the cells on the biofilm formation (Figure 8.9B). Although biofilm formation might be an issue in large scale fermentations due to high cell death, it has been suggested as a way to improve succinic acid fermentation.

Figure 8.9 (A) Biofilm formation on the anionic membrane. (B) *B. succiniciproducens* cells on the biofilm observed with scanning electron microscope (SEM)

The formation of a dense biofilm that was observed inside the electrochemical reactor might act as a protectant micro-environment for the bacterial cells. Alexandri et al. (2017) reached 45 g/L of succinic acid concentration using *B. succiniciproducens* immobilised in alginate beads and cultivated in SSL. Other researchers have used different support surfaces in order to immobilize bacterial cells, such as 50% polypropylene composites [Urbance et al. 2004], zeolite, diatomite, activated carbon, j-carrageenan, alginic acid, chitosan, agar and poly-acrylamide hydrogel [Corona-González et al. 2014], and cotton cloth [Yan et al. 2014].

In all cases, succinic acid production was increased compared to fermentations where free cells were used. A micro-environment is formed in electrochemical reactors where the cells are self-immobilised, potentially exploiting the reduced energy that is generated by the electrochemical cell. Part of the produced organic acids is extracted during the fermentation contributing to higher cell viability during the fermentation. As the main extracellular metabolite, succinic acid concentration in the fermentation broth is higher than the rest of the by-products which influences extraction rate as there is higher availability of succinic acid anions per membrane surface.

8.4. Succinic acid production coupled with *in situ* electrochemical extraction using spent sulphite liquor as carbon source

Spent sulphite liquor (SSL) was also evaluated as carbon source for the production of succinic acid using the integrated system where the bioreactor is connected with an electrochemical cell with an anion exchange selective membrane. Since the thick SSL contains many inhibitory compounds, such as LS and phenolic compounds, ultrafiltration was used to remove the majority of the inhibitory compounds prior to fermentation. Using *A. succinogenes* and *B. succiniciproducens* in ultrafiltrated SSL, succinic acid production reached 27.4 g/L and 27.8 g/L, respectively (see Chapter 6).

Figure 8.10 presents the consumption of total sugars and the production of metabolic products during fed-batch bioreactor culture carried out with *B. succiniciproducens* cultivated on ultrafiltrated SSL supplemented with 5 g/L yeast extract. The feeding solution contained mixed commercial sugars supplemented with LS and 1% (w/v) yeast extract. The final succinic acid concentration produced in this control fermentation was 15.1 g/L at 48.5 h with a yield of 0.53 g/g and a productivity of 0.31 g/L/h (Table 8.1). It should be stressed that $MgCO_3$ was not added in the fermentation medium in order to avoid blockage of the membrane. This may explain the lower fermentation efficiency compared to fed-batch fermentations using ultrafiltrated SSL (see Chapter 6). The use of $MgCO_3$ as pH regulator contributes to higher succinic acid production [Wang et al., 2012; Liu et al., 2008] providing CO_2 and Mg^{2+} ions that serve as cofactors for PEP carboxykinase, the first enzyme in the reductive branch of the TCA cycle.

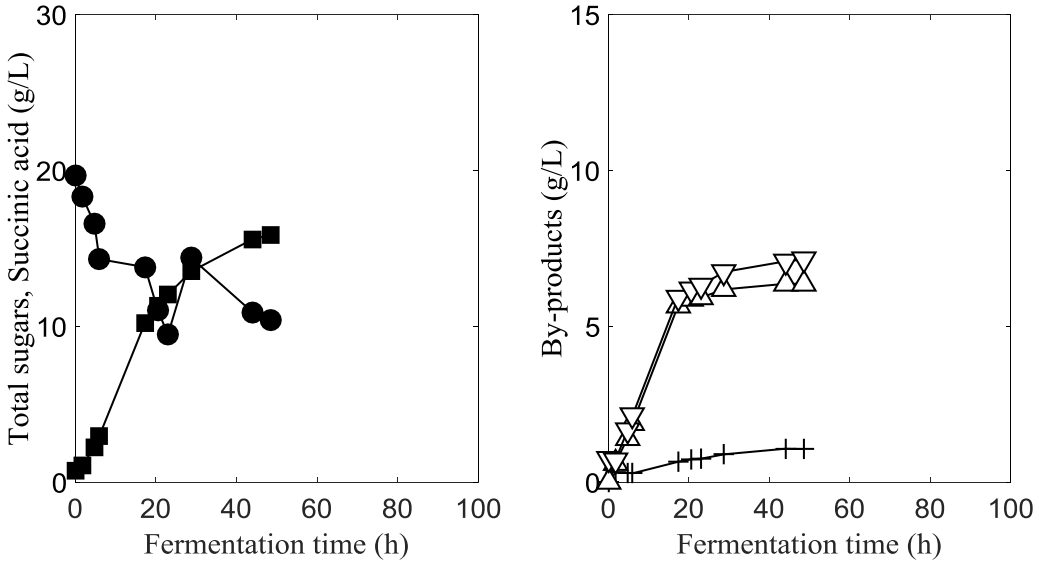

Figure 8.10 Consumption of total sugars and production of metabolic products during fed-batch bioreactor fermentations of *B. succiniciproducens* cultivated on ultrafiltrated SSL and 5 g/L yeast extract. Total sugars (●), succinic acid (■), lactic acid (+), formic acid (△), acetic acid (▽). The feeding solution used contained concentrated mixed commercial sugars (72.6% of xylose, 12.2% of galactose, 10.9% of glucose, 4.2% of mannose, and 0.1% of arabinose) supplemented with lignosulphonates and 1% (w/v) yeast extract.

Figure 8.11 presents the total sugars and metabolic products observed in the integrated system. The feeding solution contained mixed commercial sugars supplemented with LS, 1% (w/v) yeast extract and minerals. The highest succinic acid concentration reached in the fermentation broth was 19.5 g/L at 46 h (Figure 8.11A). The final succinic acid concentration extracted in the cell was 20.5 g/L at 46 h (Figure 8.11B). Compared to the control fermentation (Figure 8.10), a 45% increase to overall succinic acid production was observed. The conversion yield and productivity achieved in the integrated system were 0.61 g/g and 0.41 g/L/h, respectively. Yield and productivity were improved by 15% and 32%, respectively, as compared to the control culture. Table 8.1 presents the results from all cultures presented above.

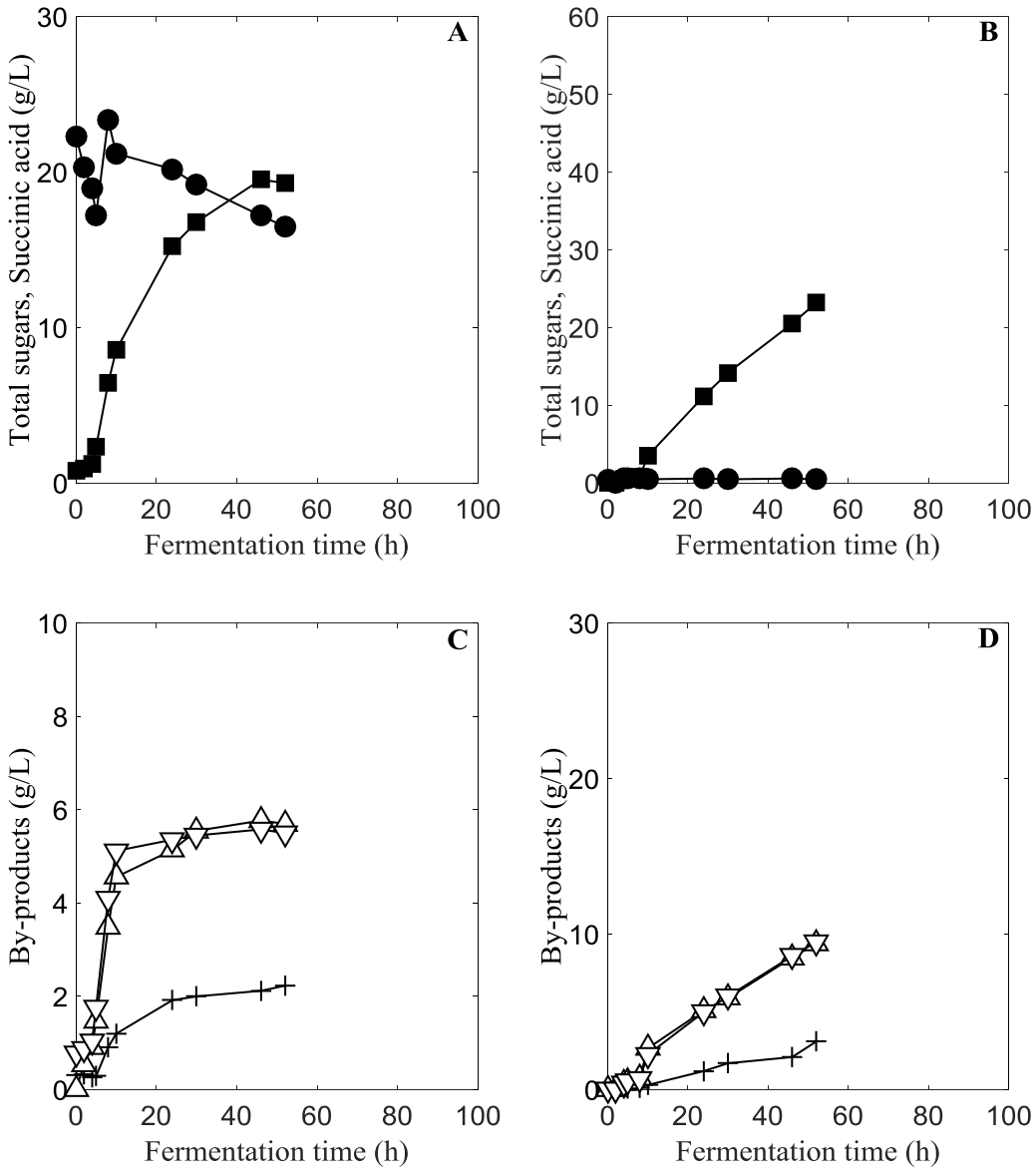

Figure 8.11 Total sugars and metabolic products during fed-batch bioreactor fermentations of *B. succiniciproducens* cultivated on ultrafiltrated SSL and 5 g/L yeast extract integrated with in-situ extraction of metabolic products using membrane electrolysis. (A and C: bioreactor; B and D: extraction cell) Total sugars (●), succinic acid (■), lactic acid (+), formic acid (△), acetic acid (▽). The feeding solution contained concentrated mixed commercial sugars (72.6% of xylose, 12.2% of galactose, 10.9% of glucose, 4.2% of mannose, and 0.1% of arabinose) supplemented with lignosulphonates, 1% (w/v) yeast extract and minerals.

Table 8.1 Results from fed-batch bioreactor cultures and fed-batch fermentations coupled with electrolytic membrane extraction using glucose, xylose and ultrafiltrated SSL

	Duration (h)	Sugars consumed (g)	Succinic acid produced (g)	Yield[1] (g/g)	Productivity (g/L/h)	LA:SA (g/g)	FA:SA (g/g)	AA:SA (g/g)	Carbon Balance[2]
Spent sulphite liquor									
Fed-batch (feed: SSL, YE)	48.5	93.26	49.68	0.53	0.31	0.07	0.42	0.43	0.839
Electrochemical reactor (feed: SSL, YE, minerals)	46	119.82	72.88	0.61	0.41	0.10	0.32	0.28	0.853
Glucose									
Fed-batch (feed: glucose, YE)	54.5	120.04	70.56	0.59	0.37	0.31	0.14	0.15	0.803
Electrochemical reactor (feed: glucose, YE, minerals)	48.5	133.39	90.68	0.68	0.46	0.23	0.14	0.17	0.870
Xylose									
Fed-batch (feed: xylose, YE)	47	123.50	69.23	0.56	0.42	0.15	0.28	0.28	0.799
Electrochemical reactor (feed: xylose, YE, minerals)	47.5	144.24	91.34	0.63	0.46	0.09	0.20	0.23	0.802
Fed-batch (feed: xylose)	72	135.70	76.57	0.56	0.30	0.17	0.23	0.23	0.773
Electrochemical reactor (feed: xylose)	29.75	84.61	47.67	0.56	0.64	0.09	0.30	0.35	0.814
Fed-batch (feed: xylose, YE, minerals)	48	30.30	13.73	0.45	0.09	0	0.34	0.55	0.721

[1] All metabolic product yields were calculated based on the quantities (g) of the organic acids produced during fermentation and removed during sampling and the quantities of individual sugars (g) consumed during fermentation, added via feeding and removed via sampling. The volumes considered included the volume of the fermentation broth, the volume of samples removed, the volume of feeding solution added and the volume of NaOH added. Biomass could not be measured as the microorganism creates biofilm.

[2] Biomass and yeast extract were not included in the carbon balance equations. Except for the carbon source (glucose or xylose), CO_2 was included as a reactant to the calculations taking into consideration that 1 mol of CO_2 is necessary for the production of 1 mol of succinic acid.

Figure 8.12 presents the succinic acid production rate achieved in control cultures and integrated fermentation and separation cultures. It can be easily observed that the production rate of succinic acid was higher in integrated cultures than the respective control cultures during the first 10 h of fermentation when SSL and glucose were used as carbon sources (Figures 8.12A and B). When xylose was used higher production rates were achieved at fermentation times higher than 10 h.

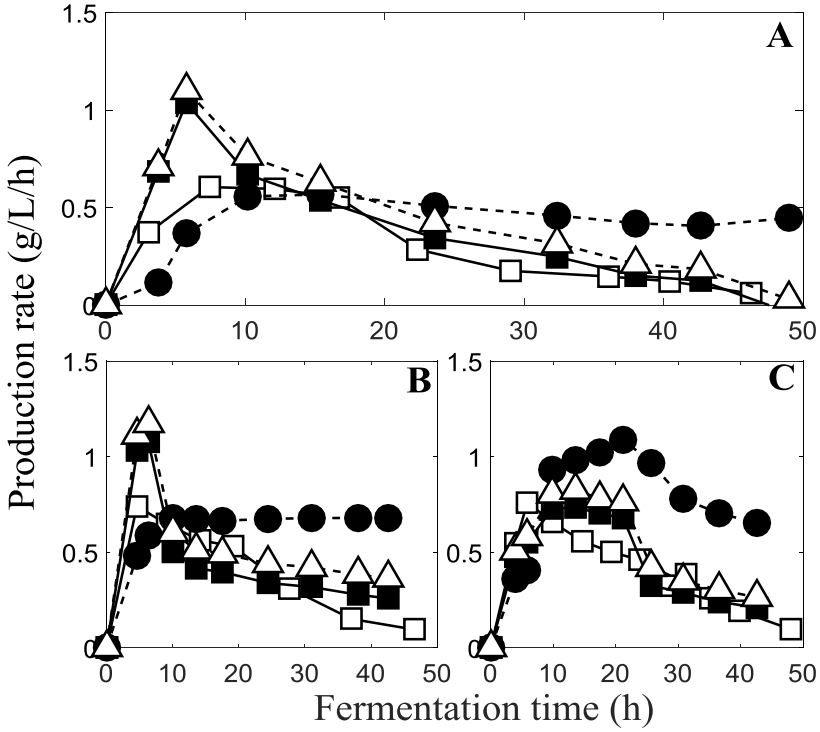

Figure 8.12 Succinic acid production rate of *B. succiniciproducens* in control fed-batch bioreactor fermentations and in fed-batch fermentations with *in-situ* extraction of metabolic products when it is cultivated on SSL (A), glucose (B) and xylose (C). Control fermentation (□), fermentation broth in electrochemical bioreactor (■), extract (●) and fermentation broth in electrochemical bioreactor plus extract (△).

Figure 8.13 presents the ratio each by-product to succinic acid (on g basis) for the three set of experiments carried out on SSL, glucose and xylose. The ratios of FA:SA and AA/SA were lower in the case when *B. succiniciproducens* was cultivated on SSL using the electrochemical bioreactor (Figure 8.12.A), while the LA:SA ratio was higher than the control culture. The LA:SA ratio was reduced in the integrated system as compared to the respective control cultures when xylose and glucose were used as carbon sources. When glucose was used, the FA:SA was similar and the AA:SA ratio was slightly increased as compared to the control culture (Figure 8.13B). All ratios were decreased in the case of xylose cultivation in

the integrated system. In general, the overall by-product formation was decreased when the integrated system was employed. As mentioned before, this could be attributed to the supply of hydrogen gas during water electrolysis that occurs inside the extraction cell.

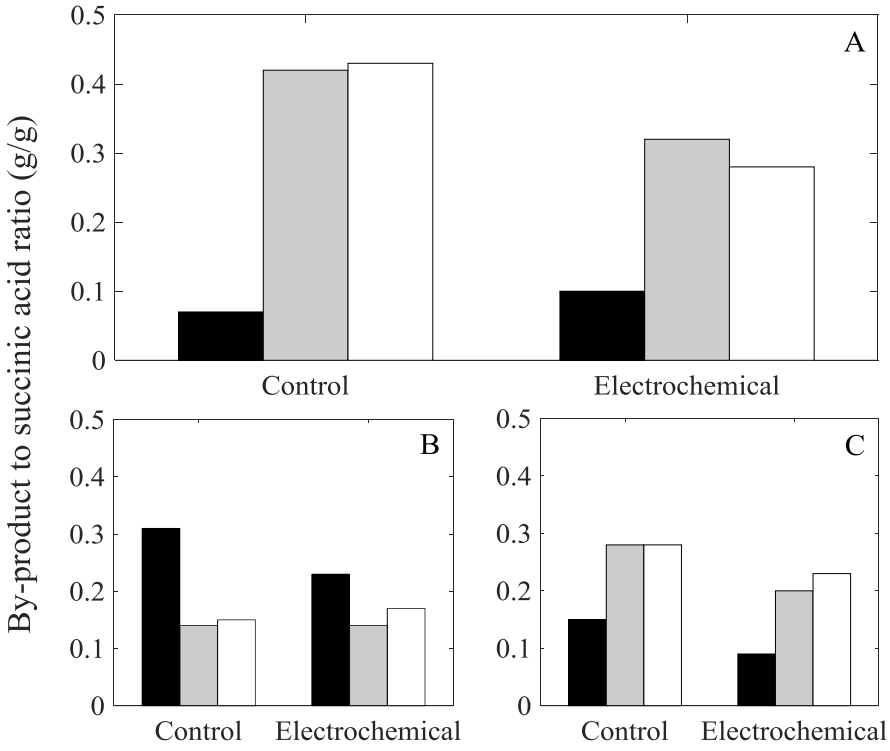

Figure 8.13 Final by-product to succinic acid ratio of *B. succiniciproducens* in control fed-batch bioreactor fermentations and in fed-batch bioreactor fermentations with in-situ extraction of metabolic products when cultivated on SSL (A), glucose (B) and xylose (C). LA:SA (black bars), FA:SA (grey bars), AA:SA (white bars).

Figure 8.14 presents the extraction rate of metabolic products in the integrated systems for all carbon sources used. The highest extraction rates for succinic acid, formic acid and acetic acid were achieved when xylose was used as carbon source. The *in-situ* extraction of organic acids is vital for the improvement of fermentation efficiency. *B. succiniciproducens* produces succinic acid from the reductive pathway of TCA cycle (Becker et al., 2013). Specifically after carbon source degradation, phosphoenolpyruvate (PEP) can either be converted to oxaloacetate (OAA), which will lead to succinic acid production through the C4 pathway (TCA cycle), or pyruvate, that could be converted into lactate, formate, acetate and ethanol via the C3 pathway (pyruvate metabolism). The production of organic acids can become inhibitory to the cells at high concentrations. The critical concentration of metabolic products for *B. succiniciproducens* growth has been determined (see Chapter 5) at 35 g/L of the organic acid mixture.

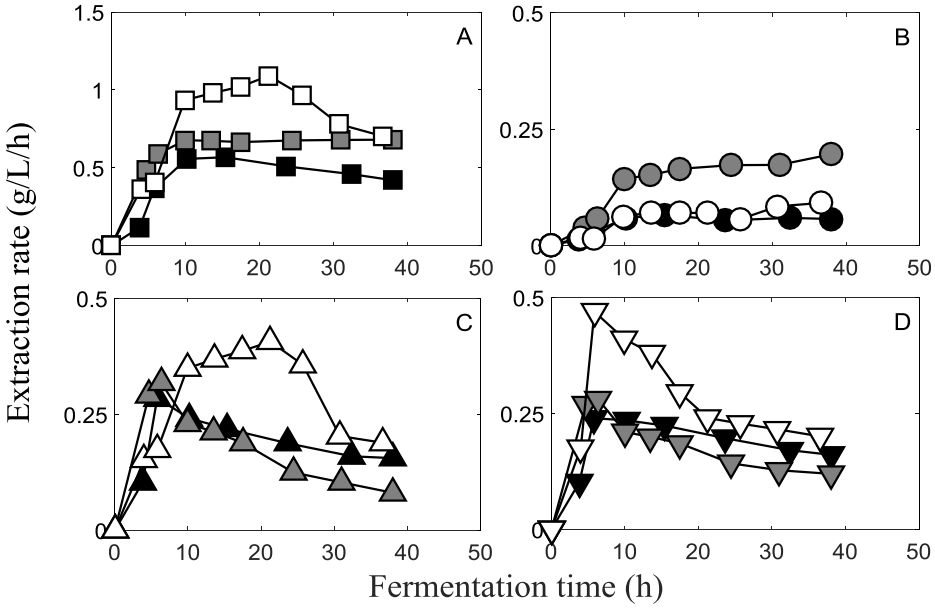

Figure 8.14 Extraction rate of the produced acids during fed-batch fermentations with *in-situ* extraction of metabolic products using a membrane electrochemical bioreactor when the carbon source used was SSL (black symbols), glucose (grey symbols) and xylose (white symbols). Succinic acid (A), Lactic acid (B), Formic acid (C), Acetic acid (D).

The utilisation of electrochemical cells on succinic acid production via fermentation has been also investigated by Park et al. (1999) with glucose and Zhao et al. (2016) with corncob hydrolysates using a cation selective membrane, where only cations can pass through the membrane. Succinic acid production by *A. succinogenes* in both cases was enhanced due to the availability of more reducing power. In this study, it is the first time to apply an electrochemical cell in succinic acid fermentation using an anion selective membrane in order to extract organic acids *in-situ*.

Another advantage of the proposed integrated process is that it can be applied in fermentations using either yeast or bacterial strains. The electrochemical bioreactor removes the charged molecules (e.g. succinate, formate, acetate anions) during fermentation. The integrated separation of anions produced during fermentation could lead to higher overall volumetric productivity due to the simultaneous removal of organic acids, the accumulation of which in the fermentation broth inhibit microbial growth. Furthermore, the succinic acid is separated in the acid form and not in the salt form. On top of that, the cells and the color do not pass through the membrane. This means that some downstream separation steps, including the removal of cells, the removal of proteins/color and acidification of the succinate salt, are

not needed. Another advantage of the integrated system is that the succinic acid concentration in the aqueous extracted solution can reach high concentrations reducing the energy requirements for evaporation.

As a conclusion, both yeast and bacterial cultures should be evaluated in the integrated system in order to identify the optimal process. Bacterial cultures have the advantage of being able to consume a greater variety of carbon sources than yeast cultures (depending always on the strain). The metabolic process involves the oxidation of a substrate and the transfer of electrons to a final electron acceptor. Fumarate is used as an electron acceptor in fumarate respiration, which is converted into succinic acid (Guettler et al., 1999) and excreted through an antiporter (Padan, 2014). Pyruvate is oxidized to release reducing power (i.e. NADH) that can be used in the C4 pathway. Thus, the provision of more reduced carbon sources as well as the supply of reducing power from external sources (e.g. electrically reduced neutral red in an electrochemical bioreactor system) could enhance the production of succinate and ATP under controlled conditions (Li et al., 2010). When H_2 or electricity is not used as additional reducing power, pyruvate should be oxidized in order to produce an electron donor for fumarate reduction resulting in decreased succinate yield and ATP synthesis via electron transport-mediated phosphorylation (Park et al., 1999).

The redox potential or oxido-reduction potential is a measure of the tendency towards reduction or oxidation. Li et al. (2010) investigated the influence of the redox potential on the fermentation profile of *A. succinogenes* NJ113 by addition of potassium ferricyanide as the oxidation agent and dithiotreitol as the reducing agent. At a redox potential level of -350 mV, succinic acid production, yield and productivity increased and by-product formation decreased compared to the rest of the redox potential levels tested (no redox potential regulation, -100, -300, -350, -400, -450 mV). This occurred due to a significantly higher NADH/NAD+ ratio observed at -350 mV (Li et al., 2010).

Xi et al. (2012) reported that biotin concentrations in the range of 2-10 mg/L result in enhanced succinic acid production when a chemically defined medium is used. Considering that succinic acid is formed from fumaric acid, the role of biotin in the pathway is to provide cytochrome b and electron transfer exits (Xi et al., 2012). Furthermore, when 5-aminolevulinic acid (a biosynthetic precursor of heme) or heme (an electron carrier that can increase the reduction potential) replaced biotin in the chemically defined medium, similar fermentation results were obtained indicating that biotin biosynthesis could be achieved from 5-aminolevulinic acid.

Conceptually, in an industrial biotechnology context, the system will consist of a classic bioreactor coupled in side-stream to a high-rate electrochemical cell performing the extractions. The key advantage towards product harvest itself is that the acid concentrate supports reactions such as crystallisation (e.g. succinic acid), phase separation (e.g. caproic acid) and esterification (e.g. acetate to ethyl acetate). Succinic acid production can theoretically be executed as a continuous process by incorporating a membrane electrolysis extraction.

Chapter 9

Conclusions and future perspective

Bio-based succinic acid production will soon evolve into a key platform chemical for the future sustainable chemical industry. It can be produced by numerous renewable resources including agricultural residues and industrial side streams. However, technological innovations are required in all processing stages including feedstock pretreatment, fermentation and downstream separation in order to reduce the overall succinic acid production cost to around 1 $/kg.

The factors contributing to the fermentative production of succinic acid were reviewed in Chapter 2 with particular focus on raw materials, culture conditions, significance of carbon dioxide availability and downstream separation and purification. The metabolic potential of succinic acid production was evaluated through discussion of the pathways involved in succinic acid production. The importance of by-product formation during fermentation that affects succinic acid production efficiency was also addressed. The prospect of integrating succinic acid production in future biorefineries was briefly assessed showing that the integration of bioprocesses in existing industrial plants would facilitate the establishment of succinic acid production. The SSL that is produced as by-product from the pulp and paper industry was highlighted as a potential feedstock for the production of succinic acid.

The experimental work was initially focused on the evaluation of the inhibitory effect of SSL components and metabolic products produced during fermentation on succinic acid production and microbial growth (Chapters 5 and 6). Based on this analysis, the lignosulphonates and the accumulation of metabolic products (e.g. succinic acid, acetic acid, formic acid, lactic acid) were the most important inhibitors as other potential inhibitors evaluated (e.g. furfural and methanol) were present in low concentrations in initial fermentation broths. Thus, it was decided that SSL pretreatment via ultrafiltration or nanofiltration should be applied in order to increase the fermentation efficiency by both *A. succinogenes* and *B. succiniciproducens*. Ultrafiltration of SSL resulted in low recovery of lignosulphonates in the retentate stream. Nanofiltration led to high recovery of lignosulphonates (95.6%) in the retentate stream. The lower losses of sugars in the retentate stream were achieved via SSL ultrafiltration. Fermentations were carried using permeate streams from both ultrafiltrated and nanofiltrated SSL with either *A. succinogenes* or *B. succiniciproducens*. The highest succinic acid concentration (33.8 g/L), yield (0.58 g/g) and productivity (0.48 g/L/h) were achieved by *B. succiniciproducens* cultures with nanofiltrated SSL. Lower succinic acid production (52.7 kg) and higher lignosulphonate recovery (306.3 kg) could be achieved via nanofiltration of 1 t of thick SSL than ultrafiltration of 1 t of SSL

which leads to the production of 71.8 kg of succinic acid and 237 kg of lignosulphonates, when *B. succiniproducens* cultures are employed in both cases.

Emphasis was given on the evaluation of the metabolic potential of *A. succinogenes* through RNA expression of the metabolic pathways involved in succinic acid production (Chapter 7). This analysis was performed in batch cultures conducted with glucose, xylose or SSL. Different sampling times were selected in order to construct cDNA libraries on lag, exponential and stationery phases of bacterial growth. The expression level of the genes was determined via RT-qPCR. The genes that were mostly affected by the presence of inhibitors in SSL, where those that encode for enzymes that participate in oxidative phosphorylation, specifically ATP synthase was overexpressed in SSL. Also genes that encode for enzymes that contribute to acetyl-CoA production were overexpressed.

In Chapter 8, a novel electrochemical bioreactor was employed in order to evaluate the integration of succinic acid production via fermentation and separation via electrochemical membrane extraction. The main advantage of this system is the fact that the charged carboxylic acids are directed via electromigration across an anion exchange membrane from the fermentation broth into a low-volume extract that contained the extracted succinic acid at higher concentrations. Potential benefits of the application of the integrated system is the improvement of fermentation efficiency, the reduced need for alkaline solution to maintain the pH value of the fermentation broth, the elimination of several downstream separation steps (e.g. bacterial mass and color removal, acidification) required in conventional succinic acid production processes. Singificant improvement on succinic acid production (up to 45%) was achieved by applying membrane electrolysis extraction.

The results presented in this PhD thesis demonstrate that SSL is a promising feedstock for succinic acid production and the integrated system deserves further optimisation. Future research initiatives should focus on:

1) the development of continuous cultures using SSL as feedstock for succinic acid production,
2) the selection of a highly efficient membrane and the appropriate configuration of the integrated system in order to optimize the production and separation of succinic acid,
3) the optimization of succinic acid separation and purification from the extracted solution derived via membrane electrochemical extraction, and
4) sustainability analysis including techno-economic evaluation and life cycle analysis of the proposed bioprocesses.

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