AGRICULTURAL UNIVERSITY OF ATHENS
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LABORATORY OF FOOD ENGINEERING, PROCESSING AND PRESERVATION OF AGRICULTURAL PRODUCTS

# "Bioprocess development for the production of 1,3-propanediol and 2,3-butanediol using food waste and crude renewable resources from biodiesel and cane sugar production processes"

A thesis submitted for the degree of

**DOCTOR OF PHILOSOPHY**

**by**

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**ATHENS 2019**

ΓΕΩΠΟΝΙΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ
ΤΜΗΜΑ ΕΠΙΣΤΗΜΗΣ ΤΡΟΦΙΜΩΝ ΚΑΙ ΔΙΑΤΡΟΦΗΣ ΤΟΥ ΑΝΘΡΩΠΟΥ
ΕΡΓΑΣΤΗΡΙΟ ΜΗΧΑΝΙΚΗΣ ΤΡΟΦΙΜΩΝ, ΕΠΕΞΕΡΓΑΣΙΑΣ & ΣΥΝΤΗΡΗΣΗΣ
ΓΕΩΡΓΙΚΩΝ ΠΡΟΪΟΝΤΩΝ

# "Ανάπτυξη βιοδιεργασιών για την παραγωγή 1,3-προπανοδιόλης και 2,3-βουτανοδιόλης με χρήση αποβλήτων τροφίμων και ακατέργαστων ανανεώσιμων πρώτων υλών από βιομηχανίες παραγωγής βιοντήζελ και επεξεργασίας ζαχαροκάλαμου"

**ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ**

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**ΑΘΗΝΑ 2019**

# PhD Thesis

**“Bioprocess development for the production of 1,3-propanediol and 2,3-butanediol using food waste and crude renewable resources from biodiesel and cane sugar production processes”**

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**“Ανάπτυξη βιοδιεργασιών για την παραγωγή 1,3-προπανοδιόλης και 2,3-βουτανοδιόλης με χρήση αποβλήτων τροφίμων και ακατέργαστων ανανεώσιμων πρώτων υλών από βιομηχανίες παραγωγής βιοντήζελ και επεξεργασίας ζαχαροκάλαμου”**

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**“Bioprocess development for the production of 1,3-propanediol and 2,3-butanediol using food waste and crude renewable resources from biodiesel and cane sugar production processes”**

# Abstract

The development of a bio-based and circular economy has been identified as a necessary step to achieve the successful transition into a sustainable economy independent of fossil resources. The development of novel sustainable technologies is required for efficient conversion of renewable raw materials into bio-based chemicals, polymers, fuels and energy. This thesis presents novel research on bioprocess development for the production of 1,3-propanediol (PDO), 2,3-butanediol (BDO) and acetoin from food waste and crude renewable resources from biodiesel and cane sugar production plants. The aforementioned bio-based chemicals could be used as additives in high-value applications (e.g. flavour and fragrances) or precursors for the production of bio-based polymers and commodity products.

Soybean cake (SBC) and crude glycerol, produced as by-products from biodiesel production processes using soybean as feedstock, were used as sole raw materials for PDO production using two bacterial strains of *Citrobacter freundii*. SBC was converted into a nutrient-rich hydrolysate by crude enzymes produced via solid state fermentation. The effect of initial glycerol and free amino nitrogen concentration on bacterial growth and PDO production was evaluated in batch bioreactor cultures showing that *C. freundii* VK-19 is a more efficient PDO producer than *C. freundii* FMCC-8. The cultivation of *C. freundii* VK-19 in fed-batch bioreactor cultures using crude glycerol and SBC hydrolysates led to PDO concentration of 47.4 g $L^{-1}$ with yield and productivity of 0.49 g $g^{-1}$ and 1.01 g $L^{-1}$ $h^{-1}$, respectively. The effect of PDO, metabolic by-products and sodium and potassium salts on bacterial growth was evaluated showing that potassium salts initially enhance bacterial growth, whereas sodium salts cause significant inhibition to bacterial growth.

Different crude glycerols, derived from a biodiesel production plant using either soybean oil alone or two mixtures of soybean oil and tallow fat as feedstocks, were evaluated for BDO production by *Klebsiella michiganensis*. The effect of nitrogen source, temperature and initial glycerol concentration on bacterial growth and BDO production was studied in shake flask cultures. Using a synthetic medium with ammonium sulphate as nitrogen source favoured BDO production. The optimum temperature was 30 ˚C. The bacterial strain *K. michiganensis* could grow and produce BDO even at high initial crude glycerol concentrations when this stream was derived from biodiesel production using only soybean oil. BDO production by *K. michiganensis* was subsequently evaluated in fed-batch bioreactor fermentations using different types of crude glycerol and various volumetric oxygen transfer coefficients ($k_La$). The highest BDO

concentration (87.6 g $L^{-1}$) was reached using crude glycerol produced from soybean oil derived biodiesel with constant $k_La$ value of 62 $h^{-1}$ during fermentation. The highest productivity (1.38 g $L^{-1}$ $h^{-1}$) with reasonable BDO concentration (76.1 g $L^{-1}$) and yield (0.41 g $g^{-1}$) were reached when the same glycerol and $k_La$ value of 77 $h^{-1}$ were used. Lower BDO production efficiency was achieved with crude glycerols derived from biodiesel production with soybean oil and tallow fats as feedstock.

The production of D-BDO and acetoin using the GRAS strain *Bacillus amyloliquefaciens* from very high polarity (VHP) cane sugar and sugarcane molasses has been evaluated. The effect of initial substrate concentration, nitrogen source, temperature, inoculum size and pH has been evaluated in shake flask cultures. Batch bioreactor cultures showed that D-BDO production (up to 28 g $L^{-1}$) with high yield (up to 0.43 g $g^{-1}$) and productivity (up to 0.93 g $L^{-1}$ $h^{-1}$) was favored at low $k_La$ values (17 – 49 $h^{-1}$). Increasing $k_La$ values (63 – 104 $h^{-1}$) caused metabolic shift towards acetoin production (up to 25.6 g $L^{-1}$) with yields up to 0.4 g $g^{-1}$ and productivities up to 1.42 g $L^{-1}$ $h^{-1}$. Fed-batch fermentation carried out at $k_La$ value of 49 $h^{-1}$ with VHP cane sugar resulted in mixed production (127.3 g $L^{-1}$) of D-BDO, meso-BDO and acetoin at 122 h with high yield (0.50 g $g^{-1}$) and productivity (1.04 g $L^{-1}$ $h^{-1}$). In a similar fed-batch fermentation with sugarcane molasses, mainly D-BDO production (48.7 g $L^{-1}$ with purity of 87.8%) was achieved at 59 h with yield of 0.40 g $g^{-1}$ and productivity of 0.83 g $L^{-1}$ $h^{-1}$. At 130 h, D-BDO was converted mainly into acetoin (55.4 g $L^{-1}$). Fed-batch cultures with sugarcane molasses could lead to either D-BDO or acetoin production with negligible by-product formation.

The GRAS strain *B. amyloliquefaciens* was also evaluated for D-BDO and acetoin production using enzymatically produced bakery waste hydrolysates in bench top and pilot scale bioreactors. Manipulation of $k_La$ during fermentation may control metabolic shift in *B. amyloliquefaciens* cells towards either D-BDO or acetoin production. Batch cultures with $k_La$ value of 64 $h^{-1}$ led to the highest D-BDO concentration (55.2 g $L^{-1}$) with a yield of 0.42 g $g^{-1}$. The highest acetoin concentration (47.4 g $L^{-1}$) with productivity of 2.16 g $L^{-1}$ $h^{-1}$ was achieved in batch cultures with the highest $k_La$ value (203 $h^{-1}$) used. It was verified that $k_La$ can be used as scale-up parameter for conducting fermentations in 50 L bioreactor scale. Fed-batch fermentation may increase further D-BDO and acetoin production efficiency.

**Keywords:** bio-based chemicals, agro-industrial by-products, bioconversion, bioprocess, food waste

# Περίληψη

Η παραγωγή προϊόντων βιο-οικονομίας αποτελεί μια νέα αναπτυξιακή στρατηγική για τη μετάβαση προς μια βιώσιμη και ενεργειακά ανεξάρτητη από ορυκτά καύσιμα οικονομία. Η ανάπτυξη καινοτόμων και αειφόρων τεχνολογιών απαιτείται για τη χρήση ανανεώσιμων πρώτων υλών προς την παραγωγή χημικών ουσιών, πολυμερών, καυσίμων και ενέργειας. Η παρούσα διδακτορική διατριβή εστιάζει στην ανάπτυξη βιοδιεργασιών βασισμένων στη βιομηχανική βιοτεχνολογία για την παραγωγή 1,3-προπανοδιόλης (PDO), 2,3-βουτανοδιόλης (BDO) και ακετοΐνης με χρήση αποβλήτων τροφίμων και ακατέργαστων ανανεώσιμων πρώτων υλών από βιομηχανίες παραγωγής βιοντήζελ και ζάχαρης από ζαχαροκάλαμο.

Σογιάλευρα και ακατέργαστη γλυκερόλη που παράγονται ως παραπροϊόντα από διεργασίες παραγωγής βιοντήζελ χρησιμοποιήθηκαν ως πρώτες ύλες για την παραγωγή PDO μέσω ζύμωσης με δύο βακτηριακά στελέχη *Citrobacter freundii*. Τα σογιάλευρα χρησιμοποιήθηκαν για την παραγωγή υδρολύματος πλούσιου σε θρεπτικά συστατικά με χρήση ακατέργαστων ενζύμων που παρήχθησαν μέσω ζύμωσης στερεάς κατάστασης. Η επίδραση της αρχικής συγκέντρωσης γλυκερόλης και του αζώτου των ελευθέρων αμινομάδων αμινοξέων και πεπτιδίων στην μικροβιακή ανάπτυξη και στην παραγωγή PDO μελετήθηκε σε καλλιέργειες διαλείποντος έργου. Ικανοποιητική παραγωγή PDO σημειώθηκε από το βακτηριακό στέλεχος *C. freundii* VK-19 συγκριτικά με το *C. freundii* FMCC-8. Η πραγματοποίηση ζυμώσεων ημι-διαλείποντος έργου με χρήση ακατέργαστης γλυκερόλης και υδρολύματος σογιάλευρου οδήγησε στην παραγωγή 47,4 g $L^{-1}$ PDO με απόδοση 0,49 g $g^{-1}$ και παραγωγικότητα 1,01 g $L^{-1}$ $h^{-1}$. Τα άλατα καλίου αρχικά αύξησαν την μικροβιακή ανάπτυξη, ενώ τα άλατα νατρίου προκαλούν σημαντική παρεμπόδιση της μικροβιακής ανάπτυξης.

Η παραγωγή BDO με χρήση διαφορετικών ακατέργαστων γλυκερολών μελετήθηκε χρησιμοποιώντας το βακτηριακό στέλεχος *Klebsiella michiganensis*. Μελετήθηκε η επίδραση της πηγής αζώτου, της θερμοκρασίας και της αρχικής συγκέντρωσης γλυκερόλης δείχνοντας ότι η ανόργανη πηγή αζώτου και η θερμοκρασία των 30 ˚C μεγιστοποιούν την παραγωγή BDO. Το βακτηριακό στέλεχος *K. michiganensis* αναπτύσσεται και παράγει BDO ακόμα και σε υψηλές αρχικές συγκεντρώσεις ακατέργαστης γλυκερόλης. Ακολούθως, μελετήθηκε η παραγωγή BDO μέσω ζυμώσεων ημι-διαλείποντος έργου που πραγματοποιήθηκαν υπό διαφορετικές τιμές $k_La$ και τύπους ακατέργαστων γλυκερολών. Η υψηλότερη συγκέντρωση BDO (87,8 g $L^{-1}$) παρήχθη με χρήση τιμής $k_La$ ίσης με 62 $h^{-1}$. Η μεγαλύτερη παραγωγικότητα (1,38 g $L^{-1}$ $h^{-1}$) με συγκέντρωση BDO ίσης με 76,1 g $L^{-1}$ και απόδοσης ίσης με 0,41 g $g^{-1}$

επιτεύχθηκε σε τιμή $k_La$ ίσης με 77 $h^{-1}$. Η χρήση ακατέργαστης γλυκερόλης προερχόμενη από μείγμα σογιελαίου και ζωικού λίπους είχε ως αποτέλεσμα τη μείωση της παραγωγής BDO.

Ακατέργαστη ζάχαρη και μελάσα μελετήθηκαν ως πρώτες ύλες για την παραγωγή D-BDO και ακετοΐνης από το βακτηριακό στέλεχος *Bacillus amyloliquefaciens*. Αρχικά, μελετήθηκε η επίδραση της πηγής αζώτου, της θερμοκρασίας, του εμβολίου, της παροχής οξυγόνου και του pH στη μικροβιακή αύξηση και στην παραγωγή μεταβολικών προϊόντων μέσω ζυμώσεων σε αναδευόμενες φιάλες. Ζυμώσεις διαλείποντος έργου σε βιοαντιδραστήρα σε μικρές τιμές $k_La$ (17 – 49 $h^{-1}$) ευνοούν την παραγωγή D-BDO, ενώ υψηλότερες τιμές $k_La$ (63 – 104 $h^{-1}$) ευνοούν την παραγωγή ακετοΐνης. Στη συνέχεια, πραγματοποιήθηκαν ζυμώσεις ημι-διαλείποντος έργου χρησιμοποιώντας ακατέργαστη ζάχαρη και μελάσα. Η χρήση διαφορετικών διαλυμάτων τροφοδοσίας οδήγησε σε διαφορετική αναλογία των ισομερών της BDO και της ακετοΐνης κατά τη διάρκεια της ζύμωσης. Η χρήση ακατέργαστης μελάσας οδήγησε στην παραγωγή κυρίως D-BDO (48,7 g $L^{-1}$ με καθαρότητα 87,8%) στις 59 h ζύμωσης με απόδοση 0,40 g $g^{-1}$ και παραγωγικότητα 0,83 g $L^{-1}$ $h^{-1}$. Στις 130 h, η D-BDO μετατράπηκε κυρίως σε ακετοΐνη (55,4 g $L^{-1}$). Πραγματοποίηση ζύμωσης με αποκλειστική χρήση μελάσας οδήγησε στην παραγωγή είτε D-BDO είτε ακετοΐνης, ενώ ταυτόχρονα δεν παρατηρήθηκε παραγωγή άλλων μεταβολικών προϊόντων.

Το βακτηριακό στέλεχος *Bacillus amyloliquefaciens* αξιολογήθηκε ως προς την παραγωγή D-BDO και ακετοΐνης με χρήση υδρολύματος από απόβλητα αρτοποιίας που παρήχθη με χρήση ενζύμων. Η μεταβολή της τιμής του $k_La$ κατά τη διάρκεια της ζύμωσης μπορεί να χρησιμοποιηθεί για να στρέψει το μεταβολισμό του *B. amyloliquefaciens* προς την παραγωγή είτε D-BDO είτε ακετοΐνης. Ζυμώσεις διαλείποντος έργου σε εργαστηριακό βιοαντιδραστήρα και τιμή $k_La$ ίσης με 64 $h^{-1}$ είχε ως αποτέλεσμα την παραγωγή της υψηλότερης συγκέντρωσης D-BDO (55,1 g $L^{-1}$) με απόδοση 0,42 g $g^{-1}$. Η μεγαλύτερη συγκέντρωση ακετοΐνης (47,4 g $L^{-1}$) και παραγωγικότητας (2,31 g $L^{-1}$ $h^{-1}$) επετεύχθη σε ζυμώσεις διαλείποντος έργου με τιμή $k_La$ ίσης με 203 $h^{-1}$. Τα πειραματικά αποτελέσματα επιβεβαίωσαν ότι το $k_La$ μπορεί να χρησιμοποιηθεί για την αύξηση κλίμακας ζυμώσεων σε βιοαντιδραστήρα 50 λίτρων. Η πραγματοποίηση ζυμώσεων ημι-διαλείποντος έργου δύναται να αυξήσει την παραγωγή D-BDO και ακετοΐνης.

**Λέξεις κλειδιά:** βιοχημικά, αγρο-βιομηχανικά απόβλητα, βιομετατροπή, βιοδιεργασία, απόβλητα τροφίμων

# Dissemination activities

**List of publications**

1. **Maina, S.**, Mallouchos, A, Nychas, G.J.E., Freire, D.M.G., de Castro, A.M., Papanikolaou, S., Kookos, I.K. and Koutinas, A., 2019 Bioprocess development for (2R,3R)-butanediol and acetoin production using very high polarity cane sugar and sugarcane molasses by a *Bacillus amyloliquefaciens* strain. Journal of Chemical Technology & Biotechnology
2. **Maina, S.**, Kachrimanidou, V., Ladakis, D., Papanikolaou, S., de Castro, A.M. and Koutinas, A.A., (Accepted). Evaluation of 1,3-propanediol production by two *Citrobacter freundii* strains using crude glycerol and soybean cake hydrolysate. *Environmental Science and Pollution Research*
3. **Maina, S.**, Stylianou, E., Vogiatzi, E., Vlysidis, A., Mallouchos, A., Nychas, G.J.E., de Castro, A.M., Dheskali, E., Kookos, I.K. and Koutinas, A., 2019. Improvement on bioprocess economics for 2,3-butanediol production from very high polarity cane sugar via optimisation of bioreactor operation. *Bioresource Technology*, 274, pp.343-352.
4. **Maina, S.**, Kachrimanidou, V., Koutinas, A., 2017. From waste to bio-based products: A roadmap towards a circular and sustainable bioeconomy. *Current Opinion in Green and Sustainable Chemistry*, *8*, pp.18-23
5. **Maina, S.**, Pateraki, C., Kopsahelis, N., Paramithiotis, S., Drosinos, E. H., Papanikolaou, S., & Koutinas, A., 2017. Microbial oil production from various carbon sources by newly isolated oleaginous yeasts. *Engineering in Life Sciences*, 17. pp.333-344.

**Conference oral presentations**

1. **Maina, S.**, Papanikolaou, S., Castro, A.M. and Koutinas A. Bioprocess development for the production of (2R,3R)-butanediol and acetoin using a newly isolated GRAS strain. "4$^{th}$ Iberoamerican Congress on Biorefineries". Jaen, Spain 24-26 October, 2018.
2. **Maina, S.**, Ladakis D., Papanikolaou S., Castro, A.M. and Koutinas A. Valorization of by-products from soybean-based biodiesel production plant for 1,3-propanediol production. "6th International Conference on Sustainable Solid Waste Management". Naxos, Greece 13-16 June, 2018.
3. Sdrallis, V., **Maina, S.**, Vlysidis, A., Ladakis, D., Papanikolaou, S., Castro, A.M. and Koutinas, A. Enhanced 2,3-butanediol production by *Enterobacter ludwigii* in fed-batch and continuous culture: Substrate and product inhibition from sugarcane. "6th International Conference on Sustainable Solid Waste Management". Naxos, Greece 13-16 June, 2018.

4. **Maina, S.**, Vogiatzi, E., Stylianou, E., Vlysidis, A., Papanikolaou, S., Castro, A.M. and Koutinas A. Enhanced 2,3-butanediol production by *Enterobacter ludwigii* from sugarcane molasses. "10th World Congress of Chemical Engineering". Barcelona, Spain 1-5 October, 2017. **Award: Best oral communication** - Topic 6: Applied Biotechnology (integrated in ECAB4)
5. **Maina, S.**, Kachrimanidou, V., Kopsahelis, N., Vlysidis, A., Koukos I. and Koutinas, A. Utilization of sunflower-based biodiesel industry by-products for the development of an integrated biorefinery concept. Bioprocessing India 2016. "Sustainable Bioprocessing Products for Food, Nutrition, Health and Environment". Mohali, India 15-17 December, 2016.
6. **Maina, S.** and Koutinas, A. Utilization of by product streams from soybean-based biodiesel production plants for 1,3-propanediol production. Cost Action TD1203. Food waste valorisation for sustainable chemicals, materials & fuels (EUBis) WG2 Workshop: "Development of pilot scale or integrated bioprocesses based on refining of food supply chain wastes". Potsdam, 29-30 September, 2015.
7. **Maina, S.**, Papadaki, A., Pateraki, C., Kopsahelis, N., Papanikolaou, S. and Koutinas, A. Microbial oil production by newly isolated yeast strains and novel industrial applications based on waste and by-product valorization. 3rd International conference on Sustainable Solid Waste Management, Tinos, 2-4 July, 2015.

**Conference poster presentations**

1. **Maina, S.**, Kachrimanidou, V., Ladakis, D., Papanikolaou, S., Castro A.M., Koutinas A. Valorization of by-products from soybean–based biodiesel production plant for 1,3-propanediol production. Bioprocessing India 2016. "Sustainable Bioprocessing Products for Food, Nutrition, Health and Environment". Mohali, India 15-17 December, 2016. **Award: 2nd prize in the Industrial Bioprocessing & Bioproducts**
2. **Maina, S.**, Pateraki, C., Papadaki, A., Paramythiotis, S., Drosinos, E., Papanikolaou, S. and Koutinas, A. Microbial oil production from various carbon sources by newly isolated oleaginous yeasts. Cost Action TD1203. Food waste valorisation for sustainable chemicals, materials & fuels (EUBis) "The future of food waste: challenges and opportunities for valorisation in Europe". Wageningen, 20-21 September, 2016.
3. Pateraki, C., Kachrimanidou, V., **Maina, S.**, Papadaki, A., Tsouko, E., Kopsahelis, N., Papanikolaou, S. and Koutinas, A. Effect of nutrient supplements derived from various industrial waste and by-products streams on succinic acid production via fermentation. 12$^{th}$

International conference on Renewable Resources and Biorefineries. Ghent, Belgium 30-31 May and 1 June, 2016.

4. **Maina, S.**, Pateraki, C., Paramythiotis, S., Drosinos, E., Koutinas A. and Papanikolaou, S. Microbial oil production from various carbon sources by newly isolated oleaginous yeasts. Greek lipid forum, Athens, 11-12 June, 2015.

**Research visits**

In 2018 I spent three months at the Department of Bioengineering, Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB) (Potsdam, Germany) within the framework of Erasmus$^{+}$ Program /International Mobility

# Acknowledgements

The experimental part of this thesis was carried out at the Laboratory of Food Process Engineering (Department of Food Science and Human Nutrition), at the Agricultural University of Athens. I gratefully acknowledge the Hellenic Foundation for Research and Innovation (ELIDEK), for providing a scholarship in the framework of the Action: "1st proclamation ELIDEK for PhD candidates" and the funding received towards my PhD from National Agency of Petoleum (ANP), Petrobras (Brazil) (project 00320-2/ 2012).

Firstly, I would like to express my sincere gratitude to my advisor Dr Apostolis Koutinas for the continuous support of my PhD study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research. Besides my advisor, I would like to thank the rest of my thesis committee: Prof. Ioannis Kookos and Dr Serafim Papanikolaou for their insightful comments and encouragement, but also for the questions which incented me to widen my research from various perspectives.

I thank my friends and labmates Dr Aikaterini Papadaki, Dr Vasiliki Kachrimanidou and Dr Erminta Tsouko for the stimulating discussions, for the sleepless nights we were working together, and for all the fun we have had in the last four years. Special thanks to Ourania Kalantzi who were always so helpful and provided me with their assistance throughout my dissertation.

I would also like to thank all the members at the Laboratory of Food Process Engineering, especially Dr Harris Papapostolou, Eleni Stylianou and Olga Psaki for the support I received from the collaborative work. My thanks also go to Dr Nikolaos Kopsahelis for his invaluable advice and feedback on my research and for always being so supportive of my work.

I am also very grateful for the time I spent at Leibniz Institute of Agricultural Engineering and Bio-economy e.V. (ATB) in Germany in the framework of collaborative work funding by Erasmus+. I met interesting people who supported me, each in different ways. Special thanks to Dr Maria Alexandri, Roland Schneider and Dr Joachim Venus.

I also thank my friends for providing support and friendship that I needed. I truly thank Athanasia for sticking by my side, even when I was irritable and depressed.

Last but not the least, I would like to thank my family for always believing in me, supporting me and encouraging me to follow my dreams.

# Table of contents

# Table of contents

# Table of contents

# Table of contents

# List of tables

# List of tables

# List of figures

# List of figures

# List of figures

# List of abbreviations

| | |
|---|---|
| 3-HPA | 3-hydroxypropionaldehyde |
| ALDC | $\alpha$-acetolactate decarboxylase |
| ALDH | aldehyde dehydrogenase |
| ALS | $\alpha$-acetolactate synthase |
| BDHs | 2,3-butanediol dehydrogenases |
| BDO | 2,3-butanediol |
| CSL | corn steep liquor |
| DA | diacetyl |
| DCW | cell dry weight |
| DHA | dihydroxyacetone |
| DHAK | hydroxyacetone kinase |
| DHAP | dihydroxyacetone phosphate |
| FAN | free amino nitrogen |
| FFAs | free fatty acids |
| GDH | glycerol dehydrogenase |
| GDHt | glycerol dehydratase |
| HOR | alcohol dehydrogenase |
| IP | inorganic phosphorus |
| $k_La$ | volumetric mass transfer coefficient, $h^{-1}$ |
| LDH | lactate dehydrogenase |
| MEK | methyl ethyl ketone |
| OUR | oxygen uptake rate, |
| PDO | 1,3-propanediol |
| PDOR | 1,3-propanediol dehydrogenase |
| PTT | polytrimethylene terephthalate |
| RQ | respiratory quotient |
| SBC | soybean cake |
| SMB | simulated moving bed |
| SSF | solid state fermentation |
| TSB | tryptic soy Broth |
| TTMP | 2,3,5,6-tetramethylpyrazine |
| VHP | very high polarity |

## Chapter 1

# Introduction

# Chapter 1

Given the global challenges with respect to $CO_2$ emissions, pollution, global warming and imminent depletion of fossil fuels, bio-based chemical production is an attractive approach towards the development of a sustainable bio-economy based on renewable feedstocks instead of fossil carbon. Advances in bioprocess innovation foster the circularity of the bio-based sector and the whole bio-economy enabling the transformation of current side streams, residues and wastes into value-added products. Global production of bio-based chemicals exceeded $60\times10^6$ t and it is expected to increase with a 16.53% CAGR over the period of 2018-2026 (Anonymous, 2009). Among the bio-based chemicals, the market for C2-C4 diols such as ethylene glycol, propanediols and butanediols is still nascent. C2-C4 diols are platform chemicals containing two hydroxyl groups with various applications including production of solvents, fuels, polymers, cosmetics and pharmaceuticals. Annually, more than $18\times10^6$ t of C2-C4 diols are produced via chemical processes using fossil resources as raw materials (Zhang et al., 2017). Biological production of diols is challenging due to the requirement for highly efficient yield from low cost feedstocks. Recent advances in metabolic engineering and fermentation efficiency have paved the way towards commercialization.

1,3-Propanediol (PDO) is a three carbon platform chemical that can be used as intermediate in solvents, adhesives, resins, detergents and cosmetic production. It is used in the manufacturing of polyethers, polyurethanes and polyesters. PDO is considered one of the most important building blocks applied in polymer industry for the synthesis of polytrimethylene terephthalate (PTT), a polyester used in fibers, textiles, carpets and coatings (Zeng and Sabra, 2011). The global PDO market demand was $146\times10^3$ t in 2014 and is estimated to reach $225.9\times10^3$ t by 2022 with 5.8% annual growth from 2015 to 2022. PTT and polyurethane are the most important end-products with the former accounting for 71.8% of total market volume of PDO and the latter growing with 5.9% annual growth over the forecast period of 2015-2022 (Anonymous, 2015a).

Conventional PDO production is based on chemical synthesis from ethylenoxide (Shell) and acrolein (DuPont). In 2004, DuPont and Genencor commercialized bio-based PDO production through the valorisation of corn-based feedstocks employing a genetically engineered *E. coli* strain (Zeng and Sabra, 2011). Commercial brands namely Susterra® and Zemea® propanediol in addition to Bio-PDO™ provide alternatives for a wide variety of markets. PTT is partially produced from bio-based PDO (27%) and mostly from petro-based terephthalic acid (Aeschelmann and Carus, 2015). Biotechnological production of PDO using glycerol as

substrate has gained attention due to the surplus generated from biodiesel industries. Under this concept, METabolic EXplorer (METEX) developed a green and sustainable fermentation for PDO production using crude glycerol. METEX is targeting a 50% and 40% share of the market for PDO in cosmetics and PTT textile, respectively (Anonymous, 2017a).

2,3-Butanediol (BDO) is another important C4 diol with great potential in the manufacturing of printing inks, perfumes, synthetic rubber, antifreeze agents, fuel additives, foods and pharmaceuticals. BDO can be converted into 1,3-butadiene that is subsequently used as intermediate chemical in the production of synthetic rubber and plastics. Diacetyl, derived from BDO dehydrogenation, can serve as a flavouring agent and food additive in food industry. Another application of BDO includes the production of methyl ethyl ketone (MEK) as effective fuel additive and solvent for resins and lacquers (Celinska and Grajek, 2009). In 2018, the global market of BDO is expected to reach 74.4 kilo t, growing at a CAGR of 3.2% from 2013 to 2018. The global market for MEK is estimated to reach $1.5\times10^6$ t by 2018, growing at a CAGR of 4% from 2013 to 2018 with paints and coating holding 57% of the overall market (Anonymous, 2015b).

Common practice for BDO production is the pyrolysis of diacetate. Nowadays, butenes from crack gases are the main raw material for BDO synthesis. A C4 hydrocarbon fraction is initially obtained after the removal of butadiene and isobutene. Chlorohydration of this fraction with an aqueous chloride solution and subsequent cyclization of chlorohydrins with sodium hydroxide lead to a butane oxide mixture with the following composition: 55% *trans*-2,3 butene oxide, 30% *cis*-2,3-butene oxide, 15% 1,2-butene oxide. Hydrolysis of the mixture (50-bar, 160-220 ˚C, reaction enthalphy *DH*= - 42 kJ/mol) results in a mixture of BDO isomers which can be separated by vacuum fractionation (Gräfje et al., 2000; Ge et al., 2016). Meso-BDO is obtained from *trans*-2-butene via *trans*-2,3-butene oxide, while racemic mixture of D- and L-BDO is formed from *cis*-2-butene via *cis*-2,3-butene oxide (Gräfje et al., 2000). Intensive demand of energy and high cost of the applied catalysts, which lead to high production cost (1600USD/t), remain the major barrier for global BDO market (Bialkowska, 2016a).

Bio-based BDO has successfully been produced by LanzaTEch from waste gas resources in an industrial setting (Simpson et al., 2014). INISTA and LanzaTech collaborate on the production of bio-based butadiene using LanzaTech's carbon monoxide-derived BDO and a direct-step process through a gas fermentation process (Anonymous, 2015c).

# Chapter 1

Successful production of bio-based chemicals that are economic competitive with petrochemical ones requires the development of effective conversion technologies. The key parameters influence the bioprocesses and determine the possibility of large-scale production are the productivity, product concentration and conversion yield (Figure 1.1).

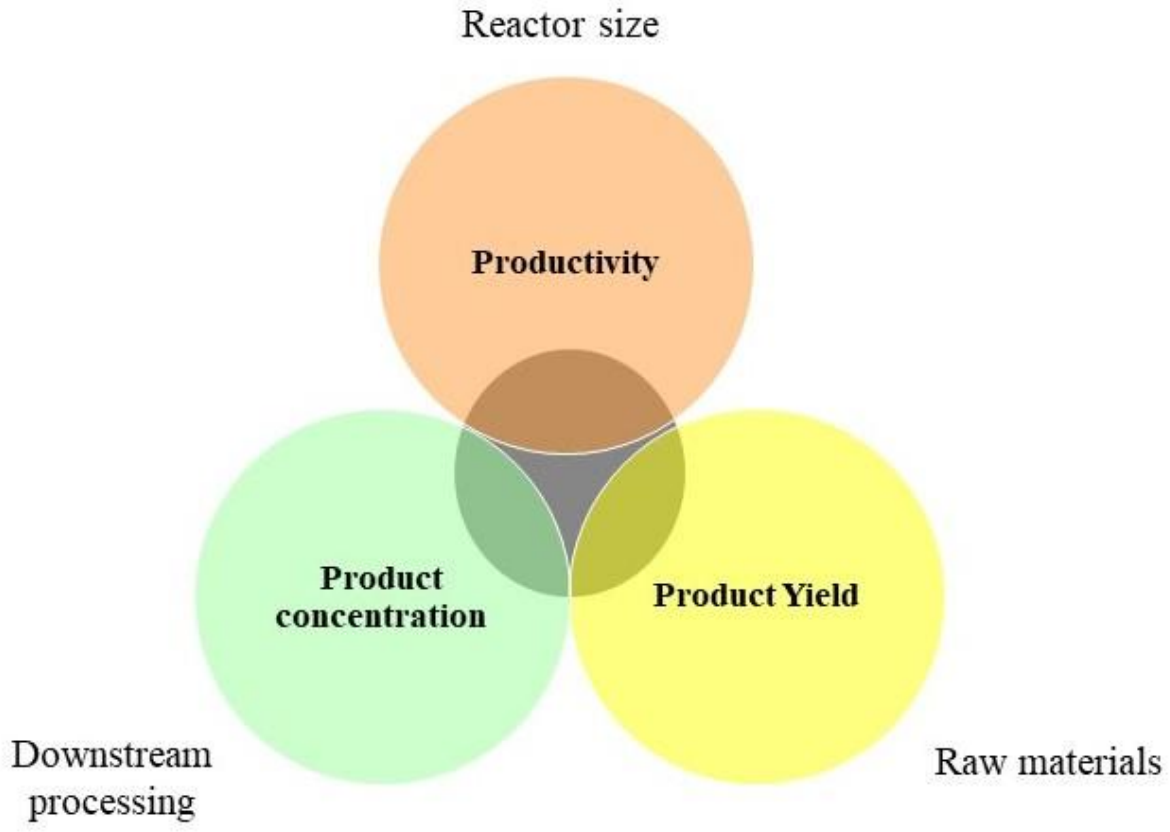


**Figure 1.1** Factors affecting the bioprocess efficiency

Maximization of the productivity leads to reduced capital and operating costs of the fermentation. A minimum productivity of 2.5 g $L^{-1}$ $h^{-1}$ is required for an economically viable bioprocess. The final product concentration is also important for the reduction of the overall downstream costs while, yield that determines the amount of substrate required, is essential for commercial production. Additionally, nutrient requirements and cost of substrate are major contributors (Werpy et al., 2004). Optimization of the microorganisms performance employed and the operational parameters influencing the bioprocesses along with the valorisation of industrial by-products and side streams can contribute to sustainable industrial production of bio-based chemicals.

## Chapter 2

# State of the art

# Chapter 2

## 2.1 State of the art for 1,3-Propanediol

### 2.1.1 1,3-Propanediol

1,3-Propanediol (PDO), also known as trimethylene glycol, 1,3-dihydroxypropane and 1,3-propylene glycol, is a platform chemical with the formula $C_3H_8O_2$ (Figure 2.1). It is a non-flammable, low toxicity liquid with boiling point of 210-212 ˚C and melting point of -28 ˚C. PDO is a valuable chemical intermediate for the manufacturing of polymers, polyurethanes, polyesters lubricants, medicines and biocides and heterocyclic compounds. Specifically, PDO is widely used in the production of polytrimethylene terephthalate (PTT), which finds applications in apparel, textile and carpets, due to its superior stretching, stretch recovery and biodegradable characteristics. It can be also applied in food, cosmetic and pharmaceutical industries (Lee et al., 2015; Kaur et al., 2012a).

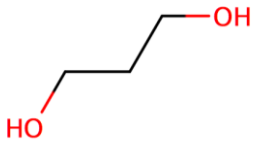


**Figure 2.1** Structure of PDO (*Source: https://pubchem.ncbi.nlm.nih.gov* )

PDO can be produced via chemical and biotechnological routes. Conversional methods for PDO synthesis include the DuPont and Shell processes (Figure 2.2). In the first, propylene is catalytically oxidised to acrolein which is hydrated at moderate temperature and pressure to 3-hydroxypropionaldehyde followed by hydrogenation to PDO. The Shell process is based on hydroformylation of ethylene oxide to 3-hydroxypropanal followed by hydrogenation to PDO (Sun et al., 2018). The conversion yield of PDO is around 65% and 80% respectively (Saxena et al., 2009).

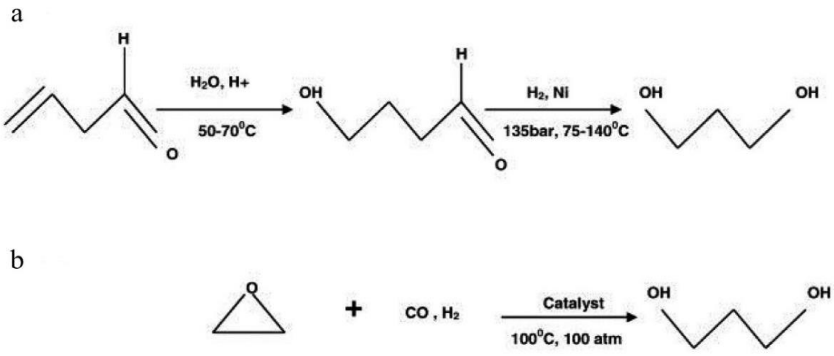


**Figure 2.2** Chemical synthesis of PDO from acrolein (a) and from ethylene oxide (b) (*Source: Przystałowska, 2015*)

Bio-based PDO is based on the valorisation of corn derived raw materials by recombinant *E. coli* strain and the fermentation of pure or crude glycerol by natural and genetically engineered strains.

### 2.1.2 Microorganisms for PDO production

Native PDO producers belong to genera of *Klebsiella*, *Clostridia*, *Enterobacter*, *Citrobacter* and *Lactobacilli*. Among these microorganisms, *K. pneumoniae* and *C. butyricum* are considered as the best producers because of their substrate tolerance, high yield and productivity. *Clostridium* species are typically anaerobic non-pathogenic microorganism, whereas *Klebsiella* are facultative anaerobe that can grow well on glycerol under aerobic, micro-aerobic, and anaerobic conditions. However, special safety precautions are required due to their pathogens or opportunistic pathogens nature. Mutated and genetically modified strains have also been studied for PDO production. Engineered strains, including native PDO producers and heterologous host (e.g. *E. coli* and *S. cerevisiae*), represent higher performance than wild-types in lab scale (Saxena et al., 2009; Lee et al., 2015). However, the stability of variant strain could not be maintained in long term or large scale. Table 2.1 presents PDO production by different bacteria species using glycerol as carbon source.

### 2.1.3 Metabolic pathway towards PDO synthesis

The pathway to PDO production using glycerol as carbon source is a coupled oxidation-reduction process. Generation of energy in the form of ATP and reducing equivalents in the form of NADH occurs in the oxidative branch, while regeneration of NAD with the formation of PDO occurs in the reductive branch. NADH is generated during glycolytic reactions (oxidative branch) which also yield several by-products (Figure 2.3).

In the reductive branch, glycerol is first dehydrated to 3-hydroxypropionaldehyde (3-HPA) catalysed by coenzyme B12-dependent glycerol dehydratase (GDHt, coded by *dhaB*) and then is reduced to PDO by NADH-dependent PDO dehydrogenase (PDOR, coded by *dhaT*) by NADH utilization. In the oxidative branch, glycerol is initially oxidized to dihydroxyacetone (DHA, coded by *dhaD*) by NAD-dependent glycerol dehydrogenase (GDH). DHA is then phosphorylated by adenosine triphosphate (ATP)-dependent dihydroxyacetone kinase (DHAK, coded by *dhaK*) to dihydroxyacetone phosphate (DHAP). DHAP enters to carbon flux towards glycolysis to form pyruvate. During fermentation process, pyruvate competes with 3-HPA for NADH-oxidoreductase to form other by-products. Different by-products are composed by

different microorganisms and process conditions employed. Butyric acid and acetic acid are the major by-products by *C. butyricym*, butanol is produced by *C. pasteurianum*, whereas ethanol, lactic acid, succinic acid and formic acid are formed by *Enterobacteria* (Lee et al., 2015; Yang et al., 2018a; Sun et al., 2018).

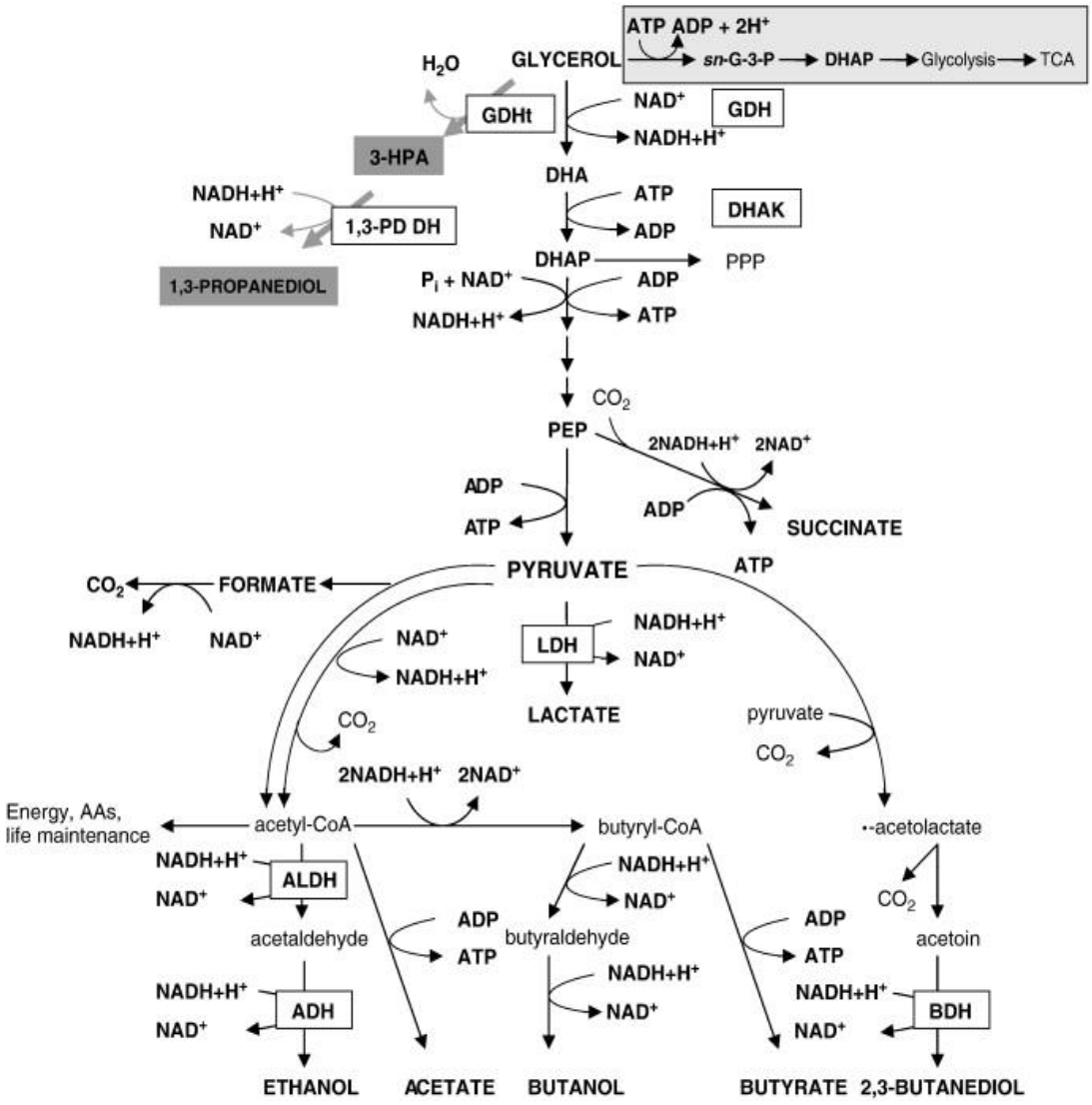


**Figure 2.3** Metabolic pathway toward PDO synthesis (*Source: Celińska, 2010*)

Abbreviations: GDHt: glycerol dehydratase, 1,3-PDO DH: 1,3-PDO dehydrogenase, GDH: glycerol dehydrogenase, DHAK: dihydroxyacetone kinase, DHA: dyhydroxyacetone, DHAP: dihydroxyacetone phosphate, PEP: phosphoenolpyruvate, AAs: amino acids, sn-G-3-P: sn-glycerol-3-phosphate, TCA: tricarboxylic acids cycle, PPP: pentose phosphate pathway, LDH: lactate dehydrogenase, ALDH: aldehyde dehydrogenase, ADH: alcohol dehydrogenase, BDH: 2,3-butanediol dehydrogenase

**Table 2.1** Production of PDO by various bacterial strains

| Strain | Glycerol | Fermentation mode | PDO ($g\ L^{-1}$) | Yield ($g\ g^{-1}$) | Productivity ($g\ L^{-1}\ h^{-1}$) | Reference |
|---|---|---|---|---|---|---|
| *K. oxytoca* M5al Δ*ldhA* | Pure | Fed-batch | 83.6 | 0.51 | 1.61 | Yang et al., 2007 |
| *K. pneumoniae* ZJU 5205 | Pure | Batch | 63.1 | 0.54 | 5.74 | Zhao et al., 2006 |
| *K. pneumoniae* M5al | Pure | Fed-batch - 5000L reactor | 58.8 | 0.44 | 0.92 | Cheng et al., 2007 |
| *K. pneumoniae* DSM 2026 | Pure | Fed-batch | 75.0 | 0.50 | 2.20 | Liu et al., 2007 |
| *K. pneumoniae* XJPD-Li | Pure | Fed-batch | 65.3 | 0.46 | 3.16 | Ma et al., 2009b |
| *K. pneumoniae* KG1 | Pure | Fed-batch | 98.8 | 0.42 | 3.29 | Zhao et al., 2009 |
| *K. pneumoniae* DSM 4799 | Pure | Fed-batch | 63.3 | 0.53 | 0.92 | Jun et al., 2010 |
| *K. pneumoniae* LX3 | Pure | Fed-batch | 71.4 | 0.49 | 2.24 | Xue et al., 2010 |
| *K. pneumoniae* ATCC 8724 | Crude | Fed-batch | 62.7 | 0.60 | 1.74 | Yang et al., 2018b |
| *K. pneumoniae* ↑*dhaT* | Pure | Fed-batch | 90.9 | 0.53 | 2.16 | Zhao et al., 2009 |
| *K. pneumoniae* LDH526 Δ*ldh*A | Pure | Fed-batch | 102.1 | 0.43 | 2.13 | Xu et al., 2009 |
| *K. pneumoniae CF ΔbudC* Δ*fdh* | Pure | Fed-batch | 75.2 | 0.47 | 2.35 | Wu et al., 2013 |
| *C. freundii* DSM30040 | Pure | Continuous two-stages | 41.5 | 0.51 | 1.74 | Boenigk et al., 1993 |
| *C. freundii* DSM 30040 | Pure | Cell immobilization | 18.9 | 0.39 | 5.70 | Pflugmacher and Gottschalk, 1994 |
| *C. freundii* ATCC 8090 | Pure | Batch | 31.5 | 0.45 | 0.35 | Barbirato et al., 1998 |
| *C. freundii* VK-19 | Crude 81% | Fed-batch | 68.1 | 0.40 | 0.79 | Metsoviti et al., 2013 |
| *C. freundii* AD970 ↑dhaT | Pure | Fed-batch | 35.6 | 0.17 | | Celinska et al., 2015 |
| *C. butyricum* DSM 5431 | Pure | Continuous cell recycle | 26.6 | | 13.30 | Reimann et al., 1998 |
| *C. butyricum* F2b | Crude 65% | Continuous | 48.1 | | | Papanikolaou et al., 2000 |
| *C. butyricum* VPI 1718 | Crude 81% | Fed-batch | 67.9 | 0.55 | 0.78 | Chatzifragkou et al., 2011 |
| | Crude 81% | Continuous | 23.4 | 0.52 | 1.87 | |
| *C. butyricum* AKR102a | Crude 55% | Fed-batch | 76.2 | 0.51 | 2.30 | Wilkers et al., 2012 |
| | Crude 55% | fb-200L reactor | 61.5 | 0.53 | 2.10 | |
| *E. coli* recombinant | Pure | Batch two-stage fermentation | 104.4 | 0.90 | 2.61 | Tang et al., 2009 |

### 2.1.4 Developments in the microbial production of PDO

Biological production of PDO is an attractive and environmental friendly approach in terms of green chemistry principles. It has been reported that energy requirements and gas emissions of bio-based PDO are nearly half comparing to the petro-based PDO (Anonymous, 2009; Urban and Bakshi, 2009). DuPont Tate & Lyle have successfully commercialized bio-PDO production in 2006 with a production capacity of 45,000 t per annum. A metabolically engineered *E. coli* stain able to produce up to 135 g $L^{-1}$ of PDO from corn-sugar with high yield and productivity (0.51 g $g^{-1}$ and 3.5 g $L^{-1}$ $h^{-1}$, respectively) is implemented for commercial manufacturing process (Nakamura and Whited, 2003).

Despite bio-PDO is already commercialized from glucose, there are also several methods for producing it with glycerol as feedstock. Glycerol, a by-product from the biodiesel production, is currently facing an over-supply on the market due to the increased biodiesel production. In the light of development approaches for value-added conversion of glycerol, PDO production form glycerol based fermentation is considered a promising approach. Apparently, a commercial implementation of glycerol-based production of PDO is under development. METabolic EXplorer and Bio-XCell are subject to construct and operate a PDO production plant using crude glycerol as feedstock (Anonymous, 2017).

The main challenge associated with bio-based PDO production lays in the development of a cost efficient bioprocess utilizing industrial side streams and by-products. Generally, fermentation of glucose is more attractive owing to the lower raw material cost. However, glycerol-based production route could be competitive with bio-based PDO from glucose, once the production process is optimized with respect to yield, productivity and cost of raw materials. Utilisation of crude glycerol generated from biodiesel industry seems promising route as it does not require additional land for crop and adds value to a by-product.

Over the last decade, significant improvements have been obtained for bioconversion of glycerol to PDO. However, several issues are needed to be addressed for bio-based production. The biggest constrains are the low yield and productivity, the formation of by-products, the cost and the pretreatment of raw materials along with the difficulties in downstream process. To overcome the barriers development of efficient strategy including process development, optimization and strain improvement is required for commercialization.

### 2.1.5 Influence of product inhibition on microbial growth and PDO synthesis

PDO synthesis is accompanied by by-product formation produced in the oxidative branch. Formation of acetic acid, ethanol and butyric acid is essential to supply energy for bacterial growth and maintenance (Zeng et al., 1994a). However, accumulation of by-products leads to low yield. It has been estimated that the maximum yield of PDO is 0.64 mol $mol^{-1}$ for *K. pneumoniae* when only acetic acid pathway is used for energy generation, whereas PDO yield is reduced when ethanol is also produced (Zeng et al., 1993).

Products accumulating during fermentation cause inhibitory effect on bacterial growth and PDO synthesis. The inhibition associated with organic acids is the result of anion specific effects on metabolism along with increased internal proton concentration. Diffusion of organic acids into the cytoplasm dissociate and disrupt the pH and anion pool of the cytoplasm. The activity of enzymes involved in protein synthesis is also disrupted resulting in reduced bacterial growth and viability. Elevated PDO concentration may cause inhibition effects rendering bacterial cells dysfunctional. The inhibitory effect of alcohols is related with modification of membrane organization by an increase in the fluidity of the membrane along with the suppression of the membrane ATPase and transport mechanisms (Colin et al., 2000).

Product inhibition has been studied by measuring the growth rate in cultures where the inhibitor was added before or during the cultivation in increasing concentration (Biebl et al., 1991). The resistance of bacteria varies between strains and the intensity of cell response differ as well. Mathematical models for inhibition effect on growth have also been proposed. These models could be used to identify the characteristic inhibition effect of an individual inhibitor in cultures with multiple products (Zeng et al., 1994a). PDO is considered the least toxic product in glycerol fermentation. *C. butyricum* can tolerate PDO concentration in the range of 60-80 g $L^{-1}$ (Colin et al., 2000; Papanikolaou et al., 2000). The addition of PDO (10-30 g $L^{-1}$) during mid-exponential phase had no significant effect on bacterial growth. Cheng et al. (2005) reported that the resistant of *K. pneumoniae* to PDO differs under aerobic and anaerobic conditions. The critical concentration of PDO was 69 and 77 g $L^{-1}$ under anaerobic and aerobic conditions, respectively. The most inhibiting effect on PDO synthesis is by 3-HPA. This compound is an intracellular intermediate that is synthesized in the reductive branch of the glycerol pathway (Celińska, 2010). Under high glycerol excess, it can be accumulated extracellular resulting in decrease GDH activity entailing lower glycerol assimilation. A concentration of 30 mM of 3-

HPA cause cessation of growth and decrease glycerol consumption in cells of *K. pneumoniae*, *C. freundii* and *E. agglomerans* (Barbirato et al., 1996).

The influence of organic acids on bacterial growth and metabolism has been rarely studied. Biebl et al. (1991) demonstrated that acetic acid and butyric acid inhibited *C. butyricum* at concentrations of 27 and 19 g $L^{-1}$, respectively. Formation of acetic acid supplies the reductive pathway with more NADH compared to the formation of butyric acid which leads to improved production of PDO (Zeng, 1996). However, the undissociated form of acetate is considered the most inhibitory metabolite for *K. pneumoniae* and *C. butyricum* (Zeng et al., 1994a). Lactic acid and ethanol are less toxic than acetic acid for *K. penumoniae* and *C. butyricum* (Cheng et al., 2005; Szymanowska and Kubiak, 2015). Under aerobic condition, the critical concentrations of lactate and ethanol were 26 and 17 g $L^{-1}$, respectively (Cheng et al., 2005).

### 2.1.6 Influence of crude glycerol

Crude glycerol originated from biodiesel industries has been used as substrate for PDO formation, although its composition may affect the final concentration and yield (Table 2.2). The composition of crude glycerol depends on the kind of feedstock used in biodiesel production, the conditions in the transesterification process and the conditions in the separate non polar phase from the polar phase. The main impurities are sodium and heavy metal ions, methanol, soap, methyl/ethyl esters, glycerides, unreacted fatty acids and other natural compounds such as phenolic antioxidants (Chatzifragkou and Papanikolaou, 2012; Samul et al., 2014). The presence of higher concentration of impurities may interfere with cell division and consequently reduce the cell viability.

Free fatty acids (FFAs) are integral components of cell structures and energy stores caused by interference with the electron transport chain and the disruption of oxidative phosphorylation. FFAs may cause leakage of cell metabolites from the cell, complete cell lysis and autolysis. Other processes that may affect the bacterial growth include inhibition of enzyme activity, impairment of nutrient uptake and generation of toxic peroxidation and autoxidation products. Growth inhibition of FFAs on bacterial growth is attributed to their physiochemical properties such as isomerism and degree of unsaturation. Generally, Gram positive bacteria are known to be more susceptible to the damaging effects of fatty acids (Desbrois and Smith, 2010). Fatty acids with high degree of unsaturation (e.g. linoleic acid) have been found to affect significantly the utilisation of glycerol by hindering the diffusion of nutrients and metabolites through the

membrane. Whereas, fatty acids with lower or no degree of unsaturation such as stearic and oleic acid are less inhibitory to bacterial growth (Venkataramanan et al., 2012). Chatzifragkou et al. (2010) have been reported that the presence of 2% (w/w) of oleic acid in *C. butyricum* culture totally inhibited bacterial growth. The application of organic solvents (hexane, heptane, octane, petroleum ether) has been proposed as method for removal of residual fatty acids. Anand and Saxena (2012) reported that cultivation of *C. freundii* on pretreated glycerol resulted in similar growth and PDO production to those obtained with pure glycerol. Treatment of diluted crude glycerol with hydrochloric acid has been used for the precipitation of fatty acids (Venkataramanan et al., 2012).

The presence of salts causes inhibitory effect on bacterial growth due to osmotic pressure, and their effect on cell membranes result in osmotic plasmolysis. This indeed was attempted by Chatzifregkou et al. (2010) who examined the impact of salts (NaCl, $K_2HO_4$ and $Na_2HPO_4$) on growth and PDO production by *C. butyricum*. The presence of NaCl at concentration of 4.5% (w/w) had an evident effect on growth and PDO synthesis, although phosphoric salts had no significantly inhibitory effects. High concentrations of monovalent salts decrease the van der Waals forces between the lipid tails in the membrane resulting in negative effect on the energetic barrier in the lipid layer of the membrane and altering the biochemical processes in the cells (Samul et al., 2012).

Alcohol affects the cell membrane by increasing the fluidity of the membrane. The effect increases with the carbon chain length and the concentration of the aliphatic alcohol. Methanol being an aliphatic alcohol of one carbon is weakly lipophilic and has the least effect on the fluidity of the membrane. Previous studies have reported a slight reduction of PDO yield with addition of methanol (Chatzifragkou et al. 2010; Venkataramanan et al. 2012).

### 2.1.7 Process development of glycerol bioconversion to PDO

The optimization of PDO fermentation could be achieved by preventing by-product formation increasing the productivity and increasing the tolerance for PDO. Various strategies have been proposed to optimize the PDO fermentation and remarkable progress has been achieved. These techniques include the evaluation of different fermentation operation modes and the application of metabolic and genetic engineering.

# Chapter 2

**Table 2.2** PDO production using different metabolites from pure and crude glycerol

| Strain | Pure glycerol | | | Crude glycerol | | | | References |
|---|---|---|---|---|---|---|---|---|
| | PDO | Yield ($g\ g^{-1}$) | Productivity ($g\ L^{-1}\ h^{-1}$) | PDO ($g\ L^{-1}$) | Yield ($g\ g^{-1}$) | Productivity ($g\ L^{-1}\ h^{-1}$) | Impurities | |
| *K. pneumoniae* DSM 2026 | 61.9 | 0.41 | 2.00 | 53.0 | 0.39 | 1.70 | Glycerol 85% | Mu et al., 2006 |
| *K. pneumoniae* DSM 4799 | 63.3 | 0.53 | 0.92 | 80.2 | 0.45 | 1.16 | Glycerol 80%,<br>Water 0.05%<br>Methanol 0.27%<br>MONG 17%<br>Sodium 13,660 ppm<br>Potassium 70 ppm | Jun et al., 2009 |
| *K. pneumoniae* DSM 4799 | 13.8 | 0.35 | 0.29 | 17.1 | 0.42 | 0.36 | Glycerol 81%<br>Water 5%<br>Methanol <0.2%<br>Salts 4.8% | Jun et al., 2010 |
| *C. butyricum* VPI 1718 | 11.3 | 0.60 | / | 11.3 | 0.57 | / | Glycerol 81%<br>Water 10-12%<br>Methanol <0.2%<br>FFAs 1%<br>Potassium 5-6% | Chatzifragkou et al., 2011 |
| *C. butyricum 2.1* | 36.2 | 0.55 | 0.39 | 21.9 | 0.35 | 0.31 | Glycerol 81%<br>Water 8%<br>Methanol 0.02%<br>MONG 4%<br>Ash 8% | Orczyk et al., 2012 |
| *C. butyricum* AKR102a | 93.7 | 0.52 | 3.30 | 76.2 | 0.51 | 2.30 | Glycerol 55%<br>Fatty acids 1%<br>Chloride 31,408 ppm | Wilkers et al., 2012 |

#### 2.1.7.1 Fermentation strategies

Batch cultivations were primarily investigated to demonstrate the feasibility for scale up of PDO fermentation. PDO concentration of 63.4 g $L^{-1}$ was achieved by *C. butyricum* using crude glycerol. Cultivation of *K. pneumoniae* resulted in 61 g $L^{-1}$ of PDO with a productivity of 1.7 g $L^{-1}$ $h^{-1}$ in batch culture (Homann et al., 1990). Despite the high PDO concentration, higher glycerol concentration inhibits the growth and PDO formation that limits further increment. Repeated batch culture, a simple and effective approach to reduce the inhibition caused by substrate and products and enhance the stability towards contamination, leads to high product concentration and productivity. PDO production of 67.8 g $L^{-1}$ with productivity of 1.04 g $L^{-1}$ $h^{-1}$ has been obtained by *Clostridium diolis* DSM 1541 in repeated batch cultivation (Kaur et al., 2012b). Similar results have been reported by Chatzifragkou et al. (2014) using crude glycerol (81% w/w) supplemented with rapeseed meal hydrolysate.

Fed-batch cultures have been applied to eliminate substrate inhibition and to obtain higher product concentrations. Hirschmann et al. (2005) reported 87 g $L^{-1}$ of PDO with a productivity of 1.9 g $L^{-1}$ $h^{-1}$ by *Clostridium* IK 124 using crude glycerol. Up to 93.7 g $L^{-1}$ could be achieved using pure glycerol by *C. butyricum* AKR102a. The implementation of crude glycerol (55% w/w) that originated from transesterification of rapeseed oil led to 76.2 g $L^{-1}$ with productivity of 2.2 g $L^{-1}$ $h^{-1}$ (Wilkens et al., 2012). Xue et al. (2010) evaluated *K. pneumoniae* LX3 in fed-batch fermentation with organic acids addition (citric acid, succinic acid and fumaric acid) resulting in 71.4 g $L^{-1}$ of PDO. The addition of organic acids significantly decreased the formation of lactic acid, ethanol and succinic acid, and was beneficial to PDO production due to less requirement for NADH. *C. freundii* has also been studied in fed-batch cultures under sterile and non-sterile conditions leading to 68.1 g $L^{-1}$ and 66.3 g $L^{-1}$, respectively (Metsoviti et al., 2013).

The highest values of productivity have been achieved in continuous cultures by *K. pneumoniae* DSM 2026 (Menzel et al., 1997). PDO production of 48.5 g $L^{-1}$ with corresponding productivity of 4.9 g $L^{-1}$ $h^{-1}$ was obtained at dilution rate of 0.1 $h^{-1}$ whereas, increasing dilution rate to 0.25 $h^{-1}$ resulted in productivity of 8.8 g $L^{-1}$ $h^{-1}$. Papanikolaou et al. (2000) investigated the production of PDO by *C. butyricum* in continuous culture. PDO concentration was 31-48 g $L^{-1}$, with a conversion yield of 0.55 g $g^{-1}$. Using a two-stage strategy, the maximum PDO obtained was between 41 to 46 g $L^{-1}$, with a maximum productivity of 3.4 g $L^{-1}$ $h^{-1}$. Continuous cultures

with recycling and immobilized cells has also been implemented in PDO fermentation (Reimann et al., 1998; Pfligmacher and Gottschalk, 1994). High productivity (8.2 g $L^{-1}$ $h^{-1}$) was obtained by *C. freundii* DSM 30040 at dilution rate of 0.5 $h^{-1}$ in immobilized continuous culture (Pflugmacher and Gottschalk, 1994). Generally, continuous cultures eliminate both substrate and product inhibition offering high values of productivity and relatively low product concentrations.

#### 2.1.7.2 Genetic engineering

Application of genetic engineering provides a potential technology to obtain better fermentation performance. Deletion of genes related to by-products accumulation and overexpression of genes related to PDO synthesis could lead to efficient PDO producers. Improved PDO concentration, yield and productivity have been achieved by inactivation of genes associated with pyruvate reduction. In oxidative branch of glycerol several by-products are formed that prevent cellular growth causing inhibitor effect on PDO synthesis. Lactate, a by-product of the reduction of pyruvate, is a major by-product in glycerol fermentation that prevents cellular growth. The fermentative lactate dehydrogenase (LDH) encoded by *ldhA* gene contributes to the synthesis of lactate. A lactate dehydrogenase deficient *K. pneumoniae* mutant was constructed resulting in 102.1 g $L^{-1}$ of PDO with productivity of 2.13 g $L^{-1}$ $h^{-1}$ with simultaneous reduction of lactic acid from 40 to 3 g $L^{-1}$ (Xu et al., 2009). Genes associated with acetyl-CoA reduction are responsible for accumulation of acetate, ethanol and butyrate. Elimination of acetate synthesis is considered the most significant target in metabolic engineering of PDO production. Deletion of the genes *poxB* encoding pyruvate oxidase, *pta* encoding phosphotransacetylase and *ackA* encoding acetate kinase has been investigated in *K. pneunomiae*. PDO production was increased by 15% compared with the parent strain whereas, acetic acid accumulation was significantly decreased (Lin et al., 2016). Inactivation of the *aldA* gene encoding aldehyde dehydrogenase (ALDH) in *K. pneumoniae* YMU2 resulted in improved PDO production of 70.5 g $L^{-1}$ with significantly reduction of ethanol and succinic acid formation and increment of yield by 96% (Zhang et al., 2006).

Overexpression of enzymes in the reductive pathway enhance PDO production and yield. In the reductive branch of glycerol dissimilation, GDHt and PDOR encoded by *dhaB* and *dhaT*, respectively are the key enzymes for PDO synthesis. Zhao et al. (2009) reported that overexpression of PDOR in *K. pneumoniae* KG1strain led to increment of yield by 27% and reduction of lactic acid, ethanol and succinic acid production by 51.8, 50.6 and 47.4%,

respectively. Simultaneous overexpression of PDOR and GDH has been investigated aiming to reduce 3-HPA accumulation and increase NADH for the cell growth and PDO formation (Chen et al., 2009). Overexpression of an endogenous NADPH-dependent alcohol dehydrogenase (HOR) in *K. pneumoniae* ACCC10082. PDO concentration and yield of the constructed strain increased by 10.4 and 9.4% respectively (Chen et al., 2011).

### 2.1.8 Downstream process

The downstream processing of PDO involves three steps: a) removal of cells and proteins, b) removal of impurities and primary separation of PDO and c) concentration and refining of PDO (Figure 2.4).

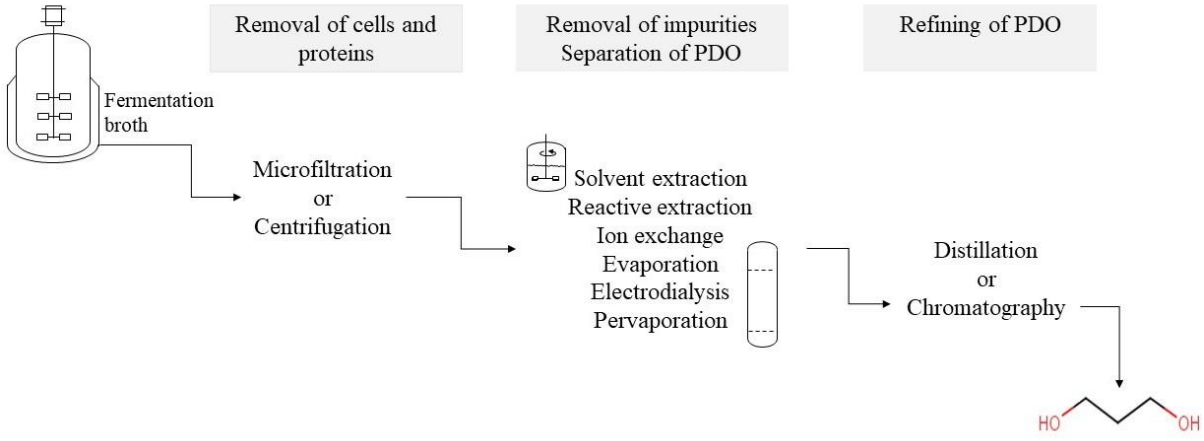


**Figure 2.4** Downstream process of PDO (*Source: Modified based on Xiu and Zeng, 2008*)

Several well-known methods are known for removal of biomass and proteins including microfiltration and high-speed centrifugation. Flocculation precipitation is another technique attracting attention in industrial scale for solid-liquid separation (Xiu and Zeng, 2008). The second stage includes the removal of impurities and separation of PDO. Until now, various methods have been proposed including solvent extraction, reactive extraction, ion exchange, pervaporation with zeolite membrane, evaporation and electrodialysis. However, all these methods have limitations in terms of relatively low product yield, cost and energy consumption. The final purification step is vacuum distillation or separation by chromatography. Chromatographic separation results in high purity and yield, though high energy consumption is needed for concentration of the final solution. Distillation consisting of four columns is the preferred system for PDO purification. For highest purity, a chemical reduction such as

hydrogenation step is optionally used prior to the final distillation column (Adkesson et al., 2011).

The purification of PDO still represents a technological challenge and remains an economical obstacle for an efficient microbial production. Classical separation methods need to be improved or combined with other technology for cost-effective separation. Moreover, applicability of the procedure for fermentation broths should be also evaluated.

## 2.2 State of the art for 2,3-butanediol

### 2.2.1 2,3-Butanediol

2,3-Butanediol (BDO), also known as 2,3-butylene glycol, 2,3-dihydroxybutaned, dimethylene glycol, dimethylethylene glycol and butane-2,3-diol, is an important building block chemical. Its chemical formula is $C_4H_{10}O_2$ with a molecular weight of 90.121 (g $mol^{-1}$). BDO is a chiral compound that can exist in three isomeric forms: (2R, 3R)- or levo- or D-BDO, (2S, 3S)- or dextro- or L-(+)-BDO and (2R, 3S)- or meso-BDO (Figure 2.5). BDO has high boiling point, ranging from 177 to 182 ˚C, and low freezing point of -60 ˚C. It can be found as a colorless and odorless liquid or in crystalline form (Celińska and Grajek, 2009; Syu, 2001).

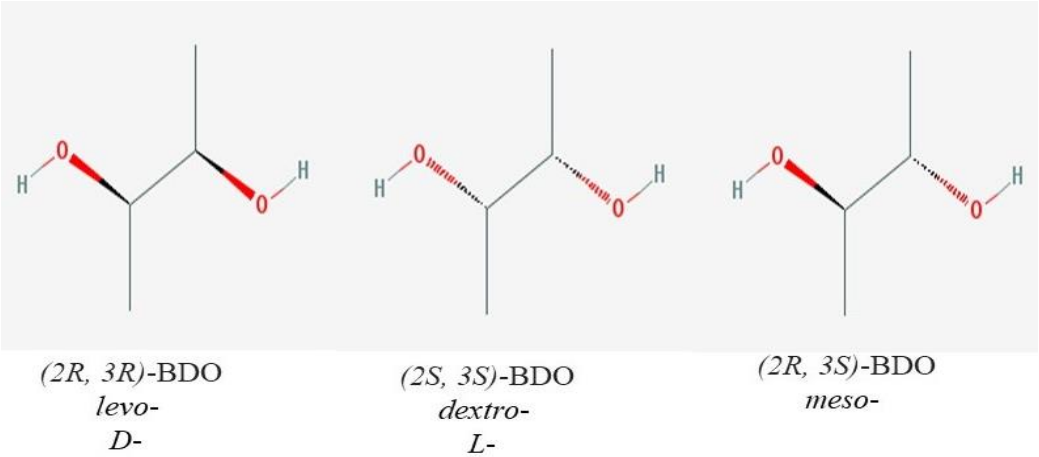


**Figure 2.5** Stereoisomers of BDO (*Source:* https://pubchem.ncbi.nlm.nih.gov)

BDO constitutes a precursor in the manufacture of bulk chemicals including 1,3-butadiene, methyl ethyl ketone (MEK), diacetyl, gamma-butyrolactone and polyurethane. Owing to its high heating value (27 000 J $g^{-1}$) and low freezing point (-60 ˚C), BDO can be used as liquid fuel, fuel additive and antifreeze agent. Other potential applications include the production of flavorings, cosmetics, printing inks, perfumes, softening and artificial rubber (Figure 2.6).

Commercially, the key downstream products of BDO have a potential global market of around 32 million tons per annum, valued at approximately $43 billion in sales (Köpke et al., 2011).

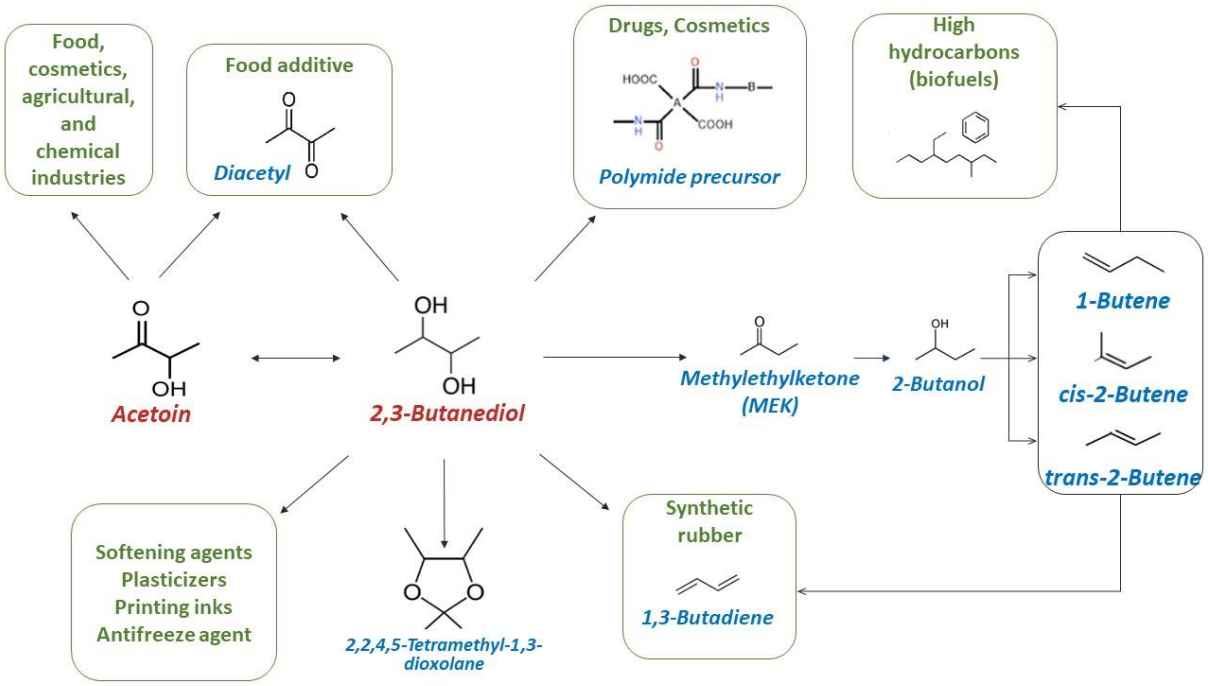


**Figure 2.6** Derivatives of BDO

### 2.2.2 Microorganisms for BDO production

Several anaerobic and facultative microorganisms are able to accumulate BDO using commercial substrates, industrial side streams and lignocellulosic materials. The most representative microorganisms that have been extensively investigated include the genera of *Enterobacter*, *Klebsiella*, *Serratia*, *Paenibacillus* and *Bacillus*. Highly efficient BDO producers are the strains *K. pneumoniae*, *K. oxytoca* and *S. marcescens* (Celińska and Grajek, 2009). Table 2.3 presents BDO production using various substrates by different strains.

*Serratia*, *Klebsiella* and *Enterobacter* are Gram-negative, facultative anaerobic, rod-shaped, non-spore-forming bacteria belonging to Enterobacteriaceae family. These genera have the potential for industrial BDO production due to resistance to bacteria pollution, relatively simple metabolic pathway, broad substrate spectrum and cultural adaptability. The highest BDO production (150 g $L^{-1}$) has been achieved by wild-type *K. pneumoniae* strain isolated from orchard soil using glucose with yield and productivity of 0.44 g $g^{-1}$ and 3.95 g $L^{-1}$ $h^{-1}$

respectively (Ma et al., 2009a). *Bacillus*, a genus of Gram-positive, endospore-forming, rod-shaped bacteria, have been used for both BDO and acetoin fermentation. The most interesting BDO producer of that genus is *B. licheniformis*, which produced 144.7 g $L^{-1}$ of BDO with yield and productivity of 0.40 g $g^{-1}$ and 1.14 g $L^{-1}$ $h^{-1}$, respectively in fed-batch culture using glucose (Jurchescu et al., 2013).

Conventional enrichment screening procedures with mutagenesis and genetic manipulation have been applied aiming to the production of pure isomer. Li et al. (2015) constructed a *D*-BDO producer *E. cloacae* strain through the deletion of *meso-bdh*, *ldh* and *frdA* genes and overexpression of *D*-BDO dehydrogenase. High D-BDO concentration of 119.4 g $L^{-1}$ with productivity of 2.34 g $L^{-1}$ $h^{-1}$ was obtained using lignocellulose derived sugars (Li et al., 2015). Highly efficient metabolically engineered *S. marcescens* strain has been developed for both BDO and acetoin production (Zhang et al., 2010a; Sun et al., 2012a).

The BDO isomers produced is dependent on the particular microorganism employed. Generally, a mixture of two BDO stereoisomers is formed by native strains. *Klebsiella* and *Enterobacter* produce L- and meso- stereoisomers of BDO, whereas a mixture of D- and meso- is generated by *Bacillus* species (Celińska and Grajek, 2009). The selection of BDO producer is largely reliant on the intended application of the alcohol, the fermentation efficiency and the safety regulations. Strains of Enterobacteriaceae family are considered pathogens or opportunistic pathogens and deletion of virulence genes should be conducted before large scale use. While, *Bacillus* species have been granted "Generally Regarded as Safe (GRAS) designation" by the US Food and Drug Administration and are considered promising for commercial operation, improvement of fermentation efficiency is required for an economic process.

**Table 2.3** BDO production by different bacterial strains

| Microorganism | Substrate | Isomer | BDO ($g\ L^{-1}$) | Yield ($g\ g^{-1}$) | Productivity ($g\ L^{-1}\ h^{-1}$) | Fermentation mode | References |
|---|---|---|---|---|---|---|---|
| | | | *K. pneumoniae* | | | | |
| CICC 10011 | Glucose | meso-, L- | 92.4 | 0.39 | 1.85 | fb | Jiayang et al., 2006 |
| CICC 10011 | Jerusalem artichoke tuber | | 84.0 | 0.29 | 2.10 | fb-SSF | Sun et al., 2009a |
| SDM *isolated from orchard soil* | Glucose | meso-, L- | 150.0 | 0.44 | 3.95 | fb | Ma et al., 2009a |
| SDM | Corncob molasses | meso-, L- | 78.9 | 0.41 | 1.29 | fb | Wang et al., 2010 |
| G31 *isolated from active slime* | Glycerol | | 70.0 | 0.39 | 0.47 | fb | Petrov and Petrova, 2010 |
| CICC 10011 | Jerusalem artichoke tuber | | 67.4 | 0.26 | 0.99 | SSF | Li et al., 2010a |
| CICC 10781 | Cheese whey powder | | 57.6 | 0.40 | 0.96 | fb | Guo et al., 2017 |
| KCTC2242 (↑*budA* ↑*budB* ) | Glucose | | 101.5 | 0.34 | 2.54 | fb | Kim et al., 2012 |
| XZF-308 (↑*nox-2*) | Glucose | | 42.8 | | | fb | Ji et al., 2013 |
| *KG1 (ΔadhE ΔldhA)* | Glucose | | 116.0 | 0.49 | 2.23 | fb | Guo et al., 2014 |
| | | | *K. oxytoca* | | | | |
| DSM3539 | Molasses | | 118.0 | 0.42 | 2.35 | R-b | Afschar et al., 1991 |
| ME-UD-3 mutant | Glucose | | 95.5 | 0.48 | 1.71 | b | Ji et al., 2009 |
| ME-XJ-8 (*ΔaldA*) | Glucose | | 90.8 | 0.48 | 1.51 | b | Ji et al., 2010 |
| M3 (*ΔpduCΔldhA*) | Crude glycerol | meso-, L- | 131.5 | 0.44 | 0.84 | fb | Cho et al., 2015a |
| M1 (↑*budC* ) | Glucose | | 142.5 | 0.42 | 1.47 | fb | Cho et al., 2015b |
| *(ΔldhA)* | Glucose | | 87.5 | 0.44 | 0.65 | fb | Kim et al., 2016a |
| KMS005-73T (*ΔadhEΔackA-ptaΔldhA*) | Glucose | | 117.4 | 0.49 | 1.20 | fb | Jantama et al., 2015 |
| *K. variicola* SW3 mutant | Crude glycerol | | 64.9 | 0.63 | 0.39 | fb-sf (shake flask) | Rahman et al., 2017 |
| | | | *S. marcescens* | | | | |
| H30 (mutant by UV and LiCl) | Sucrose | | 139.9 | 0.47 | 3.49 | fb | Zhang et al., 2010a |
| swrW (*ΔswrWΔswrA*) | Sucrose | | 152.0 | 0.46 | 2.67 | fb | Zhang et al., 2010b |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *MG1 (ΔslaC ↑bdhA)* | Sucrose | meso-, D- | 91.9 | 0.36 | 1.92 | fb | Bai et al., 2015 |
| *H30* | Sweet sorghum juice | | 109.4 | 0.42 | 1.40 | fb | Yuan et al., 2017 |
| | | | *E. cloacae* | | | | |
| DSM 30053 | Glucose | | 110* | 0.49 | 5.40 | fb | Zeng et al., 1991a |
| Dissolvens SDM | Cassava powder | meso-, D-, L- | 93.9 | 0.42 | 2.00 | fb-SSF | Wang et al., 2012 |
| EMY-68 (*ΔldhA ΔscrR*) | Sugarcane molasses | | 98.7 | 0.37 | 2.74 | fb | Jung et al., 2013 |
| EMY-70S (Δ*ldhA*Δ*scrR*Δ*cra* ↑*scrAB*) | Sugarcane molasses | | 140.0 | 0.39 | 2.59 | fb | Jung et al., 2015 |
| SDM *(ΔbdhΔptsGΔldhΔfrdA ↑galP)* | Lignocellulose derived sugars | D- | 119.4 | 0.48 | 2.34 | fb | Li et al., 2015 |
| *Raoultella ornithinolytica* B6 (↑*budABC* ) | Crude glycerol pretreated | | 78.1 | 0.42 | 0.62 | fb | Kim et al., 2017 |
| | | | *B. amyloliquefaciens* | | | | |
| *B10-127 isolated from soil* | Glucose | | 92.3 | | 0.96 | fb | Yang et al., 2011 |
| B10-127 | Glucose | | 61.4 | 0.38 | 1.71 | b (30 L) | Yang et al., 2012 |
| B10-127 | Crude glycerol: molasses (5:1) | | 83.3 | 0.42 | 0.87 | fb | Yang et al., 2013 |
| GAR (↑*dhaD* ↑*acr*) | Crude glycerol: molasses (5:1) | | 102.3 | 0.44 | 1.16 | fb | Yang et al., 2015 |
| | | | *B. licheniformis* | | | | |
| 10-1-A | Glucose | meso-, D- | 115.7 | 0.47 | 2.40 | fb | Li et al., 2013 |
| ATTV 14580 | Inulin | meso-, D- | 103.0 | | 3.40 | fb-SSF | Li et al., 2014a |
| *WX-02 (ΔgdhΔacoR)* | Glucose | meso- | 98.0 | 0.40 | 0.94 | fb | Qui et al., 2016 |
| *B. subtilis* LOCK 1086 (cloning of vhb- ↑*bdhA*) | Sugar beet molasses | | 75.7 | 0.31 | 0.66 | fb-sf | Bialkowska et al., 2016b |
| *P. polymyxa* ATCC 12321 | Corn stover hydrolysate | D- | 18.8 | 0.31 | 1.13 | continuous with cell recycling | Ma et al., 2018 |

b: batch, fb: fed-batch, R-b: repeated batch, fb-sf: fed-batch in shake flasks, fb-SSF: fed-batch with simultaneous saccharification and fermentation

### 2.2.3 Metabolic pathway towards BDO synthesis

BDO is produced from pyruvate via a mixed acid fermentation process. 3R-acetoin and 3S-acetoin are the main intermediate compounds of BDO fermentation in native strains. In bacterial metabolism, monosaccharides (hexoses and pentoses) or glycerol are first converted to pyruvate. When glucose is employed, pyruvate is formed via Embden-Meyerhof pathway. A combination of Embden-Meyerhof and pentose phosphate pathways are used when pentoses used as carbon source (Ji et al., 2011). While, in the case of glycerol, it is converted to dihydroxyacetone phosphate via glycerol-3-phosphate under aerobic conditions or via dihydroxyacetone under anaerobic conditions, which is subsequently oxidized to pyruvate (Cho et al., 2015a).

Subsequently, BDO is produced from pyruvate in a mixed acid fermentation process. The key enzymes involved in the biosynthesis of BDO from pyruvate are $\alpha$-acetolactate synthase (ALS, encoded by *budB*), $\alpha$-acetolactate decarboxylase (ALDC, encoded by *budA*) and BDO dehydrogenases (BDHs, also known as acetoin/diacetyl reductase ARs/DRs, encoded by *budC* and *gdh*) (Zhang et al., 2016). Initially, pyruvate is converted to $\alpha$-acetolactate by ALS and release one molecule of $CO_2$, then $\alpha$-acetolactate is decarboxylated into 3R-acetoin by ALDC. Under oxygen supply, $\alpha$-acetolactate is converted to diacetyl (DA) via non-enzymatic oxidative decarboxylation. Therefore, in the presence of NADH, 3R-acetoin and DA are reduced to three BDO isomer forms by different BDHs. L-BDO and D-BDO are synthesized from 3S-acetoin and 3R-acetoin by L-BDH and D-BDH respectively, whereas meso-BDO can be produced from 3R-acetoin by meso-BDH or 3S-acetoin by D-BDH. BDO can be reversibly converted to acetoin to regenerate NADH in order to maintain a constant oxidation-reduction state. The existence of various BDHs differing in their stereospecificities in native BDO producers leads to mixed formation of acetoin and BDO stereoisomers (Jia et al. 2017; He et al. 2018).

Except BDO other metabolites including succinate, formate, lactate, acetate and ethanol are synthesized, depending on microorganism and applied culture conditions. The metabolic pathway of BDO and acetoin production is presented in Figure 2.7.

The maximum theoretical yield from glucose and glycerol is 0.50 g $g^{-1}$ and 0.46 g $g^{-1}$ respectively (Jiang et al., 2014).

# Chapter 2

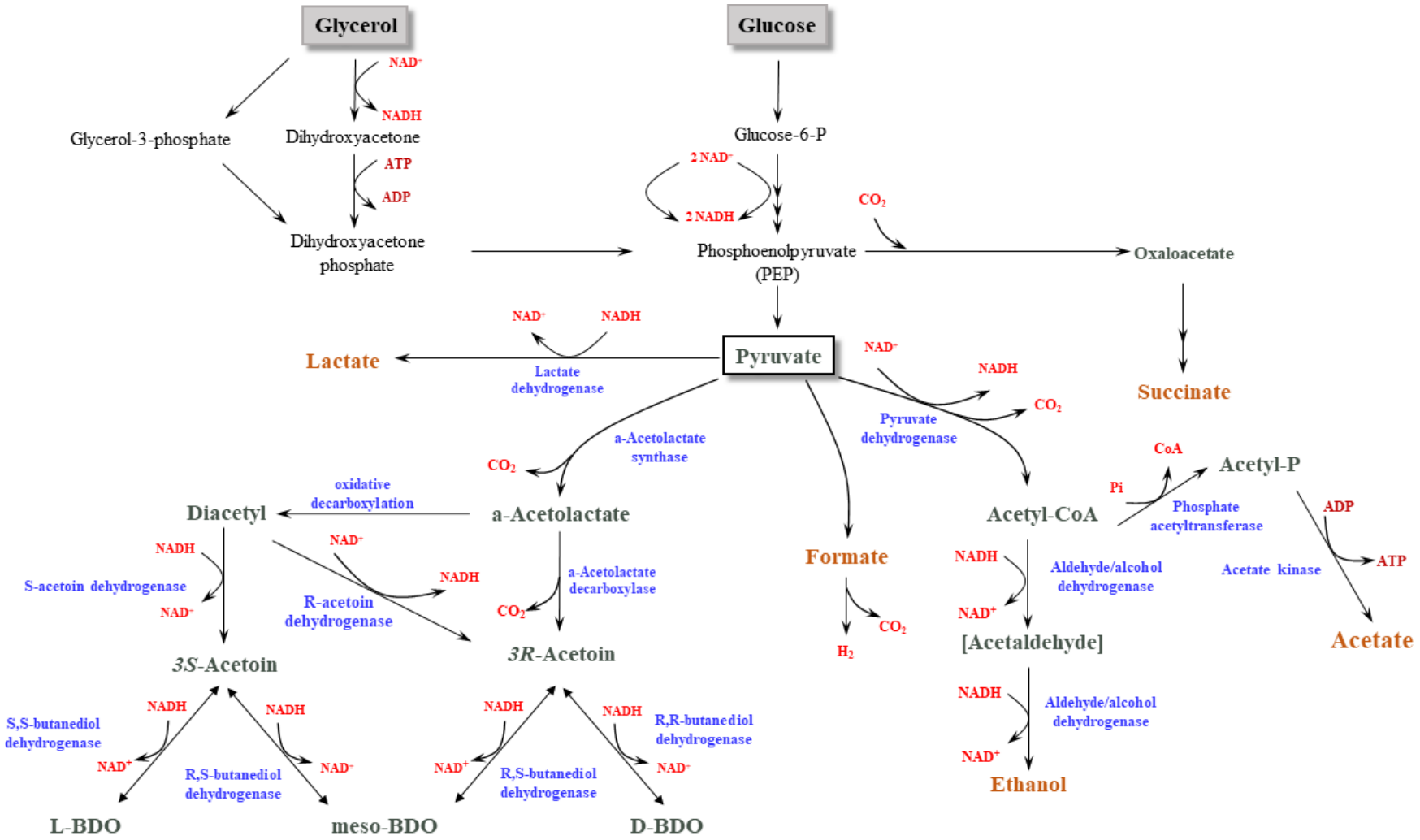


**Figure 2.7** Metabolic pathway for BDO synthesis

### 2.2.4 Biological function of BDO

The metabolic pathway of BDO participates in the regulation of the intracellular NADH/$NAD^+$ ratio in the cell owing to the reversible transformation between acetoin and BDO coupled with the $NAD^+$/NADH conversion. The biosynthesis of BDO has a vital role in preventing the intracellular acidification environment. Over acidification of environment and culture medium due to acidic product formation is prevented by the conversion of pyruvate to neutral compounds (Celińska and Grajek, 2009; Ji et al., 2011). Moreover, when carbon and energy sources have been depleted, BDO is transformed to acetoin to regulate the carbon and energy balance. In addition, acetoin biosynthesis is closely related to the branched-chain amino acid pathway and the regulation of cell life (Xiao and Xu, 2007).

### 2.2.5 Operational parameters affecting BDO synthesis

#### 2.2.5.1 Oxygen supply

Dissolved oxygen supply is considered the most critical factor in BDO fermentation affecting the distribution of metabolites produced, yield and productivity (Celińska and Grajek, 2009; Ji et al., 2011). BDO is produced under limited oxygen supply to maintain an internal redox balance of pyridine nucleotide pairs ($NAD^+$/NADH and $NADP^+$/NADPH) during glycolysis and biosynthesis. Under low oxygen supply, the respiratory chain cannot effectively regenerate the excess reducing power associated to glycolysis and NADH-consuming pathways are activated leading to the formation of by-products (Converti et al., 2003). At high oxygen supply, NADH from glycolysis is generated via respiration thus bacterial growth and $CO_2$ formation are favored. By controlling the oxygen supply toward the value oxygen demand could result in enhanced ration of acetoin to BDO. The formation of metabolites is strongly associated with oxygen availability according to the cell need to maintain the $NAD^+$/NADH balance. Therefore, a suitable oxygen supply is crucial for efficient BDO production.

Several control parameters including oxygen transfer rate, OTR (Beronio and Tsao, 1993), volumetric oxygen transfer coefficient, $k_La$ (Häßler et al., 2012; Maina et al., 2018; Rebecchi et al., 2018), oxygen uptake rate, OUR (Zeng et al., 1990a; Converti et al., 2003) and respiratory quotient, RQ (Zeng et al., 1994b; Zhang et al., 2010a), have been applied in BDO fermentation.

RQ has been used as oxygen control parameter for BDO production by *E. aerogenes* (Zeng et al., 1994b). A detailed study was carried out in batch, fed-batch and continuous mode in various

bench-top and pilot plant bioreactors. High product concentration of 102.9 g $L^{-1}$ (BDO and acetoin production of 96 g $L^{-1}$ and 6.9 g $L^{-1}$, respectively) was achieved by controlling RQ value between 4 to 4.5 in fed-batch fermentation mode. RQ was further used as control strategy in different reactors for scale-up of BDO fermentation showing that reactor hydrodynamics and initial OTR affect BDO synthesis (Zeng et al., 1994b). The same strategy has been applied in fed-batch fermentation by Zhang et al. (2010). By controlling RQ value within the range of 1 to 1.5 in the growth phase and between 1.8 to 2 in BDO production stage, improved BDO (146.6 g $L^{-1}$) produced by *S. marcescens* H30 (Zhang et al., 2010a).

Häßler et al. (2012) studied the effect of oxygen supply on BDO production by *P. polymyxa* using $k_La$ as parameter. Various $k_La$ values within the range of 19.7 to 194 $h^{-1}$ were obtained, changing agitation and aeration rates. Increasing oxygen availability caused a reduction of D-BDO with simultaneous increment of meso-BDO and acetoin formation. Moreover, low oxygen availability favor lactate and ethanol production. The highest D-BDO production of 46.1 g $L^{-1}$ was achieved at $k_La$ value of 30 $h^{-1}$.

A simple oxygen supply method based on constant agitation speed has been extensively used for BDO production. High agitation rates stimulate bacterial growth, acetoin and acetate accumulation lead to decreased BDO yields, whereas low agitation speeds favor BDO and by-product (namely succinate, lactate and ethanol) formation (Kim et al., 2016a; Priya et al., 2016; Maina et al., 2018). Cho et al. (2015) reported BDO and acetoin production of 118.5 g $L^{-1}$ and 42.1 g $L^{-1}$, respectively at 400 rpm with low by-product accumulation. Similar results have been stated by Kim et al. (2016) using *R. ornithinolytica*. BDO concentration of 68.3 g $L^{-1}$ was obtained at 400 rpm accompanied by low formation of NADH-dependent by-products. Increasing agitation rate at 500 rpm, bacterial growth and acetic acid was enhanced resulting in low BDO yield (0.19 g $g^{-1}$). On the other hand, BDO production by *B. licheniformis* was enhanced at low agitation rate (200 rpm) with high formation of formic acid, though higher agitation rates (400 rpm) resulted in equal amounts of BDO and acetoin (Li et al., 2013).

The evaluation of two-stage agitation and aeration rates has been proposed for BDO production. High oxygen supply at the growth phase, followed by low oxygen availability throughout the fermentation have been performed for BDO production (Ji et al., 2009; Yang et al., 2015; Priya et al., 2016; Qui et al., 2016). BDO of 115.7 g $L^{-1}$ with productivity of 2.4 g $L^{-1}$ $h^{-1}$ has been achieved by *B. licheniformic* using two-stage agitation speed (Li et al., 2013).

#### 2.2.5.2 pH

pH value is another important parameter that regulates the intracellular enzymatic activity and hence affects the composition of metabolites by altering the metabolic pathway. The optimal pH value of key enzymes for BDO synthesis (ALS, ALDC and BDHs) vary for different microorganisms. The activity of BDH is decreased with increasing pH values from 5 to 7.3 for *E. aerogenes* (Nakashimada et al., 2002). Moreover, the highest specific activity of BDH was at pH 6 in cultivation with *P. polymyxa* (Gao et al., 2013). ALS, which is responsible for the first step in acetoin-BDO pathway, is inactivated for pH values above 6 in *K. pneumoniae* (Stormer, 1986; Chan et al., 2016).

The metabolic function of acetoin and BDO biosynthesis is physiologically important to the microorganisms to prevent intracellular acidification. Generally, pH value above 6.5 favours the production of organic acids, yielding to decrease BDO formation. Organic acid production results in culture acidification and increment of toxic undissociated forms of acids, resulting in inhibitory effects on both bacterial growth and BDO and acetoin biosynthesis. On the other hand, pH value equal or below 6.5 leads to reduction of organic acid synthesis corresponding to enhanced BDO and acetoin formation (Celińska and Grajek, 2009).

Lee et al. (2017) reported maximum BDO production at pH value of 6.5 using *E. aerogenes*. Controlling pH value at higher levels resulted in accumulation of succinate, acetate and ethanol, whereas pH value lower than 6.5 led to decline of bacterial growth and substrate consumption rate (Lee et al., 2017). Similar optimal pH value has been demonstrated by Wong et al. (2014) using *Klebsiella* sp. In general, the optimum pH value for BDO production by *K. oxytoca* has been reported to be in the range of 5 to 6 (Xiao and Lu, 2014), while high pH values (6.8 to 7) are beneficial for enhanced bacterial growth. A two-stage pH control strategy maintaining pH at 6.0 after a natural pH drop from (pH value of around 7) neutral culture media has been also applied for BDO and acetoin production leading to increased production and yield (Sun et al., 2012a; Zhang et al., 2012; Kim et al., 2016a).

#### 2.2.5.3 Temperature

Enzymatic activity, cellular maintenance and product formation are strictly temperature dependent processes. Mesophilic species have optimum bacterial growth at temperature within the range of 25 to 45 ˚C. Acceleration of enzymatic processes has been observed with increasing temperature (Xiao and Lu, 2014).

Perego et al. (2003) have reported a progressive incense of BDO production and productivity from 34 to 37 ˚C by *B. licheniformis*. Similarly, optimal temperature for growth and BDO formation by *B. amyloliquefaciens* was at 37 ˚C. The reverse behaviour has been reported for acetoin production when temperature was increased from 25 to 40 ˚C (Yang et al., 2011). The effect of temperature on BDO production by *K. pneumoniae* and *E. aerogenes* has been examined by Barret et al. (1983). A temperature of 33 ˚C was optimal for *K. pneumoniae,* while changes in temperature within the range of 30 to 37 ˚C had little effect on BDO formation by *E. aerogenes*. In general, the optimum temperature for BDO production by Bacilliaceae strains is in the range of 34 to 37 ˚C (Li et al., 2013), whereas *Klebsiella* and *Enterobacter* strains have optimal value between 30 to 37 ˚C (Celińska and Grajek, 2009). On the other hand, the optimal temperature for growth and BDO formation by a newly isolated *R. ornithinolytica* strain was 25 ˚C (Kim et al., 2016b). Different strains may have diverse optimal temperature, therefore the optimal value should be examined individually for each strain and substrate used.

Thermophilic strains (*Geobacillus* sp. and *Bacillus licheniformis*) have been applied for BDO fermentation (Xiao et al., 2012; Li et al., 2013; Ge et al., 2016). Thermophilic fermentation process could reduce the risk of bacterial contamination and can be operated without sterilization making the process more efficient and cost-effective. Thermophilic strains have been successfully applied for simultaneous saccharification and fermentation resulting in a more cost-effective process (Li et al., 2014a). Typically, the optimum temperature of thermophilic strains is in the range of 50 to 60 ˚C (Xiao et al., 2012).

#### 2.2.5.4 Media composition

Mineral supplements are important for bacterial growth and product formation. Different media compositions could lead to alterations in the intracellular metabolic activities resulting in different fermentation products. Recent studies have indicated the important role of phosphate, acetate, $Fe^{2+}$, $Mn^{2+}$, $K^{+}$ and $Mg^{2+}$ salts on cell metabolism, substrate consumption rate and BDO production (Ma et al., 2009a; Adlakha and Yazdani, 2015). Specifically, phosphate ions improve the formation and yield of diol (Laube et al., 1984), $Mn^{2+}$ activates ALDC in the BDO pathway (Laube et al., 1984), $Fe^{2+}$ stimulates the growth and increase the consumption rate (Deshmukh et al., 2015). $K^{+}$ ions having essential role in bacterial physiology, including the

osmoregulation and maintenance of cellular pH, are important in the structure and function of various enzymes (Kinsinger et al., 2005), EDTA improves the cell permeability which may

enhance the mass transfer (Song et al., 2012). High concentrations of each supplement could result in osmotic pressure resulting in inhibitory effect upon enzymatic activity and hence product formation.

The distribution of acetoin and BDO stereoisomers is also affected by the metal ions composition. It has been demonstrated that the BDHs activity from *Corynebacterium crenatum* was increased by $Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$ and $Mn^{2+}$ ions, though $Cu^{2+}$ inhibited BDHs activity (Zhao et al., 2015). Yu et al. (2015) reported and characterized the D-BDH from *Rhodococcus erythropolis*. The $K^{+}$ cation enhanced the activity, $Na^{+}$ and $Mg^{2+}$ and $Co^{2+}$ cations slightly decrease the activity, while the enzyme activity was inhibited by $Ag^{+}$, $Cu^{2+}$ and $Fe^{2+}$. In the study with *Serratia marcescens*, it was found that the reaction of 3S-acetoin to meso-BDO could be strongly inhibited by $Fe^{2+}$ and $Fe^{3+}$, while the presence of $Mn^{2+}$ could increase the efficiency of the reaction (Zhang et al., 2014).

Acetate supplementation for enhanced BDO production has examined by several authors. It has been reported that acetate stimulates the formation of key enzymes by activating the transcriptional regulator. Moreover, acetate regulates the balance between BDO and acetoin and can inhibit the conversion of BDO to acetoin (Lee et al., 2017). Ernest and Saddler (1982) showed that growth and BDO yield by *K. pneumoniae* was increased in the presence of acetic acid (1-5 g $L^{-1}$). Acetic acid in concentration more than 10 g $L^{-1}$ inhibits the growth and BDO formation. It has been reported that undissociated form of acetic acid can penetrate into the cytoplasm of the cell resulting in acidification and depletion of ATP (Zeng et al., 1990b). Recently, sodium and potassium acetate in the range of 0-0.2 M were evaluated as sources of acetate. The optimal source for BDO production by *E. aerogenes* was potassium acetate at concentration of 0.1 M (Lee et al., 2016). On the other hand, sodium acetate at concentration of 2.02 g $L^{-1}$ was found to induce acetoin production by *P. polymyxa* (Zhang et al., 2012).

#### 2.2.5.5 Nitrogen source

Nitrogen source is necessary for bacterial growth and accumulation of BDO. Complex organic nitrogen sources including yeast extract, peptone, casamino acid and corn steep liquor (CSL) could accelerate BDO and acetoin production (Li et al., 2013; Kim et al., 2017). Yeast extract containing the essential growth micronutrients has been mainly used for BDO production.

Zhang et al. (2010) reported BDO production of 139.9 g $L^{-1}$ with productivity of 3.49 g $L^{-1}$ $h^{-1}$ using 33.4 g $L^{-1}$ yeast extract. However, the high concentration of yeast extract leads to increased production cost (Koutinas et al., 2016). CSL has been applied as an alternative and inexpensive organic nitrogen source (Ma et al., 2009a; Sun et al., 2012a; Yang et al., 2015). A mixture of yeast extract and CSL has been applied for enhanced BDO and acetoin production by Bacillaceae species (Xu et al., 2011; Tian et al., 2016) however, high concentrations are needed for enhanced production. The usage of inorganic nitrogen sources including urea and ammonium sulfate have been also evaluated. Efficient BDO production has been achieved using inorganic media composition by *E. ludwigii* (Maina et al., 2018).

#### 2.2.5.6 Substrate and product inhibition on BDO and acetoin

Initial substrate concentration along with products formation affect cell growth, BDO and acetoin production. High concentration of sugars causes osmotic stress resulting in cell membrane disruption and cell death, while organic acids may inhibit cell growth by altering intracellular pH and deactivating enzymes. Generally, BDO producers expose high tolerance to synthetic sugar concentrations up to 200 g $L^{-1}$. High specific growth rates and yields are normally obtained at lower concentration of sugars (Kim et al., 2016a). Increased initial concentration of substrate influence the metabolic pathway leading to enhanced by-products formation (Priya et al., 2016). High concentration of sugars derived from renewable resources and industrial side streams tend to inhibit cell growth due to the toxic compounds present in raw materials (Celińska and Grajek, 2009).

The inhibitory effect of fermentation products on growth of BDO producers has not been extensively studied. Kim et al. (2016) proposed a model for substrate and product inhibition for BDO production by *K. oxytoca*. Formic acid and acetic acid had the strongest inhibition effect, whereas high tolerant to succinic acid was observed. *K. oxytoca* showed high tolerance to BDO and ethanol (74 and 36 g $L^{-1}$ respectively), though acetoin strongly inhibited and completely ceased the growth at concentration of 23.6 g $L^{-1}$. A reduction of specific growth rate by 50% has been observed in *E. aerogenes* with 20 g $L^{-1}$ of ethanol (Zeng et al., 1991b). The growth of *P. polymyxa* was inhibited by 60 g $L^{-1}$ of D-BDO (Okonkwo et al., 2017a).

### 2.2.6 Fermentation mode

The reactor operation mode is an important factor for optimal process design. Various reactor operation modes, including batch, fed-batch, continuous cultures, cell recycle and immobilized cell systems, have been implemented for BDO production. Batch and fed-batch operation modes have been widely studied for BDO fermentation. Batch processes have some defects like low productivity and high substrate inhibition. Fed-batch fermentation mode has been used to overcome the effect of initial substrate inhibition and it is considered the most favourable operation mode for industrial scale. Balancing feeding strategy could lead to high final concentration. Ma et al. (2009) compared different fed-batch strategies for BDO production, including constant feed rate, pulse, constant residual glucose concentration and exponential fed-batch. Maximum BDO and acetoin production of 160 g $L^{-1}$ with productivity of 4.21 g $L^{-1}$ $h^{-1}$ was obtained by constant residual glucose concentration feeding method. A similar method was applied in BDO fermentation by *S. marcescens* (Zhang et al., 2010a). Generally, constant feeding of substrate provides suitable environment for cell metabolism and BDO production.

Continuous fermentation with cell recycle by maintaining high biomass density allows high and stable process efficiency with high values of productivity. Contamination is still the limiting factor of this technique. Improved economic viability can be achieved by continuous fermentation (Ji et al., 2011). Zeng et al. (1991) evaluated continuous culture with cell recycle system resulting in high productivity (14.6 g $L^{-1}$ $h^{-1}$), though the final production of BDO and acetoin were lower than in fed-batch mode (54 and 110 g $L^{-1}$, respectively).

A promising system employing immobilized cells has been also evaluated to increase BDO efficiency (Jurchescu et al., 2013). Maximum BDO concentration of 118.3 g $L^{-1}$ was achieved by *B. licheniformis* immobilized cells. Compared to free cells, the immobilized system showed lower BDO concentration however, the authors proposed that using proper amount of nutrients could enhance the efficiency of the bioprocess (Jurchescu et al., 2013).

### 2.2.7 Agro-industrial resources as feedstocks for BDO production

The economic viability of industrial BDO production is highly dependent on the cost of raw material (Koutinas et al., 2016). Jiang et al. (2015) reported that the substrate accounts for more than half of the total production cost of BDO. In the past decades, the carbon sources employed for BDO fermentation are mainly commercial sugars. Renewable resources have been recently used as alternative, abundant and cheap substrates for BDO production. These substrates can

be classified in three categories: agricultural residues, side streams and co-products from food industry and by-products from biofuel industries.

#### 2.2.7.1 Agricultural residues

Lignocellulose substrates including forest residues, agricultural residues (corn stover, corncob, sugarcane bagasse, sorghum stover, rice straw) and agricultural by-products (wheat bran and soybean hulls), are the most abundant agricultural biomass on earth. Lignocellulosic biomass is mainly composed of cellulose, hemicellulose and lignin. It is usually treated with chemical or physical methods to release the fermentable sugars. The hydrolysis of lignocellulose yields mixed C5 and C6 sugars with diverse ratios, depending on the type of biomass material and the pretreatment method used (Ma et al., 2018). Lignocellulosic biomass has been used as an alternative cheap substrate for BDO production.

The valorisation of corn strover hydrolysate for BDO production has received considerable attention during the recent years. Li et al. (2014) reported high BDO production of 74 g $L^{-1}$ with productivity of 2.1 g $L^{-1}$ $h^{-1}$ using corn stover hydrolysate by *B. liqueniforms*. The strain showed high tolerance to inhibitors present in lignocellulosic hydrolysate such as furfural, vanillin formic acid and acetic acid. Ma et al. (2018) studied the valorisation of corn stover hydrolysate in three fermentation modes for D-BDO using *P. polymyxa*. Fed-batch fermentation led to higher D-BDO titer of 46.12 g $L^{-1}$, however productivity was relatively low. Significantly increment of productivity was achieved in continuous fermentation reaching 1.13 g $L^{-1}$ $h^{-1}$ (Ma et al., 2018). A metabolically engineered *E. cloaca,* which could utilise glucose and xylose simultaneously, was used for production of D-BDO from corn stover hydrolysate. Up to 119.4 g $L^{-1}$ of *D*-BDO produced with a productivity of 2.34 g $L^{-1}$ $h^{-1}$ (Li et al., 2015).

Maltodrexin generated from hydrolysed corn or cassava starch was used as cheap substrate for BDO production using a metabolically engineered *K. oxytoca* strain (Chan et al., 2016). Under optimized conditions BDO reached 88.1 g $L^{-1}$ with conversion yield of 0.41 and productivity of 1.13 g $L^{-1}$ $h^{-1}$ in fed-batch fermentation mode (Chan, 2016). The valorisation of pretreated apple pomace hydrolysate has used for BDO production by Baikowska et al. (2016). Maximum BDO concentration of 51.5 g $L^{-1}$ was achieved by *B. subtilis* in fed-batch fermentation mode.

Guragain et al. (2017) evaluated the sugars derived from sorghum stalks, witchgrass and poplar biomass feedstocks for BDO production using *K. oxytoca*. Oil palm front hydrolysate has been used for BDO production by *E. cloacae,* however only 7.68 g $L^{-1}$ has been achieved in batch

culture (Hazeena et al., 2016). Sweet sorghum stalk juice containing a large amount of fermentable sugars, minerals and trace elements has been utilised for BDO production by *S. marcescens* (Yuan et al., 2017). Yeast extract along with mineral had remarkable effect on BDO production. Under optimized conditions, 109.4 g $L^{-1}$ of BDO was produced with a productivity of 1.4 g $L^{-1}$ $h^{-1}$ in fed-batch fermentation.

#### 2.2.7.2 Food industry side streams and co-products

Food waste streams are non-cellulosic raw materials that have been utilised in microbial bioconversion. Molasses, whey and starch hydrolysate after processing of cereals, maize and potatoes are feedstocks that do not require physiochemical pretreatment and usually inexpensive supplementation with small amount of minerals and nitrogen is needed for BDO production. Molasses, the residue left after the crystallization of sucrose from sugar cane juice containing around 50% fermentable sugars along with salts, proteins and vitamins, has been proven to be a suitable substrate for BDO production. Bailkowska et al. (2016) used *B. subtilis* for BDO production using molasses in fed-batch culture. BDO production was varied by using glucose and sucrose as feeding solution. The maximum BDO of 75.7 g $L^{-1}$ was achieved using molasses as carbon source and glucose as feeding solution (Baikowska et al., 2016). Jung et al. (2013) reported the highest BDO production (98.7 g $L^{-1}$) from sugarcane molasses by *E. aerogenes*. The optimization of media composition using molasses as carbon source has been studied by Dai et al. (2015) leading to increased BDO production. In fed-batch fermentation mode and under optimized conditions, 99.5 g $L^{-1}$ of BDO and acetoin were produced with a productivity of 1.66 g $L^{-1}$ $h^{-1}$ (Dai et al. 2015).

Cheese whey, a side stream from cheese production, is another renewable resource of interest for BDO production. Guo et al. (2017) optimized the media composition using cheese whey as carbon source with *K. pneumoniae* leading to 57.6 g $L^{-1}$ of BDO with conversion yield of 0.40 g $g^{-1}$.

Fruit wastes from open markets have also been used as carbon sources in BDO fermentation (Liakou et al., 2017). *E. ludwigii* produced around 50 g $L^{-1}$ of BDO, however succinic acid and lactic acid were produced as by-products resulting in yield of 0.40 g $g^{-1}$ (Liakou et al., 2017).

### 2.2.7.3 Biofuel industries by-products

The valorisation of glycerol generated as by-product from biodiesel industry for bioconversion has attached much interest. Previous studies have successfully utilised crude glycerol generated from plant oils and animal fat for BDO production by various wild and metabolically engineered strains. Two non-pathogenic bacterial strains, namely *R. planticola* and *R. terrigena* have been reported to produce up to 30 g $L^{-1}$ of BDO in batch experiment using raw glycerol (55-85 % w/w) as carbon source (Ripoll et al., 2016). Petrov and Petrova (2010) developed a forced pH fluctuation process by consecutive raising of ΔpH value at 1 every 12 hours for BDO production using pure glycerol. The concentration of BDO reached 70 g $L^{-1}$ with a conversion yield of 0.53 g $g^{-1}$ and productivity of 0.47 g $L^{-1}$ $h^{-1}$. However, *K. pneumoniae* possess the PDO synthesis pathway and PDO was generated as by-product reaching a concentration of 16.3 g $L^{-1}$. *B. amyloliquefaciens* was reported to produce 102.3 g $L^{-1}$ of BDO without by-product formation in fed-batch fermentation mode using raw glycerol supplemented with molasses as carbon source (Yang et al., 2013). The most efficient BDO producers are metabolically engineered strains by simultaneous expression of the genes involved in BDO production and inactivation of genes involved in a competitive pathway. Kim et al. (2017) demonstrated BDO production of 65.4 g $L^{-1}$ without PDO formation from crude glycerol using a *bud*ABC overexpression mutant *R. ornithinolytica*. In the same study, pretreatment of crude glycerol improved BDO production and yield by 19% and 7.7%, respectively. An adapted mutant strain *K. variicola* SW3 displayed high tolerance in crude glycerol (up to 200 g $L^{-1}$) for BDO production (Rahman et al., 2017). In fed-batch fermentation mode *K. variicola* SW3 reached 64.9 g $L^{-1}$ of BDO from crude glycerol showing that the overexpression of GDH had significant increment on *budB* and *bud*C genes. The highest BDO production (131.5 g $L^{-1}$) from crude glycerol was achieved by a metabolically engineered *K. oxytoca* strain. Deletion of *pdu*C and *ldh*A genes was improved BDO production and reduced the by-product formation (PDO and lactic acid). A mixture of L-BDO and *meso*-BDO in ration of 0.92:1 was produced by *K. oxytoca* M3 (Cho et al., 2015a). BDO stereoisomer formation in *K. pneumoniae* was investigated by Chen et al. (2014) in glycerol metabolism. The wild *K. pneumoniae* strain produced all BDO isomers with *meso*-BDO in higher concentration (ratio of meso-BDO, L-BDO and D-BDO was 2.6:0.18:1) when glycerol used as carbon source. While the *bud*C mutant showed high levels of D-BDO suggesting high glycerol dehydrogenase activity that catalysed the conversion of R-acetoin to D-BDO.

### 2.2.8 Downstream separation

The separation and purification of BDO from fermentation broth is a challenging aspect in the development of a cost efficient production. A low energy required separation process with high efficiency is necessary. The recovery of BDO is difficult due to the high boiling point, the hydrophilic nature and the presence of dissolved and solid components (Xiao and Lu, 2014; Jiang et al., 2014).

Until now, a promising commercial technique for BDO recovery is the simulated moving bed (SMB) implementing in LanzaTech fermentation plant for the production of anhydrous ethanol and BDO (Schultz et al., 2015). However, the drawbacks of SBM are the existence of strong non-linear behavior during design and optimization and the expensive equipment.

Several separation methods including distillation (Kawamura et al., 2014), pervaporation (Qureshi et al., 1994), steam stipping (Xiu and Zeng, 2008), solvent extraction (Eiteman and Gainer, 1989), salting-out (Dai et al., 2017) and reverse osmosis (Xiu and Zeng, 2008; Kawamura et al., 2014) have been reported for BDO recovery. These methods could also be applied in the acetoin preparation process due to their similar properties. However, these techniques have some drawbacks and limitation for industrial application. Steam stripping and distillation require large amounts of energy and prevents the industrial application today. Salting-out method has been applied for BDO recovery with yield of 94-99% (Jianying et al., 2011); however, it has been evaluated only in lab scale. On the other hand, solvent extraction method requires high amount of solvent and the separation efficiency is relatively low. The development of an efficient process to fulfil the requirement of separation is necessary to be integrated with other methods.

A combination of liquid-liquid extraction with distillation known as hybrid extraction distillation has been proposed as promising technique (Harvianto et al., 2018). Solvent is used to extract BDO by forming the organic phase. The organic phase is then inserted into a distillation column where the solvent is recycled back to the extractor and BDO is recovered from the top. Harvianto et al. (2018) evaluated various solvents including ethyl acetate, butyl acetate, 1-butanol, isobutanol, 2-ethyl-1-hexanol and oleyl alcohol for BDO recovery. High distribution coefficient was obtained by using oley alcohol.

A novel method combing solvent extraction and salting-out known as aqueous two-phase extraction has been applied for BDO recovery. Salting-out increases the extraction efficiency

and decreases the quantity of solvent needed for extraction. Ethanol/ammonium sulfate (Li et al., 2010b), ethanol/phosphate (Jiang et al., 2009), isopropanol/ammonium (Sun et al., 2009b) and acetone/phosphate (Sun et al., 2012b) systems have been evaluated for BDO and acetoin purification achieving high recovery coefficients.

Another recovery technique named as fermentation-derivatization-recovery was studied (Xiao et al., 2014). This method relies on the derivatization of BDO and acetoin to other valuable compounds, which subsequently are recovered and purified from a complex mash. 2,3,5,6-tetramethylpyrazine (TTMP) can be generated by the condensation of acetoin and ammonia in aqueous solution under mild conditions (Rizzi, 1988). The TTMP can be recovered and purified by crystallization. Extraction of BDO by cyclic acetal formation using formaldehyde or acetaldehyde for acetalization has been reported by Hao et al. (2006). The acetals formed are recovered by aromatic solvent extraction or distillation. This technique requires less operating time and small amount of reactant and extractant. A more attractive technic is the acid-catalyzed dehydration of BDO to MEK followed by distillation (reactive distillation technique). This method is based on the difference in the boiling points of BDO (183-184 ˚C) and MEK (79.6 ˚C). The main reaction is the formation of MEK, while side reaction is the formation of MEK-BDO ketal. The separation of the ketal and MEK is achieved by distillation. The cyclic ketal can be reconverted to BDO and MEK under acid catalysis (Othmer et al., 1952; Emerson et al., 1982; Cui et al., 2018). Under this concept, acetoin can be converted to BDO by hydrogenation or electroreduction using decorated cathodes with subsequently dehydration of BDO to MEK (Gomez et al., 2018a; Gomez et al., 2018b).

## 2.3 Agro-industrial residues and food wastes

### 2.3.1 Oilseed crops: the case of soybean

Oilseeds, including soybean, sunflower, rapeseed, cotton, coconut and palm, are important crops cultivating for food, feed and industrial uses. Oilseed crops are classified as group four after cereals, vegetables and melon and fruits and nuts according to FAO (2013).

Soybean (*Glycine max* (L.) Merrill), a bushy, leguminous plant, native of South-East Asia, is one the most widely produced oilseed around the world, with a production of about 56% of the world's total oilseeds (Pratap et al., 2012). In 2017/2018 worldwide soybean production was estimated at 346.92 $10^6$ t. US, Brazil and Argentina are the largest soybean producer accounting for 82% of global production (Anonymous, 2018a). Soybean production is expected to increase

rapidly in order to meet the demand for soy in the feed and energy sector. Recently, FAO suggests an increase to 515 $10^6$ t by 2050, others estimate a 2.2% increase per year until 2030 (WWF, 2014).

Soybean seeds typically contain around 18-20% oil, 40% protein, 17% cellulose and minor contents of sugars, ash and fibre. It can be consumed directly as human food products or it can be crushed into meal and oil. Globally, approximately 87% of soybean is crushed into meal and oil with the remaining 13% used for direct human consumption (Thoenes, 2006).

The soybean crushing process generates about 78.5% of soy meal, 19% oil and 2.5% lost in processing (Brown-Lima et al., 2010). Extraction of oil from soybean seed is conducted by mechanical press, solvent extraction, supercritical fluid extraction and microwave and ultrasound assisted oil extraction. The most common process practice is the solvent extraction (Figure 2.8). The first step in the solvent-extraction process involves cleaning, cracking, conditioning and flaking. Prior to the conditioning step, hulls are removed by aspiration in order to increase the protein content of the meal. The flakes are conveyed either directly to the extractor or to an expander. Subsequently, the flakes are washed with hexane to solubilize the soybean lipid material and through a series of step the hexane oil mixture is separated. The oil-rich extract called miscella is evaporated and the solvent is recycled to the extractor. Subsequently, the oil is refined to obtained the soybean oil. During the refining process lecithin can be removed. The soybean flakes with the oil removed are called spent flakes. The spent flakes are conveyed to a desolventizer toaster for removing undrained hexane. From the desolventizer-toaster, the meal passes through a dryer-cooler to get a moisture content of 13-14% and cooled for storage. The meal is then screened and ground with a hammer mill to produce a uniform particle size prior to shipment to the end user. The meal from dehulled soybean contains less than 1.5% crude fat and about 48% protein (Koc et al., 2011). In some cases, the hulls are added back and soybean meal contains different quantity of protein and fiber (Banaszkiewicz, 2011).

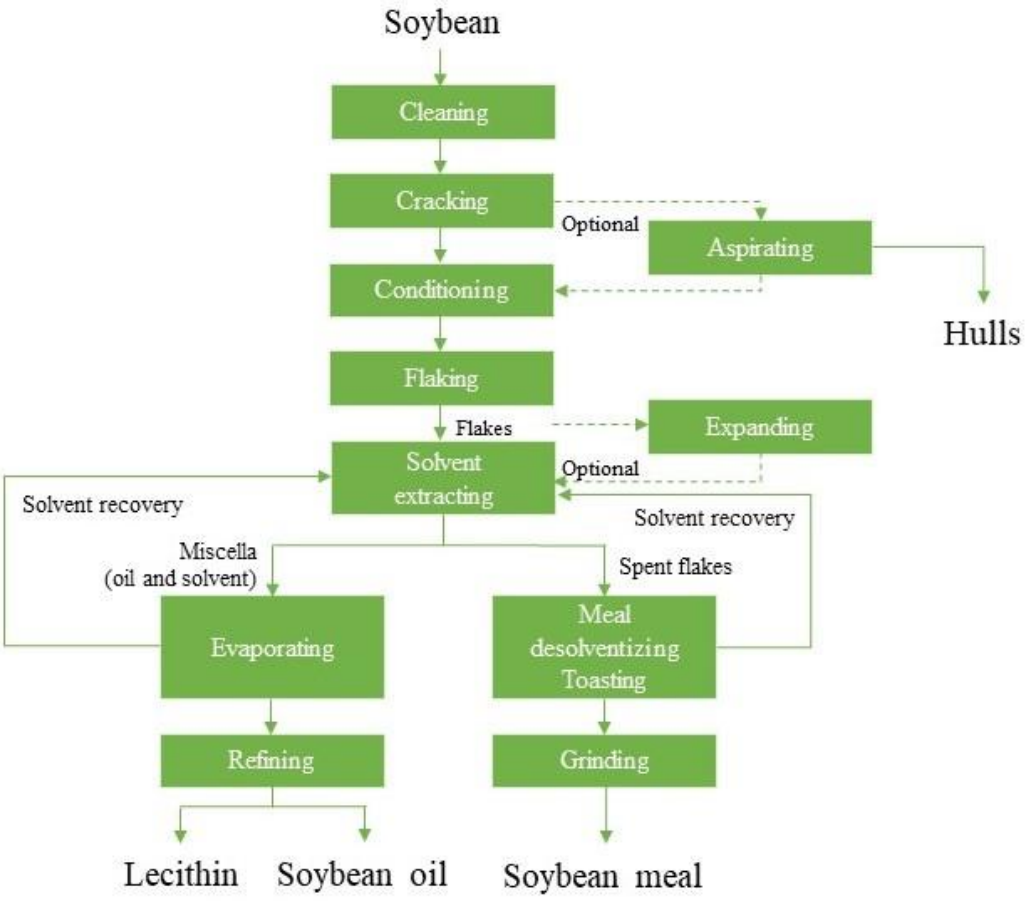


**Figure 2.8** Solvent extraction process of soybean

Soybean meal is generally used for animal feed due to the high protein content. It can be also used for the production of soybean protein concentrate and isolate which are applied in the food industry and in some industrial products such as plastics, adhesives and coatings. Soybean oil is primary employed for the production of food products. While a portion of oil is applied as feedstock for biodiesel production (Figure 2.9).

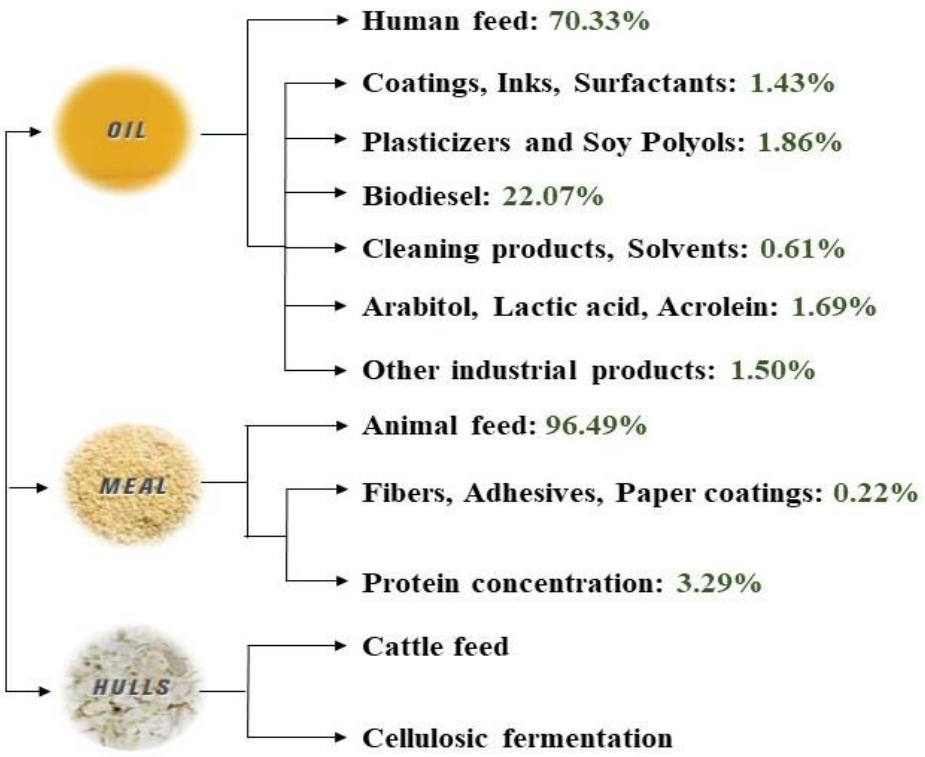


**Figure 2.9** Soy-based products (*Source: Board, 2016*)

### 2.3.2 Crude glycerol

Glycerol, also known as propane-1,2,3-triol, glycerine, trihydroxypropane, glyceritol or glycidic alcohol, is an oily, odorless, colorless liquid with syrupy-sweet taste (Quispe et al., 2013). Glycerol can be generated as a by-product from the transesterification reaction of triglycerides in the production of free fatty acids and fatty esters in biodiesel industries. It is also formed as by-products from the saponification of triglycerides in the production of free fatty acids and fatty esters and from the hydrolysis reaction of fats and oils in oleochemical plants. Synthetic glycerol can be obtained from propylene (Quispe et al., 2013; Tan et al., 2013). Around 25% of total glycerol demand was met by the petrochemical synthesis and the other fraction was obtained from soap manufacturing. Owing to the increased biodiesel production, currently 60-65% of total glycerol is produced as a by-product from biodiesel sector (Ciriminna et al., 2014).

Glycerol finds application in various industries such as pharmaceuticals, personal care, food and beverages, tobacco, alkyd resins and polyether polyols, whereas compounds of glycerol are used to preserve lotions, inks, fruits and lubricants. The global glycerol market was valued at

USD 2.19 $10^9$ in 2015 and is projected to reach USD 3.12 $10^9$ by 2021, while it is expected to grow at a CAGR of 6.8% over the forecast period of 2016 to 2021 (Anonymous, 2017b).

Crude glycerol supply is dependent on biodiesel production. During triglyceride transesterification, glycerol separates from oil phase as a by-product. Around 1 kg of glycerol is produced for every 9 kg of fatty acid methyl ester. Increased biodiesel production results in the accumulation of glycerol, which leads to a price decline (Figure 2.10). It has been projected that the global biodiesel production will reach almost 39 $10^6$ liters by 2024, which implied that approximately 4.2 $10^6$ gallons of crude glycerol would be produced (OECD/FAO, 2015). It is estimated that by 2015 biodiesel production will be increased by 3.5% per year (Monteiro et al., 2018).

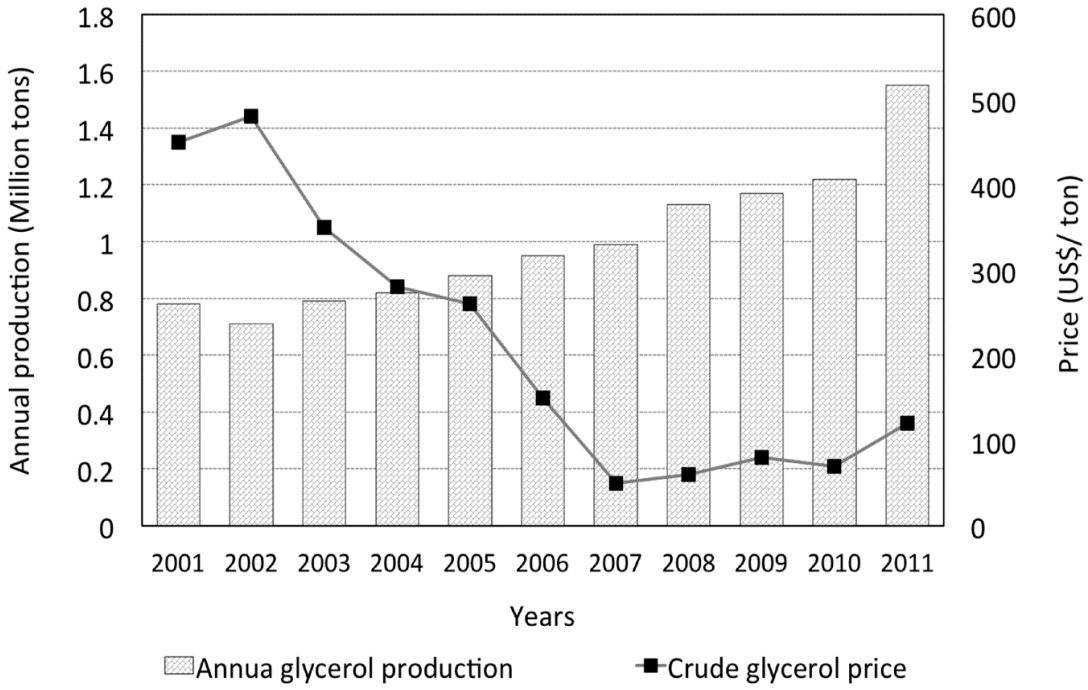


**Figure 2.10** Glycerol production and price (*source: Liu and Gao, 2018*)

Crude glycerol produced after the reaction contains impurities such as alcohol, spent catalyst, ash, water, methanol and fatty acid. Each component varies in content depending on the reaction conditions as well as the raw material used. Purification of crude glycerol is required for use in food, pharmaceutical and cosmetic industries. Various methods have been proposed including distillation, filtration, chemical treatment, adsorption, ion-exchange, extraction and crystallization. However, refining crude glycerol to a high degree is very an expensive process

especially for small and medium sized biodiesel producers. The approximate cost of crude glycerol is 0.33 US $/kg and 0.58 US$.kg to obtain purer glycerol (Quispe et al., 2013).

New opportunities for converting crude glycerol into value-added chemicals have emerged, promoting the commercial viability of biodiesel and further development. Chemical conversion, combustion, composting, anaerobic digestion and animal feed have been proposed for utilisation of glycerol. Recently, much attention has been paid to biochemical processes for the production of high added-value products through fermentation process (Table 2.4).

**Table 2.4** Potential chemicals from glycerol through bioconversion (Adapted from Monteiro et al., 2018 and Begnato et al., 2017)

| Product | Uses |
|---|---|
| Hydrogen | Alternative energy to the use of fossil fuels |
| Ethanol | Food and chemical industries |
| PDO | Chemical intermediate in the manufacture of polyethers, polyesters and polyurethanes |
| Butanol | Potential liquid fuel. Solvent |
| Propionic acid | Chemical product used in cellulose plastic, gerbicides and perfumes |
| Succinic acid | Application in agricultural, food and pharmaceutical industries |
| Erythritol | Applications in the chemical, cosmetic, food and pharmaceutical sectors |
| Mannitol | Food and pharmaceutical applications |
| BDO | Plastics, anti-freeze solution, methyl ethyl ketone, 1,3-butadiene, diacetyl |
| Dihydroxyacetone | Skin care products |
| Lactic acid | Food industry, intermediate in polyester resins and polyurethane |
| Citric acid | Agro-industrial products |
| PHB | Production of polymers |
| Arabitol | Food industries |

### 2.3.3 Sugarcane industry co-products

Sugarcane (*Saccharum officinarum*) is a major crop cultivated in tropical and sub-tropical countries like Brazil, China, India and Thailand for the production of cane sugar (Cheavegatti-Gianotto et al., 2011). In 2017/2018, approximately 191.81 $10^6$ t of sugar produced worldwide with sugarcane accounts for about 80% of the total production. Brazil is the leading global sugar cane producer, with a production of 38.87 $10^6$ t. Brazil is expected to remain the main sugarcane producer by 2027, producing 34% of the world's sugarcane which will be used for 20% of global sugar production and 88% of global sugarcane based ethanol production (Anonymous,

2018b). Other products of the processing include bagasse (25-30% cane) after crushing of sugarcane, mud (3-5% cane) after clarification and molasses (3.5-5% cane) after centrifuge.

The process flow diagram for typical cane sugar production is shown in Figure 2.11. Initially, the cane is mechanically grinding and milling. The crushed cane is mixed with water known as imbibition water to enhance the extraction of the juice. Bagasse is generated as residue of cane stalks left after the crushing and extraction of juice from sugarcane. The juice from the mills is strained to remove large particles and then clarified. In raw sugar production, clarification is done exclusively with heat and lime. The lime is added to neutralize the organic acids and temperature raised to 95 ˚C. A heavy precipitate forms, known as mud, is separated from the juice in the clarifier through filtration. Subsequently, clarified juice goes to the evaporators for the concentration of the juice. The concentrated juice called syrup passes through vacuum pans for crystallization. From the crystallizer, the masticate is transferred to high-speed centrifuge, in which molasses (A) is separated from raw sugar. The raw sugar is sent back through the crystallizing and centrifuging processes two or three more times. Finally, the raw sugar is sent to the refinery for the production of white sugar (de Souza Dias et al., 2015; Sahu, 2018).

The very high polarity (VHP) raw sugar with minimum sucrose content of 99.4% was invented by Brazil. VHP sugar is made by processing the raw cane juice by boiling until crystallization of sucrose. The juice and the crystal sucrose form are allowed to cool and they are separated by centrifuge. During the centrifugal process, the crystals are separated from the remaining liquid content, which is molasses. The molasses drawn off in the first centrifugal process are called ‘first molasses’, and still contain a significant amount of sugar. First molasses is then put through the entire process once more. The sugar crystals produced at each stage of the process are a light brown colour and is called ‘VHP cane sugar’.

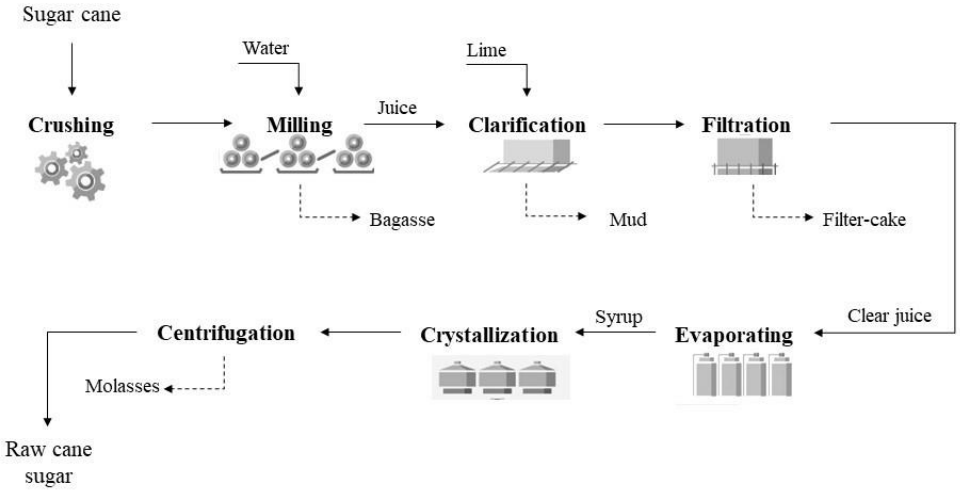


**Figure 2.11** Process flow diagram for cane sugar production

Bagasse, the residual woody fiber of the cane contains around 45-50% water, 2-5% dissolved sugar and 40-45% fibers. It is mainly used as fuel for the boilers and lime kilns to produce electricity, the manufacturing of paper and paperboard products and ethanol production. Mud containing 75-80% moisture, 2-5% sugar and 5-10% fibers is mainly used as fertilized and source of sugarcane wax (Sahu, 2018).

Molasses is an important co-product of the sugarcane processing industry. Around 2.5-4% of molasses is produced from one tone of sugarcane. It is mainly composed of sugars, such as fructose (9%), glucose (7%) and sucrose (40-50%), non-sugar organic matter (9-12%), proteins, inorganic components and vitamins. Its composition is influenced by sugarcane variety and maturity, season of production, climate, practices employed at the processing plant, and storage conditions (Sindhu et al., 2016). Molasses is used as raw material in the alcohol industry, bakery yeast, bioethanol, chemical solvent and cattle feed. Moreover, it has been investigated for fermentative production of various bio-based chemicals including lactic acid, succinic acid, ethanol and BDO.

### 2.3.4 Food wastes: the case of bakery industry

Food waste is organic waste discharged from food processing plants and the retail market (Lin et al., 2014; Kiran et al., 2015). Nearly 88 million t of food wastes including fresh vegetables, fruits, meat, bakery and dairy products are generated annually in the EU with associated cost estimated at 143 $10^6$ € (Anonymous, 2016). Bakery is one of the major food industry in the world with revenue of \$1.805 $10^6$ in 2018. In EU, bread and bakery products amount to \$50.540

$10^6$ in 2018 and it is expected to grow annually by 0.8% (Anonymous, 2018c). Bakery wastes are a combination of different wastes including sweet bread, cakes, dough, pies and croissants. Hence, their composition is highly variable. They can be formed during the manufacturing process, storage and supply of bread. Approximately 5% of total bakery products produced are lost or wasted (Chandrasekaran, 2012). The major conventional waste management and valorisation strategies for food wastes include the production of animal feed, fertilizer and biogas through anaerobic digestion (Lin et al., 2014). Alternatively, food wastes, rich in macromolecules, mainly starch and protein, as well as several macronutrients, could be a valuable resource for the formulation of fermentative feedstock and the production of chemicals and fuels (Maina et al., 2017). Bakery wastes have been successfully applied for enzymes, poly-lactic acid, succinic acid and bio-colorant production (Zhang et al., 2013a; Pleissner et al., 2015; Haque et al., 2016).

## Chapter 3

# Objectives

# Chapter 3

The transition from the current economy based on fossil raw materials toward a sustainable bio-based economy requires the interplay of research and innovation strategies for the production of bio-based chemicals, energy and fuels. Renewable resources including industrial side streams from existing industrial sectors could be efficiently utilised.

Sustainable bioprocess development plays a key role in the bio-economy sector. The development of sustainable bioprocesses requires the application of efficient and robust upstream and downstream processes. Regarding the upstream process, selection of appropriate microorganism producing the desired molecules at high efficiency, selection of suitable feedstock and evaluation of fermentation parameters affecting the fermentation efficiency, are important factors that should be optimised. Under this concept, this thesis explores the production of 1,3-propanediol (PDO) and 2,3-butanediol (BDO) utilizing food wastes from the bakery industry and crude renewable resources from biodiesel and cane sugar production processes.

The thesis is organized into two sections. In the first section (Chapter 5), the by-products from a soybean based biodiesel production process were evaluated as nutrient complete fermentation feedstocks suitable for PDO production via fermentation. Two wild-type *Citrobacter freundii* strains were evaluated and compared. The inhibitory parameters (PDO, by-products and salts) affecting bacterial growth were also studied for both strains.

In the second section (Chapters 6-8), three case studies were carried out for BDO production via fermentation using different renewable resources. The effect of various fermentation parameters including substrate concentration, temperature, pH and oxygen supply was investigated in batch shake flask and bioreactor cultures. Then, fed-batch fermentations were performed under the optimum conditions. Chapter 6 focuses on the evaluation of various crude glycerol streams originated from biodiesel production processes for the production of BDO by *Klebsiella michiganensis*. Chapter 7 presents the evaluation of sugar-based substrates for BDO production by *Bacillus amyloliquefaciens*. In Chapter 8, hydrolysis of bread waste was investigated for the production of rich fermentation feedstock that was subsequently utilised for BDO production by *Bacillus amyloliquefaciens*.

# Chapter 3

The main objectives of this thesis are presented below (Figure 3.1):

- Production of crude enzyme consortia via solid state fermentation using soybean cake
- Hydrolysis of soybean cake using crude enzymes for the production of nutrient rich feedstock
- Evaluation of crude glycerol for PDO production by wild-type *C. freundii* VK-19 and *C. freundii* FMCC-8 strains
- Investigation of inhibitory parameters affecting bacterial growth of *C. freundii* strains
- Evaluation of fermentation parameters affecting BDO production using crude glycerol as substrate
- BDO production in fed-batch fermentation using *K. michiganensis* using different types of crude glycerol under various $k_La$ values
- Evaluation of fermentation parameters affecting D-BDO and acetoin production by *B. amyloliquefacients* using sugar-based substrates from sugar cane mills
- BDO production in fed-batch fermentations with *B. amyoliquefaciens* evaluating the effect of different feeding media
- Hydrolysis of bread wastes using commercial enzymes for the production of fermentation feedstock for D-BDO and acetoin production
- Valorisation of bread waste hydrolysate for D-BDO and acetoin production under various $k_La$ values
- Evaluation of the feasibility to scale up D-BDO and acetoin production using $k_La$ values as scale-up parameters

# Chapter 3

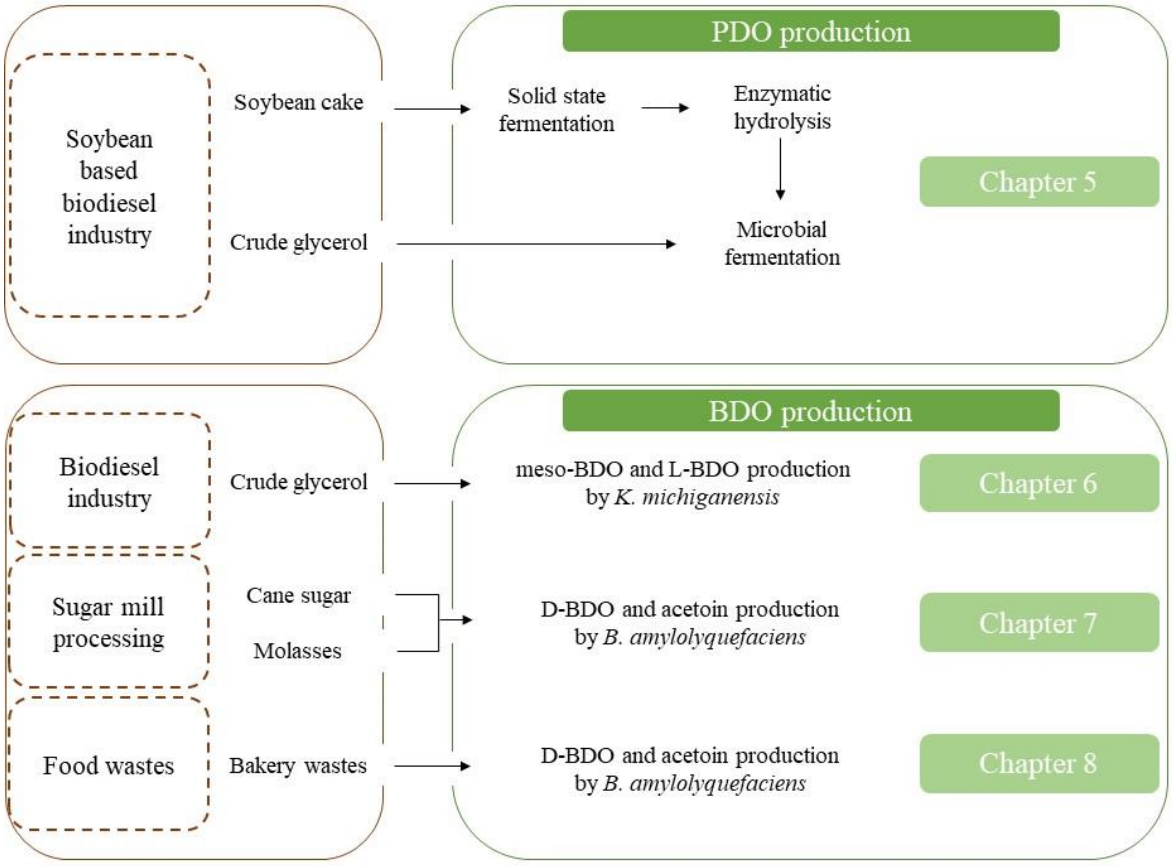


**Figure 3.1** Objectives of the study

## Chapter 4

# Materials and methods

# Chapter 4

## 4.1 Crude side streams

### 4.1.1 Soybean cake

Soybean cake (SBC) was provided by BSBios (Passo Fundo, Rio Grande do Sul, Brazil) as a by-product of soybean based biodiesel production process. SBC was utilised as substrate for the production of crude enzyme consortia through solid state fermentation (SSF) with the fungal strain *Aspergillus oryzae*. Subsequently, SBC was employed for the production of a nutrient rich hydrolysate that was evaluated as fermentation feedstock for the production of PDO by two *Citrobacter freundii* strains (Chapter 5).

### 4.1.2 Crude glycerol

Different types of crude glycerol were obtained from the biodiesel plant Petroleo Brasileiro (Petrobras, Brazil). The types of crude glycerol streams were produced from biodiesel production processes using 100% soybean oil (100-So), 90% soybean oil with 10% tallow fat (90-So:10-Tf) and 65% soybean oil with 35% tallow fat (65-So:35-Tf). A pretreated crude glycerol stream was evaluated in microbial bioconversions obtained by overnight decanting of the original crude glycerol in separation funnels**.** 100-So crude glycerol was used as substrate for PDO production (Chapter 5), while 100-So, 90-So:10-Tf and 65-So:35-Tf crude glycerols were evaluated as carbon sources for BDO production by *Klebsiella michiganensis* (Chapter 6).

### 4.1.3 Very high polarity cane sugar and sugarcane molasses

VHP cane sugar and sugarcane molasses were provided by the sugarcane industry Cruz Alta (Guarani, São Paulo, Brazil). The composition of VHP cane sugar and sugarcane molasses was characterized and subsequently evaluated as carbon source for BDO production by *Bacillus amyloliquefaciens* (Chapter 7).

### 4.1.4 Bakery wastes

Sugar bakery (Sbw) and bread wastes (Bw) were obtained from a local bakery store in Potsdam, Germany. The Sbw and Bw were dried at 60 ˚C and were milled using a cutter mill (Grindomix GM 200, Retsch, Germany) with 1 mm mesh size. Sbw and Bw were used for the production of nutrient-rich hydrolysates that were subsequently utilised for BDO production by *B. amyloliquefaciens* (Chapter 8).

### 4.1.5 Commercial supplements

Glucose, glycerol, yeast extract, bacterial peptone, meat extract, CSL, Tryptic Soy Broth (TSB), NaCl, KCl, $Na_2HPO_4$, $KH_2PO_4$, $K_2HPO_4$, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $(NH_4)_2HPO_4$, KOH, EDTA, $CaCl_2 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, NaOH, HCl, $H_2SO_4$, Tween 80, Antifoam 204, acetic acid, succinic acid, formic acid, lactic acid and PDO were purchased from Sigma.

## 4.2 Microorganisms

### 4.2.1 The fungal strain *Aspergillus oryzae*

An industrial strain of *Aspergillus oryzae* isolated from soy sauce starter at the company Amoy Food LTD (Hong Kong) has been kindly provided by Professor Colin Webb (University of Manchester, UK). *A. oryzae* was employed in SSFs for the production of crude enzyme consortia essentials for the hydrolysis of SBC. The strain was maintained in the form of spores in sand at 4 ˚C. Germination of spores was carried out with phosphate-buffered saline (PBS) solution containing (in g $L^{-1}$): 8 NaCl, 0.2 KCl, 1.44 $Na_2HPO_4$, 0.24 $KH_2PO_4$. Prior to each experiment, *A. oryzae* spores were sporulated and stored on slopes containing 30 g $L^{-1}$ SBC, 20 g $L^{-1}$ wheat bran and 20 g $L^{-1}$ agar. The slopes were used to inoculate Erlenmeyer flasks composed of the same solid substrate aiming to increase the spore concentration used as inoculum in SSF.

### 4.2.2 Bacterial strains and pre-culture conditions

#### 4.2.2.1 Bacterial strains for PDO production

The bacterial strains *Citrobacter freundii* FMCC-8 and *C. freundii* VK-19 isolated from minced beef (Doulgeraki et al., 2011) were employed for PDO fermentation. Inoculum was conducted in 250 mL Erlenmeyer flasks containing 80 mL of TSB, with pH of 7.0 ± 0.5, containing (in g $L^{-1}$): 2.5 dextrose, 17 enzymatic digest of casein, 3 enzymatic digest of soybean meal, 5 NaCl and 2.5 $Na_2HPO_4$. The pre-culture was cultivated at 30 ˚C for 18 h in a rotary shaker at 180 rpm.

#### 4.2.2.2 Bacterial strains for BDO production

The bacterial strains *K. michiganensis* and *B. amyloliquefaciens*, kindly provided by Professor George-John Nychas, were used for BDO production. The strains have been isolated from foodstuff at the Laboratory of Food Microbiology and Biotechnology in the Department of

Food Science and Human Nutrition of the Agricultural University of Athens. The identification and characterization of the strains were conducted by the Belgian Co-ordinated Collection of Microorganisms (BCCM-LMG). The bacterial strains were stored in cryopreservation vials at -80 ˚C containing liquid culture and pure glycerol in 50% v/v ratio.

The pre-culture medium for *B. amyloliquefaciens* contained (in g $L^{-1}$): 10 glucose, 5 yeast extract, 10 bacterial peptone and 5 NaCl. The pre-culture medium for *K. michiganensis* was TSB. The pre-cultures were prepared by inoculating one cryopreservation vial to a 500 mL Erlenmeyer flasks containing 200 mL of pre-culture medium and incubated at 40 ˚C for *B. amyloliquefaciens* and at 30 ˚C for *K. michiganensis* on a rotary shaker at 180 rpm for 18-20 h.

## 4.3 Production of SBC hydrolysate

### 4.3.1 Solid state fermentation

SSFs of SBC were carried out for the production of crude enzyme consortia by *A. oryzae*. To achieve high spore concentration, initially *A. oryzae* was sporulated in Erlenmeyer flasks on solid medium (5% SBC, 5% wheat bran and 2% agar). Sterilised distilled water (10 mL) and Tween 80 (0.01% v/v) were added into each slope and the surface was scratched with a wire loop. Subsequently, 2 mL of this spore suspension were added on the surface of the solid medium in each flask. Incubation was performed at 30 ˚C in an orbital shaker for 4 days. Then, 50 mL of sterilized distilled water, Tween 80 (0.01% v/v) and glass beads (4 mm diameter) were added in each flask to obtain the fungal spore suspension.

SSFs were performed in 250 mL Erlenmeyer flasks with 5 g (db) sterilized SBC. A fungal spore suspension of $2\times10^6$ per mL was used as inoculum. The SSFs were incubated at 30 ˚C for 48 h.

### 4.3.2 Enzymatic hydrolysis of SBC

After 48 h of SSF, the fermented solids were suspended in sterilized distilled water and macerated using a kitchen blender followed by vacuum filtration in order to obtain the crude enzyme extract. The enzyme extract was added in 1 L Duran bottle containing unprocessed SBC. The final initial SBC solid concentration was 50 g $L^{-1}$, while an initial proteolytic activity of 5.32 U $mL^{-1}$ was used. The Duran bottles were placed in a water bath at 45 ˚C while agitation was achieved with magnetic stirrers.

Samples were collected at random intervals and the solids were separated via centrifugation (10 min, 3000 g). The supernatant was used for the analysis of free amino nitrogen (FAN) and

inorganic phosphorus (IP). Hydrolysis experiments, as well as subsequent analysis of their products, were carried out in duplicates and the results presented as average ± standard deviation.

The SBC hydrolysates, used as fermentation media for PDO production, were obtained by vacuum filtration following filter-sterilization using 0.2 μm filter unit (Polycap TM AS, Whatman Ltd.).

## 4.4 Production of bakery waste hydrolysates

Enzymatic hydrolyses of Sbw and Bw were carried out using commercial enzymes in 10 L BIOSTAT bench-top reactor (Sartorius AG, Germany) and in 50 L reactor (B. Braun Biotech, Germany). Well homogenized grinded substrate was added into the 10 L reactor together with distilled water to final concentration of 24% (db, w $v^{-1}$) in the case of Sbw and 26% (db, w $v^{-1}$) in the case of Bw. The initial Bw concentration used in the 50 L reactor was 24.5% (db, w $v^{-1}$). A liquefaction step was initially carried out at pH value of 6 where an enzyme dosage of bacterial endo-amylase (BAN 240 L) was added to aqueous suspensions of Sbw and Bw. The slurry was heated at 80 ˚C for 2 h. Then, pH and temperature decreased to 4.5 and 52 ˚C, respectively. The liquefied starch slurry was subjected to saccharification process with addition of glucoamylase (STARGENTM 002) and simultaneous addition of protease (FERMGEN) in order to hydrolyse the protein content of the two substrates. Samples were taken at variable intervals during enzymatic reaction for determination of glucose production. When the glucose concentration stopped increasing, the hydrolysate was centrifuged or filtered and it was used as carbon source for D-BDO production by *B. amyloliquefaciens*.

## 4.5 Bacterial fermentations for PDO production

### 4.5.1 Bioreactor fermentations

Batch and fed-batch fermentations were carried out in a 1 L bioreactor (New Brunswick Scientific, USA) with a working volume of 0.8 L. Fermentations were inoculated with 10% (v/v) of inoculum in exponential growth phase. The incubation temperature was 30 ˚C, agitation rate was 180 rpm and pH value was regulated at 7.0 using 5 M NaOH and 5M HCl solution.

Different sets of batch bioreactor experiments were performed to optimize the initial concentrations of crude glycerol and FAN. In the batch bioreactor trials evaluating the effect of initial glycerol concentration, crude glycerol was used as the sole carbon source in varying

concentrations supplemented with the nutrient medium that consisted of (in g $L^{-1}$): 5 bacteriological peptone, 5 meat extract, 2.5 yeast extract, 2 $K_2HPO_4$, 0.84 $MgSO_4 \cdot 7\ H_2O$ and 0.06 $MnSO_4 \cdot H_2O$ (Metsoviti et al., 2013).

In the batch bioreactor trials, where SBC hydrolysate was evaluated as nitrogen and mineral supplement, the SBC hydrolysate was diluted in order to achieve different initial FAN concentrations. Crude glycerol was used as carbon source using the optimum concentrations identified in the previous set of experiments.

Four fed-batch fermentations were carried out using the two *C. freundii* strains, where pure and crude glycerol were used as carbon sources supplemented with the modified medium based on Matsoviti et al. (2013). Fed-batch experiments were also conducted using crude glycerol and SBC hydrolysate using the optimum FAN concentration identified in batch fermentations. When glycerol concentration was lower than 10 g $L^{-1}$, a concentrated crude glycerol solution (500 g $L^{-1}$) was added into the medium in order to maintain the glycerol concentration in the range of 10-15 g $L^{-1}$.

### 4.5.2 Evaluation of bacterial growth in microplate reader incubator

The inhibition caused by various organic acids along with PDO, $K^+$ and $Na^+$ salts on *C. freundii* VK-19 and *C. freundii* FMCC-8 growth was evaluated using a 96-well microtiter plate system (Infinite M200-PRO, TECAN). Acetic acid, succinic acid, formic acid, lactic acid, PDO, $K_2SO_4$ and NaCl were used in inhibition trials at varying initial concentrations ranging from 1 to 90 g $L^{-1}$. The final volume of the culture was 200 μL and the inoculum size was 10% (v/v). The fermentation medium contained pure glycerol (20 g $L^{-1}$), peptone (5 g $L^{-1}$), meat extract (5 g $L^{-1}$), yeast extract (2.5 g $L^{-1}$), $K_2HPO_4$ (2 g $L^{-1}$), $MgSO_4 \cdot 7\ H_2O$ (0.84 g $L^{-1}$) and $MnSO_4 \cdot H_2O$ (0.06 g $L^{-1}$). Mineral oil (50 μL) was added after inoculation in order to maintain anaerobic conditions. Incubation was carried out at 30 ˚C with shaking intervals (180 rpm) that lasted for 5 sec every 10 min. Growth was assessed by measuring the optical density at 650 nm, while the specific growth rate was calculated using the optical density of six independent replicates within the linear logarithmic growth phase of the culture according to equation 1.

$$\mu = \frac{ln_{OD_2} - ln_{OD_1}}{t_2 - t_1} \qquad (1)$$

# Chapter 4

## 4.6 Bacterial fermentations for BDO production by *K. michiganensis*

### 4.6.1 Shake flask fermentations

Different sets of shake flask fermentations were carried out evaluating the effect of nitrogen sources, temperature, type of crude glycerol and initial concentration of glycerol. All fermentations were conducted in 500 mL Erlenmeyer flasks with working volume of 100 mL with 10% (v/v) inoculum using the fermentation medium with the following composition (in g $L^{-1}$): 30 glycerol, 6 $(NH_4)_2HPO_4$, 0.45 KOH, 0.51 EDTA, 0.3 $MgSO_4 \cdot 7H_2O$, 0.09 $CaCl_2 \cdot 6H_2O$, 0.0225 $FeSO_4 \cdot 7H_2O$, 0.0075 $ZnSO_4 \cdot 7H_2O$ and 0.0038 of $MnSO_4 \cdot H_2O$ (Pirt and Callow, 1958). The experiments were carried out at 180 rpm and initial pH value of 6.5. Samples, were periodically taken under aseptic conditions to analyse glycerol consumption, metabolites production and bacterial growth. The data presented as average ± standard deviation of duplicate experiments.

In experiments where the effect of nitrogen source was evaluated, ammonium sulphate (7.2 g $L^{-1}$), yeast extract (5 g $L^{-1}$), CSL (10 g $L^{-1}$), mixture of yeast extract and CSL (5 and 10 g $L^{-1}$, respectively) and mixture of yeast extract and ammonium sulphate (2.5 and 7.2 g $L^{-1}$, respectively) were used as nitrogen source in the above medium. The optimal nitrogen source was subsequently used in all subsequent fermentations. The evaluation of temperature on cell growth and BDO production was carried out in shake flask cultures using 5 different temperatures (27, 30, 37, 40 and 44 ˚C).

The different types of pretreated crude glycerol (100-So, 90-So:10-Tf and 65-So:35-Tf) were investigated using 30 g $L^{-1}$ of glycerol concentration. Crude glycerol pretreatment was carried out via overnight decanting in separation funnels. Then, the effect of initial glycerol concentration (using the crude glycerol stream 90-So:10-Tf) on BDO production efficiency was investigated in shake flask cultures by varying the initial glycerol concentrations (35.7, 62.0, 75.2, 115.9 and 135.9 g $L^{-1}$).

### 4.6.2 Fed-batch fermentations

The optimum conditions identified in the previous set of experiments were used in fed-batch fermentations to investigate the effect of volumetric oxygen transfer coefficient ($k_La$) on BDO production efficiency using either pure or different types of pretreated crude glycerol (100-So, 90-So:10-Tf and 65-So:35-Tf). The experiments were performed in a 6.7 L bench top bioreactor

(Ralf Advanced, Bioengineering, Switzerland) containing 2 L of fermentation medium (Figure 4.1). The inoculum and aeration were 10% (v/v) and 1 vvm, respectively. The bioreactor was equipped with sensors for pH, temperature and dissolved oxygen. Air was supplied by injecting sterile filtered air (0.2 μm) through a ring sparger at the bottom of the vessel. The experiments were performed at 30 ˚C. The pH value was maintained at 6.5 ± 0.1 with addition of 10 M NaOH or 10% $H_2SO_4$. Antifoam 204 (Sigma) was used when needed. A concentrated feeding solution of pure or crude glycerol (700 g $L^{-1}$) was fed into the bioreactor when the glycerol concentration was around 5-10 g $L^{-1}$.

When pure glycerol was used, one fermentation was carried out at agitation cascade (150-400 rpm) corresponding to $k_La$ value of 46-62 $h^{-1}$ and one fermentation was performed at constant agitation rate of 400 rpm corresponding to $k_La$ value of 62 $h^{-1}$. In the case of 100-So crude glycerol as carbon source, four fed-batch fermentations were carried out at agitation rates of 150-400, 400, 500 and 600 rpm corresponding to $k_La$ values of 46-62, 62, 77 and 83 $h^{-1}$. Constant agitation rates of 400, 500 and 600 rpm were evaluated using 90-So:10-Tf crude glycerol and 65-So:35-Tf crude glycerol as presented in Table 4.1.

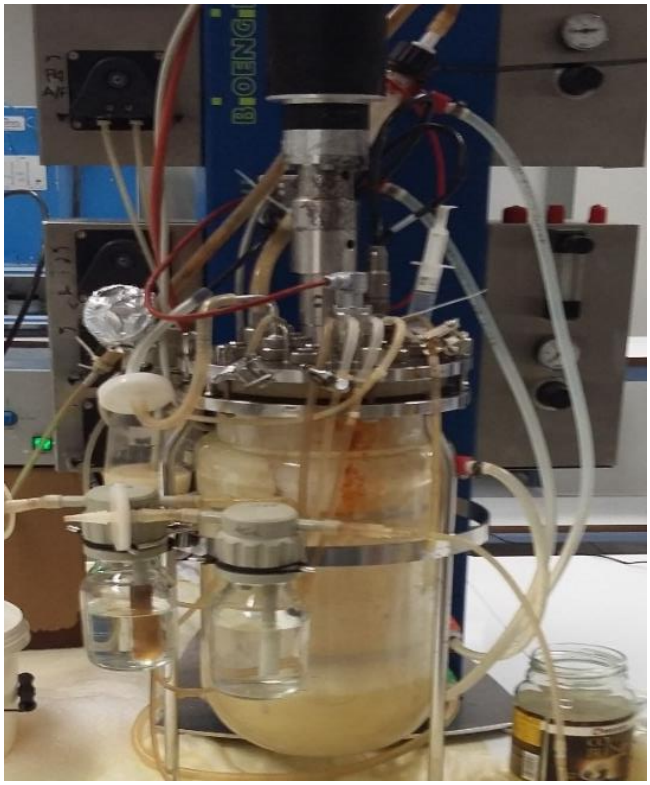

**Figure 4.1** Bioreactor used for BDO production by *K. michiganensis*

**Table 4.1** Fed-batch fermentations with *K. michiganensis* on different types of glycerol

| Type of glycerol | Agitation rate (rpm) | 150-400 | 400 | 500 | 600 |
|---|---|---|---|---|---|
| | $k_La$ ($h^{-1}$) | 46-62 | 62 | 77 | 83 |
| pure | | ■ | ■ | | |
| 100-So | | ■ | ■ | ■ | ■ |
| 90-So:10-Tf | | | ■ | ■ | ■ |
| 65-So:35-Tf | | | ■ | ■ | |

## 4.7 BDO production using sugar-based substrates

### 4.7.1 Shake flasks and bioreactor experiments in batch mode

Preliminary experiments were initially performed in shake flask fermentations for the evaluation of fermentative parameters including initial substrate concentration, nitrogen source, temperature and inoculum size. The effect of VHP cane sugar concentration was investigated by applying VHP cane sugar concentration from 30 to 220 g $L^{-1}$ using the fermentation medium with the following composition (in g $L^{-1}$): 0.5 $KH_2PO_4$, 2 $K_2HPO_4$, 0.3 KCl, 0.025 $MnSO_4 \cdot H_2O$ and 15 yeast extract (Adlakhaand Yazdani, 2015). Therefore, different organic nitrogen sources, including yeast extract (5 and 10 g $L^{-1}$), bacterial peptone (5 and 10 g $L^{-1}$) and CSL (5 and 10 g $L^{-1}$), were used to replace the yeast extract from the fermentation medium above. The study of initial VHP cane sugar concentration and nitrogen source was performed at initial pH of 6.8, inoculum size of 10% (v/v) and temperature of 30 ˚C. Regarding the effect of temperature on growth and BDO production, the experimental sets were incubated at temperature within the range of 27 to 44 ˚C. The effect of inoculum size in the range of 5-25% was evaluated at optimized conditions of initial cane sugar concentration, nitrogen source and temperature. Finally, the effect of sugarcane molasses concentration, in range of 6-30% (w/w), was studied under optimized fermentative parameters. All shake flask experiments were carried out in 500 mL Erlenmeyer flask containing 100 mL of fermentation medium. Cultures were incubated on a rotary shaker at 180 rpm. Substrate was autoclaved separately and was added to fermentation media under sterilized conditions. Samples were collected periodically to determine the cell growth and the concentrations of sugars, BDO and organic acids. All experiments were performed in duplicated and the results presented as average ± standard deviation.

Batch experiments were conducted to optimize the oxygen supply and pH value. The effect of oxygen supply was studied by varying the agitation speed with constant aeration rate of 1 vvm corresponding to $k_La$ values in the range of 17-132 $h^{-1}$. In this set of experiments, fermentation pH was controlled at 6.8. Subsequently, pH values of 5.5, 6, 6.4 and 7 were evaluated in batch experiments for BDO production at optimized conditions identified in the previous set of experiments. The batch fermentations were conducted in 3.6 L bench top bioreactor (Labfors 4, Infors HT, Switzerland) with working volume of 1 L with initial cane sugar concentration in the range of 70-90 g $L^{-1}$, yeast extract concentration of 15 g $L^{-1}$, temperature of 40 ˚C and inoculum size of 15%. pH was adjusted with 5M NaOH and 10% $H_2SO_4$ solutions.

### 4.7.2 Fed-batch fermentations

The optimized operational parameters were considered in fed-batch cultures using VHP cane sugar and sugarcane molasses as carbon source. The feeding solution used in fermentations with VHP cane sugar was either mixture of glucose and fructose or VHP cane sugar. Fed-batch fermentation was also initiated with molasses followed by usage of VHP cane sugar and molasses as feeding medium. The feeding solution of VHP cane sugar was 700 g $L^{-1}$, while the concentration of solution with molasses was 400 g $L^{-1}$. The feeding solution was fed into the bioreactor at different pulses, when the residual total sugar concentration decreased to 40 g $L^{-1}$. The experiments were carried out in 3.6 L bench top bioreactor (Labfors 4, Infors HT, Switzerland) with working volume of 1 L, $k_La$ value of 49 $h^{-1}$, 40 ˚C, pH of 6.0 and 15% inoculum size.

## 4.8 BDO production using bakery waste hydrolysates

### 4.8.1 Batch fermentations in 5 L bench top bioreactor

Batch fermentations were carried out for the evaluation of bakery waste hydrolysate as substrate for BDO production and the effect of oxygen supply on BDO synthesis. Fermentations were carried out at 40 ˚C in 5 L bench top bioreactor (Sartorius AG, Germany) with working volume of 3 L. The inoculum, aeration rates were 15% (v/v) and 1 vvm, respectively. The pH was constantly maintained at 6.0 by automatic addition of 5 M NaOH and 5% $H_2SO_4$. Antifoam 204 (Sigma) was used when needed.

Initially, commercial glucose as well as Sbw and Bw hydrolysates were evaluated as carbon sources supplemented with medium with the following composition (in g $L^{-1}$): 15 yeast extract,

0.5 $KH_2PO_4$, 2 $K_2HPO_4$, 0.3 KCl and 0.025 $MnSO_4 \cdot H_2O$. The initial glucose concentration in the fermentations carried out with commercial glucose was 110 g $L^{-1}$, whereas the initial glucose concentration in bakery hydrolysates was around 120 g $L^{-1}$.

The effect of oxygen supply was performed at constant agitation speeds of 200, 300, 400, 500 and 600 rpm corresponding to $k_La$ values of 26 $h^{-1}$, 64 $h^{-1}$, 113 $h^{-1}$, 157 $h^{-1}$ and 203 $h^{-1}$. The initial glucose concentration of filtrated Bw hydrolysate was 120-130 g $L^{-1}$. The fermentation medium was supplemented with nitrogen and minerals with the same composition as reported before.

### 4.8.2 Batch fermentation in 50 L pilot scale bioreactor

Batch fermentation on pilot scale was performed in 50 L bioreactor (B. Braun Biostat UD) with working volume of 30 L (Figure 4.2). Agitation and aeration were 1 vvm and 300 rpm, respectively in order to get similar $k_La$ value (116 $h^{-1}$) with the fermentation conducted in the bench-top bioreactor at 400 rpm. The fermentation parameters were the same as reported previously.


**Figure 4.2** 50 L bioreactor

### 4.8.3 Fed-batch fermentation in 6.7 L bench top bioreactor

A fed-batch fermentation was carried out in 6.7 L bioreactor (Ralf Advanced, Bioengineering, Switzerland) containing 3 L of fermentation medium (Figure 4.2). Agitation and aeration were accordingly adjusted in order to acquire a similar $k_La$ value (110 $h^{-1}$) to the fermentation conducted in 50 L pilot scale bioreactor. The fermentation medium and other operating parameters were the same as reported previously.

## 4.9 Analytical methods

### 4.9.1 Determination of physicochemical characteristics of raw materials

The total Kjeldahl nitrogen (TKN) concentration of soybean meal, molasses and bakery wastes was determined using a Kjeltek TM 8100 distillation unit (Foss, Denmark). Elemental determination of crude side streams (glycerol, VHP cane sugar and molasses) and SbW and WB hydrolysates was conducted using an inductively coupled plasma optical emission spectrometer (ICP-OES, Ultima 2, Horiba Jobin Yvon) based on the ASTM-D 1976 method. Ash, moisture, lipid, acid detergent fibre (ADF), acid detergent lignin (ADL) and neutral detergent fiber (NDF) were determined based on the AOAC official methods 942.05, 930.15, 920.39 and 973.18, respectively. The results of ADL, ADF and NDF were expressed as cellulose (ADF-ADL), hemicellulose (NDF-ADF) and lignin (ADL) content. Starch content was determined by the ISO 10520:1997 standard method. Extraction of total non-polar compounds from the original crude glycerol and the fractions generated after decanting was carried out with petroleum ether (Hu et al., 2012). The fatty methyl esters in the organic fraction were quantified by GC-FID using the methodology reported by Kachrimanidou et al. (2014).

### 4.9.2 Growth determination

Bacterial growth was determined by cell dry weight (DCW). Cells were collected by centrifugation (9000 × g, 10 min, 4 ˚C) in a Hettich Universal 320-R (Germany) and washed twice with distilled water. The sediment was dried at 105 ˚C for 24 h and then the sample was cooled down in a desiccator until constant weight was obtained.

### 4.9.3 Substrate and metabolites determination

Substrate consumption, acids and diol formation were monitored using HPLC Shimandzu UFLC XR unit with an RI detector equipped with a Phenomenex Rezex ROA column, with 300

mm length and 7.8 mm internal diameter. A 10 mM $H_2SO_4$ solution was used as mobile phase at flow rate of 0.6 mL per min and column temperature of 65 ˚C. Prior to analysis samples were appropriately diluted and filtered through a 0.2 μm Whatman membrane filters. The concentration of sugars was also determined with a Shodex SP0810 column. Pure water was used as mobile phase at flow rate of 1 mL per min and temperature of 80 ˚C. The concentration of sugars, glycerol and metabolites was determined by calibration curves with correlation coefficient ($R^2$) equal or more than 0.98%.

### 4.9.4 Determination of acetoin and BDO isomers

The quantities and the ratio of acetoin and BDO stereoisomers forms (D-, L- and meso-) were determined by gas chromatography using a chiral capillary column (HP-Chiral B). Prior to GC analysis, the supernatant was extracted with ethyl acetate after the addition of 1,3-propanediol as the internal standard. The operation conditions were as follows: helium was used as carrier gas, the column oven temperature was maintained at 50 ˚C for 2 min and it was then increased to 170 ˚C at a rate of 20 ˚C $min^{-1}$. The injection and the detector temperature were both 230 ˚C. The injection volume was 1 mL. The concentration of the products was determined by calibration curves.

### 4.9.5 FAN and IP determination

FAN concentration in hydrolysis and fermentation samples was determined by the ninhydrin colorimetric method promulgated in the European Brewery Convention (Lie, 1973). IP was assayed by the ammonium molybdate spectrophotometric method (Harland and Harland, 1980).

### 4.9.6 Determination of protease activity

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

### 4.9.7 Estimation of $k_La$ value

The $k_La$ values of the bioreactors were estimated in a system without microbial population based on the methods reported by Lee and Tsao, 1979 and Aroniada et al., 2018. The estimation was based on the measurement of dissolved oxygen (DO) in aeration and step response experiments in a cell-free system. The probe step response is obtained by initially immersing the DO probe

in a vessel where nitrogen is sparged, while the cell-free fermentation medium is sparged with air so that the liquid is saturated in oxygen. Then, the DO probe is instantly removed from the oxygen-free medium and immersed in the oxygen-saturated bioreactor and the DO readings ($i(t)|_{step}$) of the probe are recorded. The aeration response is obtained by sparging air into in an initially free of oxygen bioreactor and the readings of the probe are recorded ($i(t)|_{aeration}$) until saturation in oxygen is achieved. The two responses are then normalized between with respect to the probe reading at saturation and then the volumetric mass transfer coefficient is estimated using the following equation (2) (Lee and Tsao, 1979).

$$\frac{1}{k_L \mathrm{a}} = \int_0^{\infty} \left( \left. \frac{i(t)}{i_{\infty}} \right|_{step} - \left. \frac{i(t)}{i_{\infty}} \right|_{aeration} \right) dt \qquad (2)$$

According to Equation (2) the inverse of the volumetric mass transfer coefficient is equal to the area between the two normalized responses.

# Evaluation of 1,3-propanediol production by two *Citrobacter freundii* strains using crude glycerol and soybean cake hydrolysate

# Chapter 5

## 5.1 Introduction

The escalating environmental impact caused by the utilisation of fossil resources for the production of fuels and chemicals and the need for the development of innovative technologies to tackle the economic recession are the main reasons pushing towards the transition to a sustainable circular economy. Within this context, first generation biobiesel production processes could evolve into novel biorefineries incorporating the production of bio-based chemicals and polymers (Zabaniotou et al., 2018). Industrial biodiesel production is predominantly carried out via transesterification of vegetable oils or animal fats with methanol. Biodiesel production from soybean leads to the production of crude glycerol and soybean cake (SBC) as the main side streams.

The glycerol content in crude glycerol streams varies in the range of 66-90% (Chatzifragkou et al., 2012). The main impurities found in crude glycerol streams are sodium or potassium salts depending on the catalyst used, water, methanol, unreacted fatty acids and mono-, di-, and tri-glycerides. Moreover, significant quantities of minerals such as calcium, magnesium, phosphorous are also present in crude glycerol streams (Kaur et al., 2012b). Although glycerol purification could be employed to upgrade its use in pharmaceutical and cosmetic applications, this is hindered due to high processing costs. Crude glycerol has been widely evaluated for the production of various bio-based chemicals. The development of a two-stage bioprocess, including solid state fermentation (SSF) and enzymatic hydrolysis of oilseeds, for the formulation of rich fermentation feedstock exhibits an alternative and cost-effective strategy for utilisation of oilseed meals (Kachrimanidou et al., 2014). The nutrient rich hydrolysates along with crude glycerol could be valorised in bioconversion for the production of citric acid, succinic acid and 1,3-propanediol (PDO) (Chatzifragkou et al., 2014).

Under this concept, one of the main objectives of this chapter is the evaluation of crude glycerol containing sodium salts and defatted SBC produced by biodiesel production plants using soybean seeds for the production of PDO by two *Citrobacter freundii* strains. SBC was used in two-stage bioprocess for the formulation of complex nutrient supplements required by *C. freundii* strains for the production of PDO. The effect of initial concentration of crude glycerol and SBC hydrolysate were evaluated in batch fermentations. Fed-batch experiments were performed for PDO production using commercial and rich SBC hydrolysate supplemented with glycerol (either pure or crude). The inhibitory effect of fermentation by-products as well as

sodium and potassium salts on microbial growth of *C. freundii* strains was undertaken in order to identify the major factors influencing PDO production.

## 5.2 Characterization and analysis and analysis of raw materials

### 5.2.1 Crude glycerol

The composition of crude glycerol is mainly affected by the origin of oil feedstock, the type of catalyst used in the biodiesel production processes, the transesterification efficiency and the recovery process used (Zubaida et al., 2018). Glycerol generated from biodiesel plants can be classified into two categories based on the purity *i.e.* crude glycerol with purity of 60-80% (w/w) and refined glycerol with 99.1-99.8% (w/w) purity (Lee et al., 2015). Impurities of crude glycerol from biodiesel production consists of residual methanol, unreacted fatty acids, methyl esters of fatty acids, glycerides and water. Significant quantities of inorganic salts and micro-nutrients such as calcium, magnesium, phosphorous could be encountered in crude glycerol streams (Kaur et al., 2012a).

The crude glycerol used in this study was generated from biodiesel production plants using soybean seeds (100-So) and the composition is presented in Table 5.1.

**Table 5.1** Composition of crude glycerol

| Component | Crude glycerol 100-So | Crude glycerol 100-So after decanting |
|---|---|---|
| Glycerol (w/w, %) | 77.7 | 79.4 |
| Water (w/w, %) | 14.0 ± 0.35 | 12.8 ± 0.19 |
| Ash (w/w, %) | 5.32 | 5.21 |
| Methanol (w/w, %) | - | - |
| Monoglyceride (w/w,%) | 2.95 | 1.1 |
| Ca (ppm) | 8.8 | 2.4 |
| Fe (ppm) | 0.55 | 0.74 |
| K (ppm) | 13 | 8.8 |
| Mg (ppm | 4.9 | 0.9 |
| Na (ppm) | 21000 | 22000 |
| P (ppm) | 54 | 56 |

### 5.2.2 Soybean cake

Defatted SBC is the residue from the soybean processing industry generated after oil extraction. The composition of SBC is influenced by cultivation practices, soybean variety and application

of dehulling and/or oil separation through solvent extraction processes implemented on industrial scale. Typically, SBC contains high amount of protein in the range of 40-49%. The composition of SBC is presented in Table 5.2. The protein of SBC contains considerable quantities of lysine, isoleucine, valine and leucine. The nutrient-rich nature of SBC creates a suitable substrate for microbial fermentation.

**Table 5.2** Composition of SBC

| Components | Content |
|---|---|
| Moisture (%, wet basis) | 13.0 ± 0.40 |
| Protein (wt, %, TKN × 6.25) | 47.0 ± 1.20 |
| Ash (wt, %) | 6.5 ± 0.09 |
| Lipids (wt, %) | 2.2 ± 0.20 |
| Cellulose (wt, %) | 24.2 ± 0.90 |
| Hemicellulose (wt, %) | 18.1 ± 1.60 |
| Lignin (wt, %) | 2.1 ± 0.08 |

## 5.3 Production of SBC hydrolysate via two-stage bioprocess

Defatted SBC, containing significant amount of protein, can be hydrolysed into assimilable components by microbial strains to formulate complex nutrient supplements. The hydrolysis of SBC macronutrients could be conducted using commercial or crude enzymes. The fungal strain *Aspergillus oryzae*, a well-known producer of protease, phytase, xylanase and cellulose, has been extensively used for the production of crude enzyme consortia via SSF (Kachrimanidou et al., 2013). Hence, in this study, the defatted SBC hydrolysate was produced by a two-stage bioprocess involving SSF of *A. oryzae* for the production of crude enzyme consortia that were subsequently employed for the hydrolysis of SBC.

SSFs of SBC were carried out at initial moisture content of 65% and uncontrolled pH (Kachrimanidou et al., 2013). Figure 5.1 presents the profile of proteolytic activity during SSF of SBC. The proteolytic activity increased during fermentation reaching 271 U $g^{-1}$ at 58 h.

Enzymatic consortia produced by *A. oryzae* were used to hydrolyse the protein of SBC into amino acids and peptides along with the release of inorganic phosphorus (IP). The hydrolysis of SBC was carried out at 45 ˚C with an initial SBC solid concentration of 50 g $L^{-1}$. Figure 5.2 presents the profile change of free amino nitrogen (FAN) and IP concentration during SBC hydrolysis. FAN and IP concentrations were increased during hydrolysis up to 1.7 g $L^{-1}$ and

132 mg $L^{-1}$, respectively, at 48 h. The TKN to FAN conversion yield was 41%, including the total quantity of SBC used in both SSF and hydrolysis.

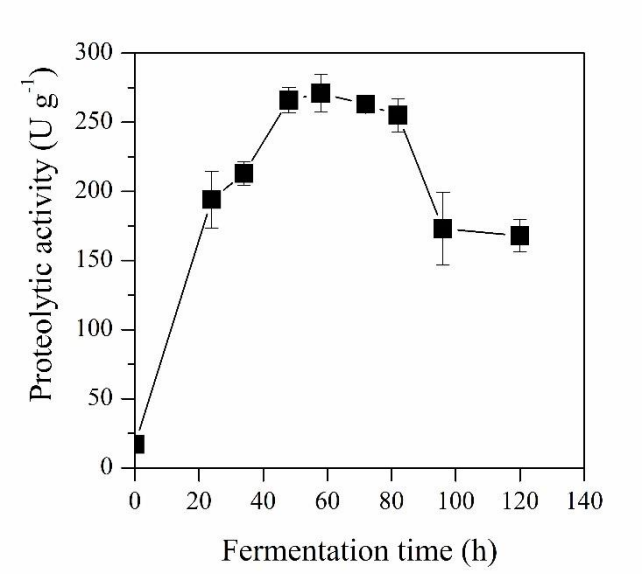


**Figure 5.1** Proteolytic activity (U $g^{-1}$ fermented solids) produced by *A. oryzae* during SSF

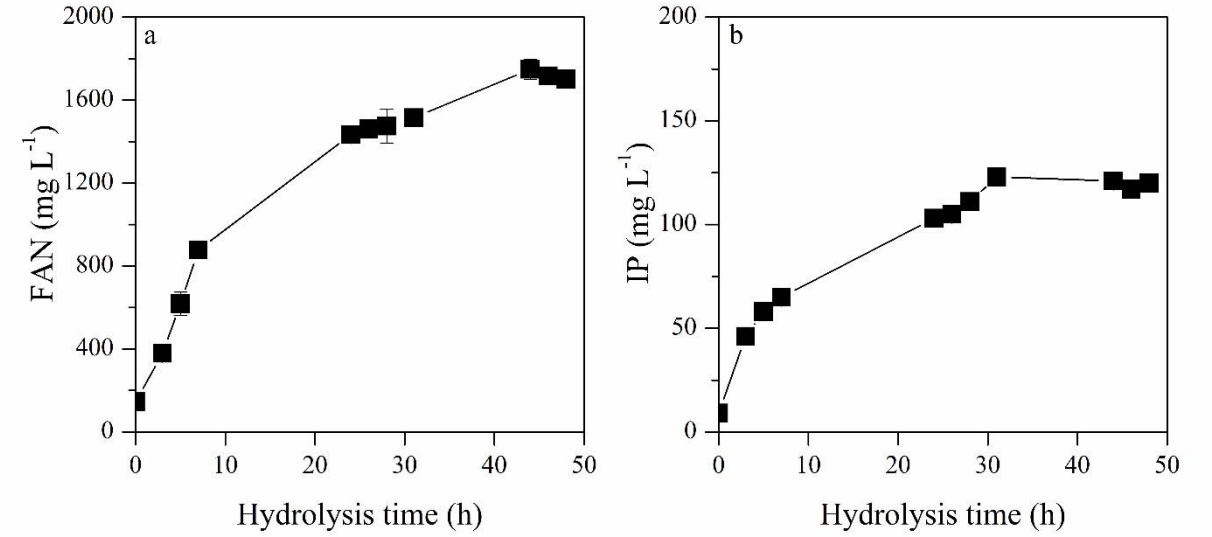


**Figure 5.2** Production of FAN (a) and IP (b) during the enzymatic hydrolysis of SBC

### 5.4 Effect of initial crude glycerol concentration on PDO production

The composition of crude glycerol is mainly affected by the origin of oils/fats, the conditions of the transesterification reaction and the biodiesel purification methodologies used. The impurities present in crude glycerol can inhibit microbial growth and PDO production (Kaur et al., 2012c; Samul et al., 2014). The bacterial strains *C. freundii* VK-19 and *C. freundii* FMCC-8 can efficiently utilise crude glycerol for PDO production (Metsoviti et al., 2012; Metsoviti et

al., 2013). Although *C. freundii* VK-19 has been cultivated in three different crude glycerols by Metsoviti et al. (2013) without major differences in PDO production efficiency, this study evaluated the cultivation of both strains on the crude glycerol derived from soybean oil transesterification because it contained sodium salts. The crude glycerols evaluated by Metsoviti et al. (2013) contained potassium salts. Different types of crude glycerol can influence cell proliferation and metabolite formation (Anand and Saxena, 2012).

The effect of different initial glycerol concentrations on cell growth and PDO production by the two *C. freundii* strains was evaluated in batch fermentations (Table 5.3).

**Table 5.3** Effect of initial crude glycerol concentration on growth and PDO production by *C. freundii* VK19 and *C. freundii* FMCC-8 strains during batch fermentations

| Initial glycerol ($g\ L^{-1}$) | DCW ($g\ L^{-1}$) | PDO ($g\ L^{-1}$) | Yield ($g\ g^{-1}$) | Productivity ($g\ L^{-1}\ h^{-1}$) | Acetic acid ($g\ L^{-1}$) | Succinic Acid ($g\ L^{-1}$) | Formic Acid ($g\ L^{-1}$) | Lactic Acid ($g\ L^{-1}$) | Ethanol ($g\ L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| *C. freundii* VK-19 | | | | | | | | | |
| 31.6 | 1.7 | 14.7 | 0.47 | 0.82 | 5.6 | 2.6 | 1.4 | 4.3 | 1.5 |
| 53.0 | 2.9 | 26.3 | 0.50 | 1.01 | 8.0 | 3.4 | 3.1 | 3.8 | 3.5 |
| 71.8 | 3.3 | 31.3 | 0.45 | 1.08 | 8.4 | 1.9 | 2.3 | 6.8 | 0.5 |
| 90.5 | 4.3 | 37.2 | 0.43 | 0.66 | 8.1 | 3.0 | 1.6 | 15.8 | 2.2 |
| 136.2 | 4.5 | 40.2 | 0.37 | 0.47 | 6.6 | 7.3 | 0 | 21.7 | 2.8 |
| *C. freundii* FMCC-8 | | | | | | | | | |
| 20.9 | 3.5 | 10.7 | 0.51 | 0.63 | 4.2 | 1.7 | 0.8 | 1.6 | 0.8 |
| 31.3 | 3.0 | 15.0 | 0.49 | 0.60 | 4.0 | 1.7 | 1.1 | 2.8 | 0.0 |
| 41.6 | 3.0 | 18.6 | 0.45 | 0.60 | 5.0 | 2.2 | 1.0 | 4.2 | 1.3 |
| 52.0 | 2.8 | 13.9 | 0.45 | 0.31 | 3.3 | 1.6 | 0.0 | 3.3 | 0.0 |

PDO concentration increased with increasing glycerol concentration for the bacterial strain *C. freundii* VK-19, reaching a PDO concentration of 40.2 g $L^{-1}$ when an initial glycerol concentration of 136.2 g $L^{-1}$ was used. Increasing initial glycerol concentration led to gradual increase in by-product formation thus reducing the glycerol to PDO conversion yield. The highest PDO yield (0.50 g $g^{-1}$) and productivity (1.01 g $L^{-1}$ $h^{-1}$) was achieved in the case that the initial glycerol concentration was 53 g $L^{-1}$. In the fermentations carried out with the strain *C. freundii* FMCC-8, the highest PDO concentration (18.6 g $L^{-1}$) was achieved when an initial glycerol concentration of 41.6 g $L^{-1}$ was used. The highest yield (0.51 g $g^{-1}$) and productivity (0.63 g $L^{-1}$ $h^{-1}$) were achieved when the initial glycerol concentration was 20.9 g $L^{-1}$. Increasing initial glycerol concentration resulted in decreasing DCW in the case of *C. freundii* FMCC-8

contrary to *C. freundii* VK-19 where DCW was increased with increasing initial glycerol concentration. Thus, *C. freundii* VK-19 is more tolerant to increasing crude glycerol concentrations than *C. freundii* FMCC-8. Similar results have been reported by Metsoviti et al. (2013) when *C. freundii* VK-19 was cultivated on crude glycerol containing potassium salts and a purity of 81%, where the highest yield (0.53 g $g^{-1}$) and productivity (1.53 g $L^{-1}$ $h^{-1}$) were achieved at an initial glycerol concentration of 40 g $L^{-1}$.

The optimum initial glycerol concentration varies for different bacterial strains. For instance, the optimum initial glycerol concentration for *Klebsiella pneumoniae* is in the range of 60-80 g $L^{-1}$ (Sattayasamitsathit et al., 2011). Papanikolaou et al. (2004) reported that initial glycerol concentration up to 90 g $L^{-1}$ did not exhibit inhibition on bacterial growth and PDO production when *Clostridium butyricum* was cultivated in continuous cultures. In general, *Clostridium butyricum* strains show high tolerance in crude glycerol (up to 150 g $L^{-1}$) (Wilkens et al., 2012). The bacterial strains *C. freundii* and *Pantoea agglomerans* achieved the optimum PDO production when the initial glycerol concentration was 20 g $L^{-1}$ (Casali et al., 2012).

## 5.5 Fed-batch cultures using pure and crude glycerol supplemented with commercial nutrients

Fed-batch fermentations were carried out with both strains on either pure or crude glycerol supplemented with commercial nutrients in order to identify the PDO production efficiency of the two strains. Based on the results presented in Table 5.3, the initial glycerol concentration used was 50 g $L^{-1}$ and 20 g $L^{-1}$ in the case of *C. freundii* VK-19 and *C. freundii* FMCC-8, respectively.

In the case of *C. freundii* VK-19, the highest PDO concentrations were 55.6 g $L^{-1}$ and 47.2 g $L^{-1}$ when pure and crude glycerol were used, respectively (Figures 5.3 and 5.4). The cultivation of *C. freundii* VK-19 on crude glycerol resulted in lower yield (0.38 g $g^{-1}$) and productivity (0.73 g $L^{-1}$ $h^{-1}$) than pure glycerol. In the fed-batch fermentation carried out with *C. freundii* FMCC-8, the PDO concentration (44.4 g $L^{-1}$) was higher when pure glycerol was used (Figure 5.4). However, in the case of pure glycerol, the yield achieved by *C. freundii* FMCC-8 was very low due to the production of high by-product concentration. The use of crude glycerol led to lower PDO and DCW in the case *of C. freundii* FMCC-8 (Figure 5.5).

The use of crude glycerol generally leads to lower PDO production efficiency than pure glycerol (Wilkens et al., 2012; Otte et al., 2009; Hirschmann et al., 2005). PDO production with *Clostridium butyricum* decreased by 11% when crude glycerol of 65% purity was implemented (Petitdemange et al., 1995). Various pre-treatment methods have been proposed aiming to overcome this bottleneck, including treatment with hydrochloric acid and/or organic solvents in order to remove fatty acids. Anand and Saxena (2012) have evaluated the effect of pre-treated crude glycerol with various solvents on microbial proliferation and PDO production by *C. freundii* reaching similar yields to pure glycerol.

It is worth noting that implementation of different types of glycerol resulted in different combinations of by-product formation for both bacterial strains. When *C. freundii* VK-19 was cultivated on pure glycerol, the main by-products were lactic acid (9.7 g $L^{-1}$), acetic acid (7.6 g $L^{-1}$) and formic acid (6.8 g $L^{-1}$). When the same strain was cultivated on crude glycerol, lactic acid concentration was increased to 20.8 g $L^{-1}$ and acetic acid and formic acid concentrations were decreased. The implementation of pure glycerol in *C. freundii* FMCC-8 cultures led to high concentrations of formic acid (16.3 g $L^{-1}$) and acetic acid (15.0 g $L^{-1}$), whereas the utilisation of crude glycerol led to lower by-product concentrations. Wilkers et al. (2012) reported a shift in organic acid formation when crude glycerol generated from rapeseed oil was used in fed-batch fermentation of *C. butyricum*. When crude glycerol was utilised, butyric acid concentration decreased around 38%, while lactic acid formation increased from 1 to 6 g $L^{-1}$ as compared to the utilisation of pure glycerol.

Table 5.4 presents PDO production reported in literature-cited publications by different *C. freundii* strains. Generally, PDO production by *C. freundii* strains is relatively low using either pure or crude glycerol. The highest PDO production has been reported by Metsoviti et al. (2013) employing crude glycerol (85.5% w/w) derived from transesterification of mixtures of edible fatty materials, such as soybean oil and cottonseed oil, in fed-batch fermentation mode. In this study, the implementation of crude glycerol resulted in lower production of PDO, while *C. freundii* VK-19 proved to be more efficient than *C. freundii* FMCC-8.

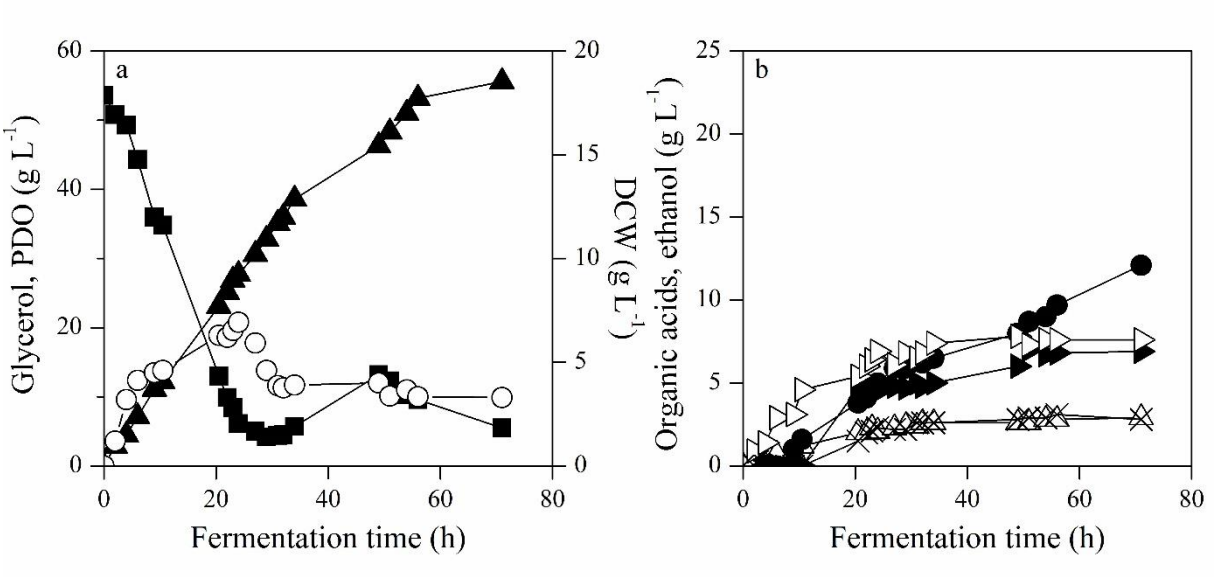


**Figure 5.3** Profile change of PDO (▲), DCW (o) and glycerol (■) (a) along with organic acids and ethanol formation (b) during fed–batch fermentation using pure glycerol and commercial supplements by *C. freundii* VK-19. Acetic acid (▷), succinic acid (△), lactic acid (●), formic acid (►) and ethanol (×)

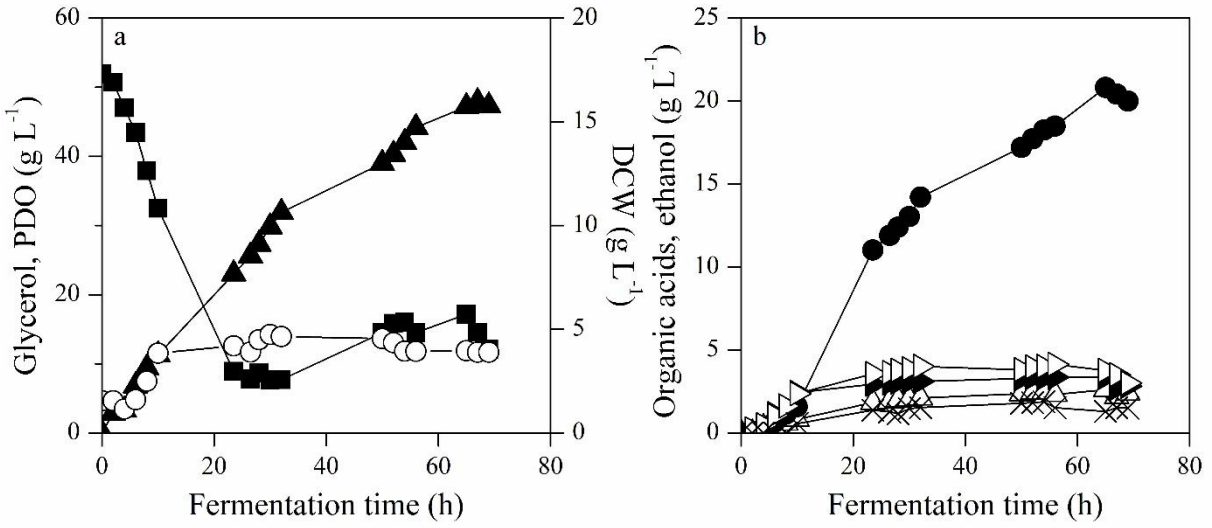


**Figure 5.4** Profile change of PDO (▲), DCW (o) and glycerol (■) (a) along with organic acids and ethanol formation (b) during fed–batch fermentation using crude glycerol and commercial supplements by *C. freundii* VK-19. Acetic acid (▷), succinic acid (△), lactic acid (●), formic acid (►) and ethanol (×)

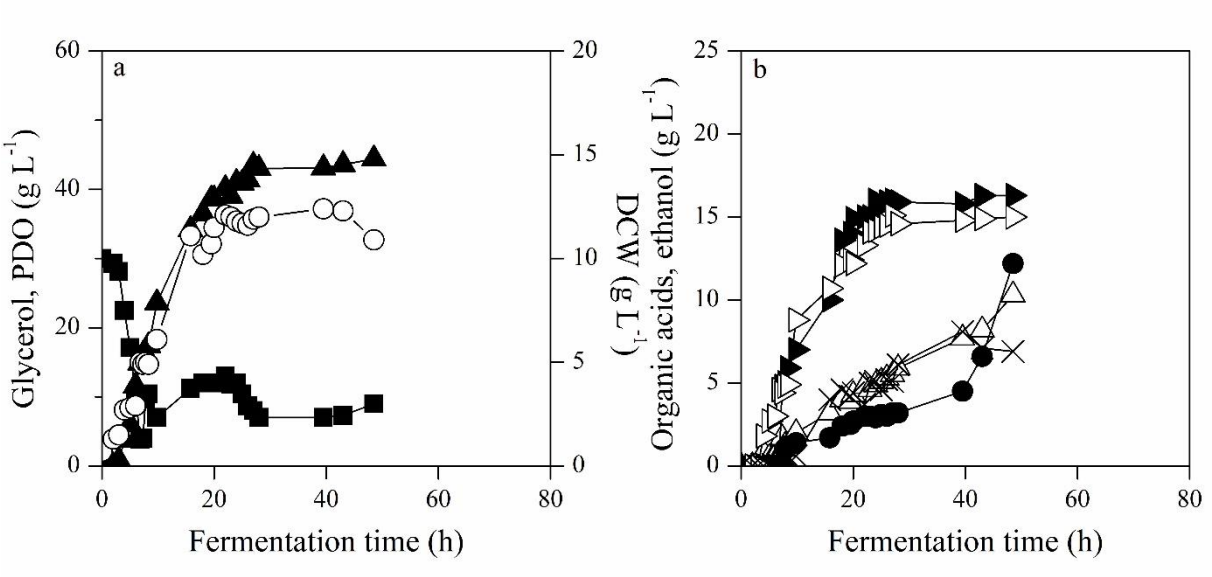


**Figure 5.5** Profile change of PDO (▲), DCW (o) and glycerol (■) (a) along with organic acids and ethanol formation (b) during fed–batch fermentation using pure glycerol and commercial supplements by *C. freundii* FMCC-8. Acetic acid (▷), succinic acid (△), lactic acid (●), formic acid (►) and ethanol (×)

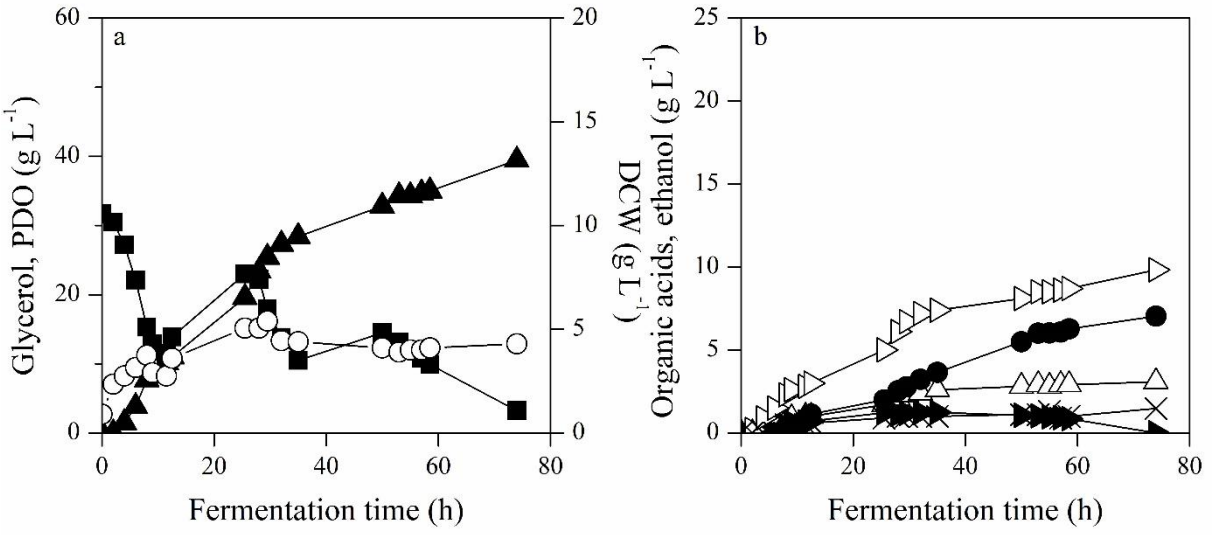


**Figure 5.6** Profile change of PDO (▲), DCW (o) and glycerol (■) (a) along with organic acids and ethanol formation (b) during fed–batch fermentation using crude glycerol and commercial supplements by *C. freundii* FMCC-8. Acetic acid (▷), succinic acid (△), lactic acid (●), formic acid (►) and ethanol (×)

**Table 5.4** PDO production by *C. freundii* strains

| Strain | Type of glycerol | PDO (g $L^{-1}$) | Yield (g $g^{-1}$) | Productivity (g $L^{-1}$ $h^{-1}$) | Fermentation mode | References |
|---|---|---|---|---|---|---|
| *C.freundii* ATCC 8090 | pure | | 0.53<br>0.45 | | Batch | Barbirato et al., 1998 |
| *C. freundii* | pure | 25.4 | | | Anaerobic bottles | Anand et al., 2012 |
| | crude* | 23.0 | | | | |
| *C. freundii VK-19* | crude 85.5%, w $w^{-1}$ | 39.9 | 0.49 | 1.53 | Batch | Metsoviti et al., 2013 |
| | | 68.1 | 0.40 | 0.79 | Fed-batch | |
| *C. freundii* DSM 30039 | pure | 13.2 | 0.58 | 0.66 | Batch | Homann et al., 1990 |
| *C. freundii* Go | | 10.5 | 0.54 | 0.58 | | |
| *C. freundii* Zu | | 12.0 | 0.53 | 0.92 | | |
| *C. freundii* K2 | | 12.4 | 0.53 | 0.89 | | |
| *C. freundii* Zu | | 28.1 | 0.53 | 1.34 | | |
| *C. freundii* K2 | | 26.0 | 0.52 | 1.37 | | |
| *C. freundii* VK-19 | pure | 55.6 | 0.44 | 0.99 | Fed-batch | This study |
| | crude 79.4%, w $w^{-1}$ | 47.2 | 0.38 | 0.73 | | |
| *C. freundii* FMCC-8 | pure | 44.4 | 0.28 | 0.92 | | |
| | crude 79.4%, w $w^{-1}$ | 35.5 | 0.53 | 0.60 | | |

* pre-treated with hexane

## 5.6 Effect of initial FAN concentration on PDO production

SBC hydrolysate was evaluated as a sole nitrogen and nutrient supplement in batch fermentation for PDO production by *C. freundii* strains. The effect of initial FAN concentration on microbial proliferation and PDO synthesis was studied using crude glycerol as carbon source at optimum initial concentration as identified previously. The results from batch fermentation using different initial FAN concentrations for *C. freundii* strains are demonstrated in Table 5.5.

An initial FAN concentration of 400 mg $L^{-1}$ proved to be optimum for *C. freundii* VK-19 cultures resulting in PDO concentration of 27.9 g $L^{-1}$, yield of 0.53 g $g^{-1}$ and productivity of 1.07 g $L^{-1}$ $h^{-1}$. When the initial FAN concentration was 337 mg $L^{-1}$, microbial growth and PDO production efficiency was much lower. Low PDO concentration (15.2 g $L^{-1}$), yield (0.27 g $g^{-1}$) and productivity (0.5 g $L^{-1}$ $h^{-1}$) were also achieved when the initial FAN concentration was 799 mg $L^{-1}$. The fermentations carried out with *C. freundii* VK-19 using 492 mg $L^{-1}$ and 626 mg $L^{-1}$ of initial FAN concentration resulted in similar final PDO concentration and yield. In the case of *C. freundii* FMCC-8 cultivation on SBC hydrolysate, the PDO production efficiency was

similar within the range of the different initial FAN concentrations used. The results presented in Table 5.5 demonstrate that the SBC hydrolysate can provide the essential nutrients required by both strains to sustain microbial growth and PDO production.

**Table 5.5** Effect of initial FAN concentration on growth and PDO production by *C. freundii* VK-19 and *C. freundii* FMCC-8

| Initial FAN (mg $L^{-1}$) | DCW (g $L^{-1}$) | PDO (g $L^{-1}$) | Yield (g $g^{-1}$) | Productivity (g $L^{-1}$ $h^{-1}$) | Acetic acid (g $L^{-1}$) | Succinic Acid (g $L^{-1}$) | Formic Acid (g $L^{-1}$) | Lactic Acid (g $L^{-1}$) | Ethanol (g $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| *C. freundii* VK-19 | | | | | | | | | |
| 337 | 2.6 | 14.6 | 0.27 | 0.43 | 4.0 | 3.3 | 3.9 | 8.1 | 5.0 |
| 400 | 2.9 | 27.9 | 0.53 | 1.07 | 8.7 | 3.8 | 3.1 | 3.8 | 3.5 |
| 492 | 2.4 | 23.0 | 0.45 | 0.96 | 8.5 | 3.4 | 1.3 | 6.6 | 3.3 |
| 626 | 3.9 | 24.8 | 0.48 | 0.88 | 7.6 | 2.5 | 1.1 | 7.1 | 1.9 |
| 799 | 2.7 | 15.2 | 0.27 | 0.50 | 5.3 | 5.7 | 0.0 | 5.9 | 8.7 |
| *C. freundii* FMCC-8 | | | | | | | | | |
| 326 | 2.3 | 11.1 | 0.56 | 0.93 | 4.5 | 1.8 | 0.8 | 1.8 | 1.2 |
| 437 | 3.4 | 9.8 | 0.53 | 0.82 | 4.2 | 1.7 | 0.8 | 3.1 | 1.3 |
| 642 | 3.7 | 11.2 | 0.55 | 1.12 | 5.1 | 2.4 | 1.0 | 2.1 | 1.3 |
| 920 | 2.8 | 12.5 | 0.56 | 0.73 | 5.3 | 2.5 | 0.5 | 2.7 | 0.0 |

Compared to the fermentations carried out with commercial nutrients and the same initial glycerol concentration (Table 5.3), the results obtained using SBC hydrolysate were improved for both *C. freundii* strains. This demonstrates that SBC hydrolysate beyond supply of necessary nutrients for growth and product formation, it can also improve PDO production efficiency.

## 5.7 Fed batch fermentations

Based on the results obtained with crude glycerol and SBC hydrolysate in batch cultures, *C. freundii* VK-19 and *C. freundii* FMCC-8 were further evaluated in fed-batch cultivations. Fed-batch experiments were conducted by adding a concentrated crude glycerol solution (500 g $L^{-1}$) when the glycerol concentration was around 10 g $L^{-1}$, in order to maintain the glycerol concentration in the range of 10 to 20 g $L^{-1}$.

Figure 5.7 presents the profile change of glycerol, PDO, DCW and by-products during fed-batch fermentation of *C. freundii* VK-19 carried out with crude glycerol and SBC hydrolysate. The initial glycerol and FAN concentration were 50 g $L^{-1}$ and 400 mg $L^{-1}$, respectively. The

highest PDO concentration (47.4 g $L^{-1}$) was achieved with a conversion yield and productivity of 0.49 g $g^{-1}$ and 1.01 g $L^{-1}$ $h^{-1}$, respectively. A gradual increase of by-products formation, mainly lactic acid and acetic acid, was observed during fermentation. Comparing the results with the fed-batch fermentations conducted with crude glycerol and commercial nutrients, the use of SBC hydrolysate increased the yield and productivity by 28.9% and 38.4%, respectively. Metsoviti et al. (2013) reported a PDO concentration of 68.1 g $L^{-1}$ when crude glycerol containing potassium salts was used in *C. freundii* VK-19 fed-batch fermentations. Thus, a lower PDO concentration was achieved in this study with the strain *C. freundii* VK-19 when crude glycerol containing sodium salts was used.

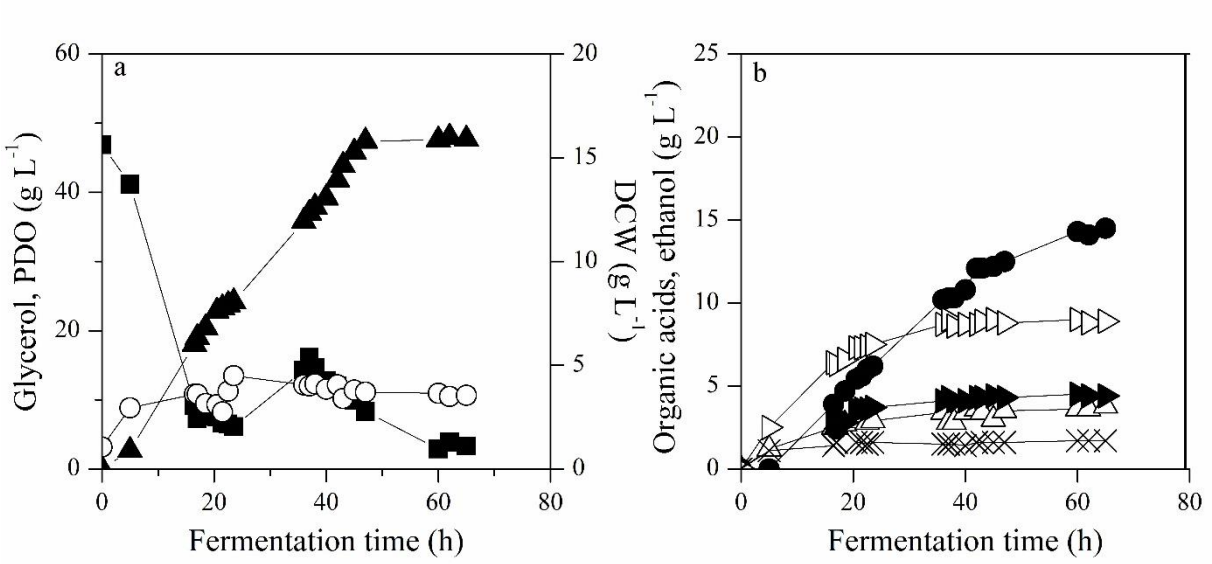


**Figure 5.7** Profile change of PDO (▲), DCW (o) and glycerol (■) (a) along with organic acids and ethanol formation (b) during fed–batch fermentation using crude glycerol and SBC hydrolysate by *C. freundii* VK-19. Acetic acid (▷), succinic acid (△), lactic acid (●), formic acid (►) and ethanol (×)

The bacterial strain *C. freundii* FMCC-8 was also evaluated in fed-batch experiments with SBC hydrolysate and crude glycerol at an initial glycerol concentration of 20 g $L^{-1}$ and an initial FAN concentration of 600 mg $L^{-1}$ (Figure 5.8). Production of PDO reached 37.7 g $L^{-1}$ with conversion yield of 0.55 g $g^{-1}$. By-product formation was observed throughout fermentation. Acetic acid (9.8 g $L^{-1}$) and lactic acid (6.5 g $L^{-1}$) were the predominant by-products at 55 h. Lactic acid production rate was increased after 36 h. By-products formation and the accumulation of impurities have led to a relatively low productivity (0.69 g $L^{-1}$ $h^{-1}$). This strain is less efficient than *C. freundii* VK-19 on PDO production.

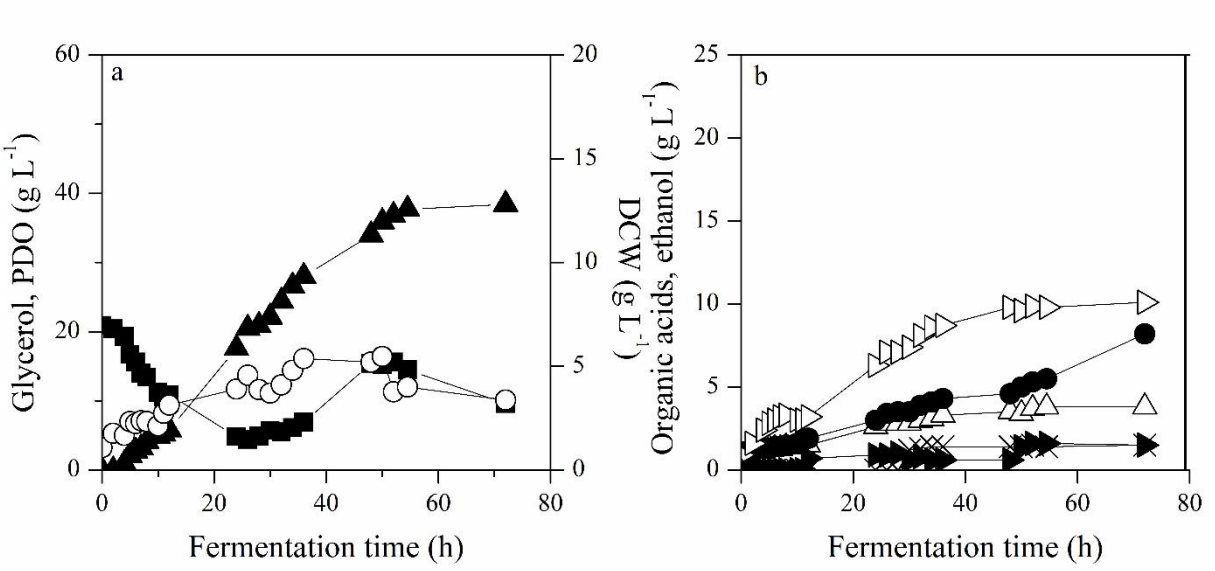


**Figure 5.8** Profile change of PDO (▲), DCW (o) and glycerol (■) (a) along with organic acids and ethanol formation (b) during fed–batch fermentation using crude glycerol and SBC hydrolysate by *C. freundii* FMCC-8. Acetic acid (▷), succinic acid (△), lactic acid (●), formic acid (►) and ethanol (×)

## 5.8 Effect of metabolic products, $Na^+$ and $K^+$ on bacterial growth

The inhibitory level of metabolic products on cell growth has been studied so far for *Klebseilla pneumoniae* and *C. butyricum*. In the case of *C. butyricum,* PDO, acetic acid and butyric acid showed significant inhibition on cell growth in a pH auxostat at pH 6.5 (Biebl et al., 1991). PDO, 2,3-butanediol and acetic acid showed a higher inhibition on microbial cells of *K. pneumoniae* (He et al., 2013).

The effect of organic acids and PDO on microbial proliferation of *C. freundii* VK-19 and *C. freundii* FMCC-8 was evaluated in microtiter plate experiments. Figures 5.9 and 5.10 present the normalized specific growth rate ($\mu_{max}$) achieved with each metabolic product in various concentrations for *C. freundii* VK-19 and *C. freundii* FMCC-8.

In the case of *C. freundii* VK-19, the addition of lactic acid, formic acid and acetic acid at concentrations higher than 20 g $L^{-1}$ resulted in a linear $\mu_{max}$ reduction. More specifically, the specific growth rates were decreased by 78.0, 69.4 and 67.4% when 20 g $L^{-1}$ lactic acid, formic acid and acetic acid were used, respectively. On the other hand, *C. freundii* VK-19 exhibited a significant tolerance against PDO and succinic acid even at concentrations higher than 60 g $L^{-1}$. Initial concentrations of PDO and succinic acid between 1 to 50 g $L^{-1}$ resulted in less than

15% reduction on specific growth rate. In the case of *C. freundii* FMCC-8, the inhibition of organic acids on cell growth was evident. In particular, the growth was terminated when the initial concentrations of formic acid, lactic acid, acetic acid and succinic acid were 20, 35, 40 and 60 g $L^{-1}$, respectively. *C. freundii* FMCC-8 was more tolerant to PDO in high concentrations. At PDO concentrations up to 40 g $L^{-1}$, the specific growth rate was decreased around 15%, while initial concentrations between 50 to 90 g $L^{-1}$ resulted in stronger inhibition on bacterial growth. Comparatively, both strains demonstrated similar inhibition behaviour on growth concerning lactic acid, acetic acid and formic acid. The effect of succinic acid and PDO on the proliferation of *C. freundii* strains was less pronounced even at higher concentrations. The fermentation end-products are accumulated in the fermentation broth causing a synergistic impact on cell growth inhibition (Pateraki et al., 2016). This phenomenon can possibly explain the experimental results obtained from fed-batch fermentations where the presence of organic acids along with PDO hindered microbial proliferation, hence the production of PDO.

Impurities of crude glycerol such as methanol, salts and fatty acids could also affect cell growth and also the synthesis of metabolites. Several studies have evaluated the effect of each component present in crude glycerol on bacterial growth and PDO production (Chatzifragkou et al., 2010; Venkataramanan et al., 2012; Samul et al., 2014). Chatzifragkou et al. (2010) studied the effect of impurities present in crude glycerol on PDO production by *C. butyricum*. The presence of NaCl and methanol in the growth medium did not affect cell growth and PDO production during the batch process, while the double bond from long-chain fatty acids negatively affected the bioconversion process. Monovalent salts at high concentrations have been reported to inhibit bacterial growth induced by the osmotic pressure and the swelling effect by weakening van der Waals forces on the lipid membrane interface (Petrache et al., 2006). Cell membrane modifications could affect the biochemical processes in microbial cells, including the transportation of nutrients across the membrane due to the change on the energetic barrier within the lipid layer (Marquis 1968; Venkataramanan et al., 2012).

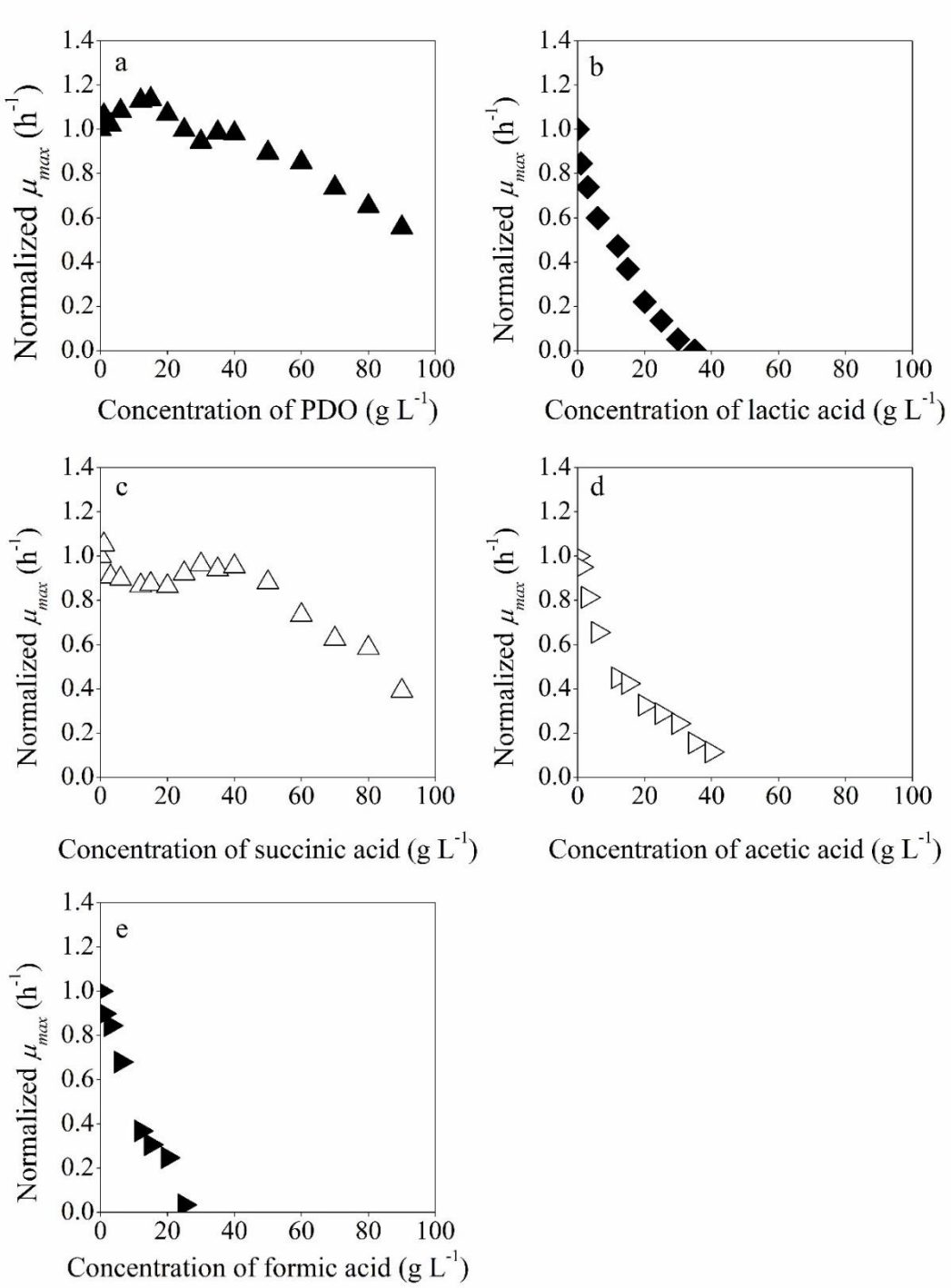


**Figure 5.9** Effect of PDO (a), lactic acid (b), succinic acid (c), acetic acid (d) and formic acid (e) on specific growth rate of *C. freundii* VK-19

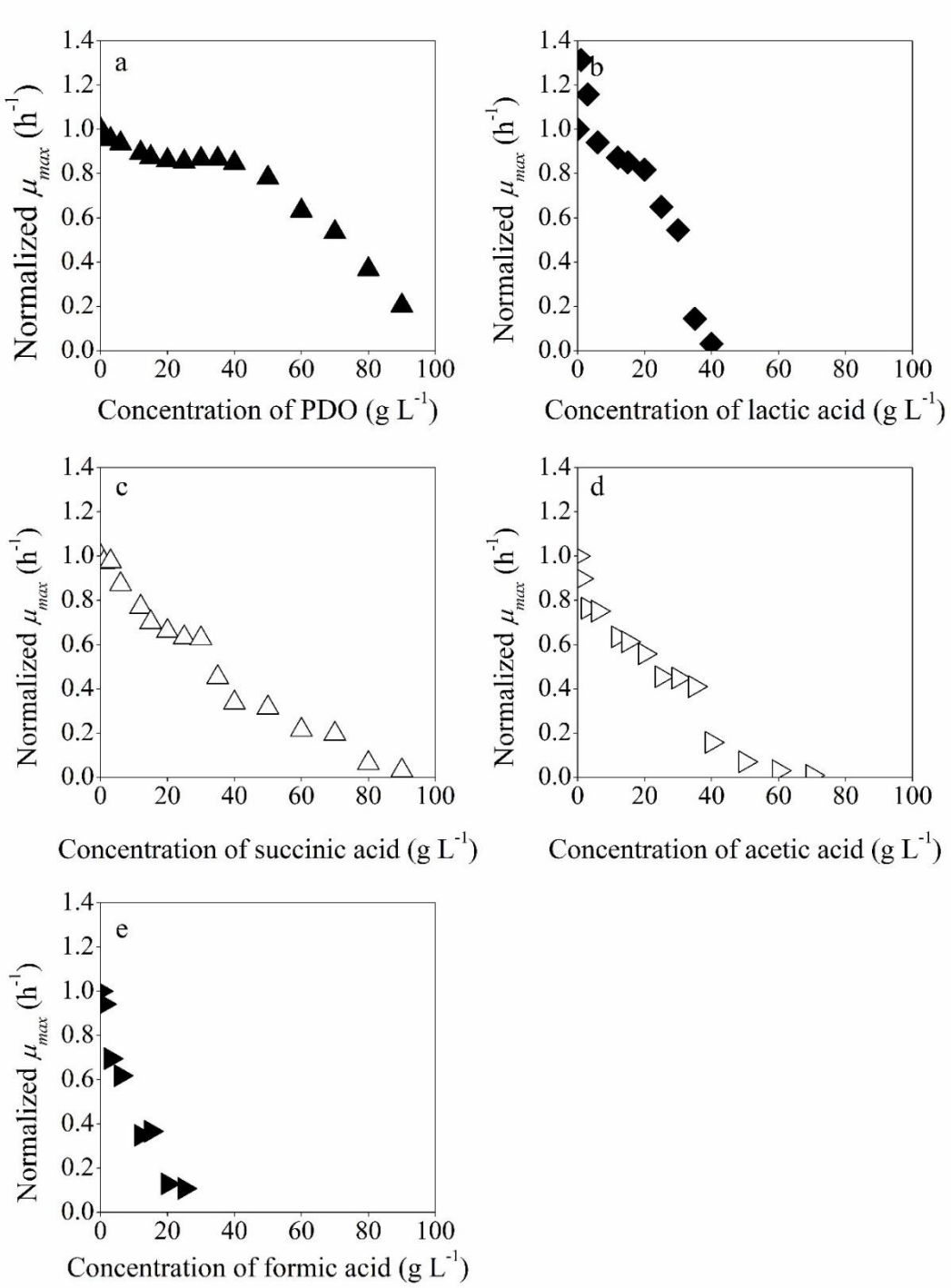


**Figure 5.10** Effect of PDO (a), lactic acid (b), succinic acid (c), acetic acid (d) and formic acid (e) on specific growth rate of *C. freundii* FMCC-8

The effect of $Na^+$ and $K^+$ salts on the two strains of *C. freundii* was evaluated in microtiter plate experiments. Figure 5.11 presents the specific growth rate obtained for both strains in microtiter plate fermentations carried out in the presence of $Na^+$ and $K^+$ salts added in the form of NaCl and $K_2SO_4$ in various concentrations. The addition of $K_2SO_4$ initially enhanced bacterial

growth. At all concentrations used, the presence of potassium salts did not inhibit bacterial growth for both strains. On the other hand, a linear reduction of $\mu_{max}$ was observed with increasing concentrations of $Na^+$. These results demonstrate that the use of crude glycerol that contains sodium salts results in lower PDO production efficiency than the use of crude glycerols containing potassium salts. This explains the lower production efficiency achieved in this study than the one reported by Metsoviti et al. (2013) where crude glycerol with potassium salts was used. However, the PDO production efficiency reported in this study is among the highest reported in literature-cited publications when *Citrobacter* strains are used.

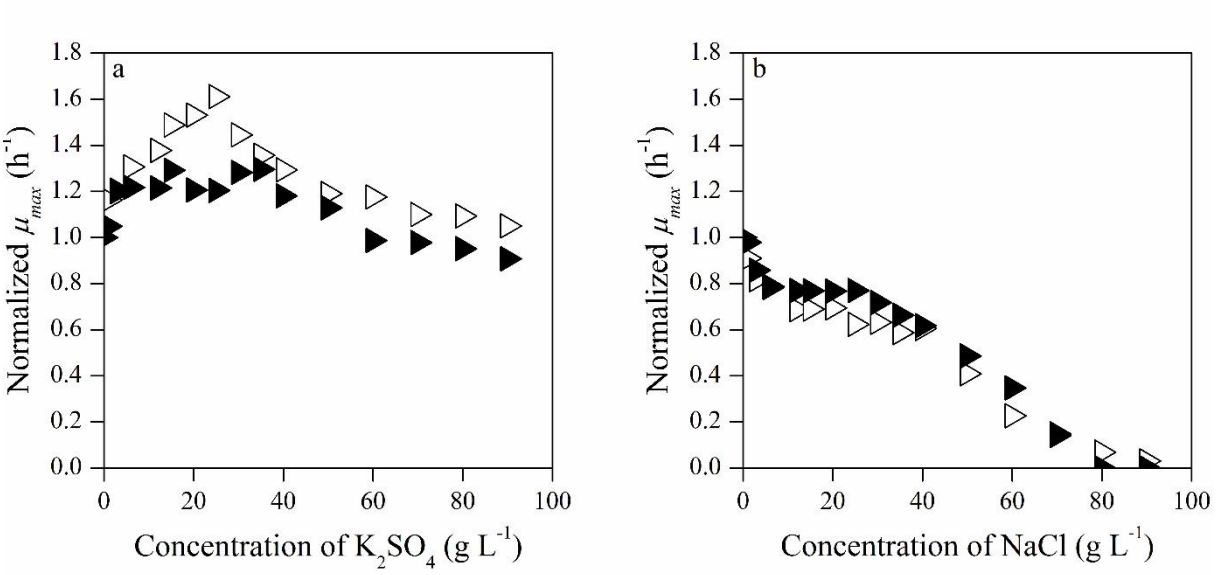


**Figure 5.11** Effect of $K^+$ (a) and $Na^+$ (b) salts on specific growth rate for *C. freundii* VK-19 (filled symbols) and *C. freundii* FMCC-8 (unfilled symbols)

## 5.9 Conclusions

In the present study the by-products generated from soybean-based biodiesel plant were evaluated for PDO production. Crude glycerol and SBC hydrolysates were implemented in fed-batch cultures using *C. freundii* VK-19 and *C. freundii* FMCC-8. The effect of inhibitors was also investigated, indicating an inhibition from lactic acid, formic acid and acetic acid in concentrations higher than 20 g $L^{-1}$ for both strains. *C. freundii* strains were more tolerant to succinic acid and PDO even in high concentrations. The effect of sodium and potassium salts on bacterial growth was also evaluated demonstrating enhanced bacterial growth when $K^+$ salts are used, whereas $Na^+$ salts showed high cell growth inhibition.

# Bioprocess development for the production of 2,3-butanediol by a newly isolated strain of *Klebsiella michiganensis* using different types of crude glycerol

# Chapter 6

## 6.1 Introduction

The development and demonstration of innovative technologies for bio-based production of intermediate chemicals could deliver better overall economics, sustainability and higher performance than using conventional petrochemical feedstocks and processes. The production of bio-based 2,3-butanediol (BDO) could be improved by valorising agro-industrial side streams and crude renewable resources. Crude glycerol, generated as a by-product from biodiesel production plants, shows a high potential as a promising fermentation feedstock. Nevertheless, the variability on its composition significantly affects the fermentation efficiency. The evaluation and optimization of fermentation parameters using crude glycerol could lead to cost-effective production of bio-based chemicals.

Based on the aforementioned arguments, the objective of this chapter is the evaluation of different types of crude glycerol on BDO production by the newly isolated bacterial strain *Klebsiella michiganensis*. The main fermentation parameters studied were type of nitrogen source, temperature, type of raw glycerol and initial crude glycerol concentration. Their effect on bacterial growth and BDO production was initially evaluated in shake flask fermentations. Oxygen supply was studied at various volumetric oxygen mass transfer coefficients ($k_La$) values in fed-batch fermentations using either pure or crude glycerol generated via transesterification of either soybean oil or soybean oil mixed with tallow fat at different ratios.

## 6.2 Characterization of crude glycerols

Crude glycerol from biodiesel plants has a glycerol content ranging from 20 to 92% (w $w^{-1}$). It also contains other components including soap, free fatty acids, methyl esters of fatty acids, glycerides and methanol. The amount of each component depends on the oil feedstock and the process employed for biodiesel synthesis. The presence and the concentration of these impurities can effect the growth of bacterial cells and consequently lead to decreased production of metabolites.

In this study, three different types of crude glycerol were used that were produced by biodiesel production processes using different oil and fat feedstocks. The first crude glycerol (100-So) was produced from a biodiesel production process where only soybean oil was used as feedstock. The second crude glycerol (90-So:10-Tf) was produced from a biodiesel production process employing a mixture of 90% soybean oil and 10% tallow fat, while the third crude glycerol (65-So:35-Tf) was produced from a process employing a mixture of 65% soybean oil

with 35% tallow fat. Each crude glycerol stream was pre-treated overnight in separation funnels in order to remove impurities and increase the glycerol content. This pretreatment approach has been also carried out in the fermentative production of poly(3-hydroxybutyrate) (PHB) using the bacterial strain *Cupriavidus necator* that led to increased production efficiency during fermentation possibly due to the significant removal of non-polar compounds and FAME from the crude glycerol used (Kachrimanidou et al., 2014). Pre-treated crude glycerol streams were evaluated in this study in microbial bioconversions for the production of BDO. The composition of initial and pre-treated crude glycerols is presented in Table 6.1 and the fatty acids contained in the non-polar fraction of pretreated crude glycerol is presented in Table 6.2.

**Table 6.1** Composition of initial and pre-treated crude glycerol streams

| Component | 100-So | 100-So* | 90-So:10-Tf | 90-So:10-Tf* | 65-So:35-Tf | 65-So:35-Tf* |
|---|---|---|---|---|---|---|
| Glycerol (%, w $w^{-1}$) | 77.8 | 79.4 | 75.8 | 81.0 | 81.8 | 83.4 |
| Water (%, w $w^{-1}$) | 14.0 | 12.8 | 11.6 | 11.4 | 17.8 | 17.1 |
| Total non-polar fraction extracted with petroleum ether) (%, w $w^{-1}$) | - | 0.11 | - | 0.19 | - | 0.17 |
| Methanol | - | - | - | - | - | - |
| Ca (ppm) | 8.8 | 6.4 | 2.7 | 2.7 | 24.0 | 23 |
| Fe (ppm) | 0.55 | 0.59 | 0.69 | 0.69 | 0.83 | 0.6 |
| K (ppm) | 13 | 8.8 | 9.8 | 9.8 | 33 | 33 |
| Mg (ppm) | 4.9 | 0.9 | 0.83 | 0.83 | 1.1 | 1 |
| Na (ppm) | 21000 | 21000 | 21000 | 21000 | 17000 | 17000 |
| P (ppm) | 54 | 56 | 56 | 56 | 97 | 94 |

* pre-treated crude glycerol obtained after overnight decanting

**Table 6.2** Fatty acid composition in the non-polar fraction of pre-treated crude glycerol streams

| | Saturated | | | Monounsaturated | | Polyunsaturated | |
|---|---|---|---|---|---|---|---|
| | Palmitic | Stearic | Myristic | Oleic | Palmitoleic | Linoleic | Linolenic |
| % | C16:0 | C18:0 | C14:0 | C18:1 | C16:1 | C18:2 | C18:3 |
| 100-So* | 35.0 | 5.8 | 0.0 | 41.0 | 0.0 | 17.7 | 0.0 |
| 90-So:10-Tf* | 32.1 | 6.6 | 0.3 | 42.6 | 0.3 | 17.2 | 0.1 |
| 65-So:35-Tf* | 31.9 | 8.7 | 1.0 | 42.9 | 1.1 | 12.7 | 0.4 |

## 6.3 Evaluation of BDO production in shake flask fermentations

### 6.3.1 Effect of nitrogen source

Complex nutrient supplements including yeast extract and corn steep liquor (CSL) could enhance BDO production efficiency during fermentation. Yeast extract, containing essential growth micronutrients, has been mainly used for enhanced BDO production. CSL has been applied as inexpensive nitrogen source to replace expensive complex organic nutrient supplements (Li et al., 2013, Kim et al., 2017). In this study, yeast extract and CSL have been evaluated as organic nitrogen sources, while ammonium sulphate has been evaluated as inorganic source of nitrogen (Figure 6.1). The same initial glycerol (around 30 g $L^{-1}$) and minerals concentrations have been used in all shake flask fermentations presented in Figure 6.1. The composition of the mineral is the same as the one reported by Pirt and Callow (1958). The concentration of ammonium sulphate (7.2 g $L^{-1}$) used in shake flask fermentation is the same as the one used by Pirt and Callow (1958). The low yeast extract concentration (5 g $L^{-1}$) used was chosen considering that yeast extract is an expensive nutrient supplement that should be minimal in order to develop cost-competitive industrial bioprocesses. A higher concentration of CSL (10 g $L^{-1}$) as this nutrient supplement is cheaper than yeast extract. Two combinations of CSL and yeast extract as well as yeast extract and ammonium sulphate were also evaluated in order to evaluate the synergistic effect of different sources of nitrogen.

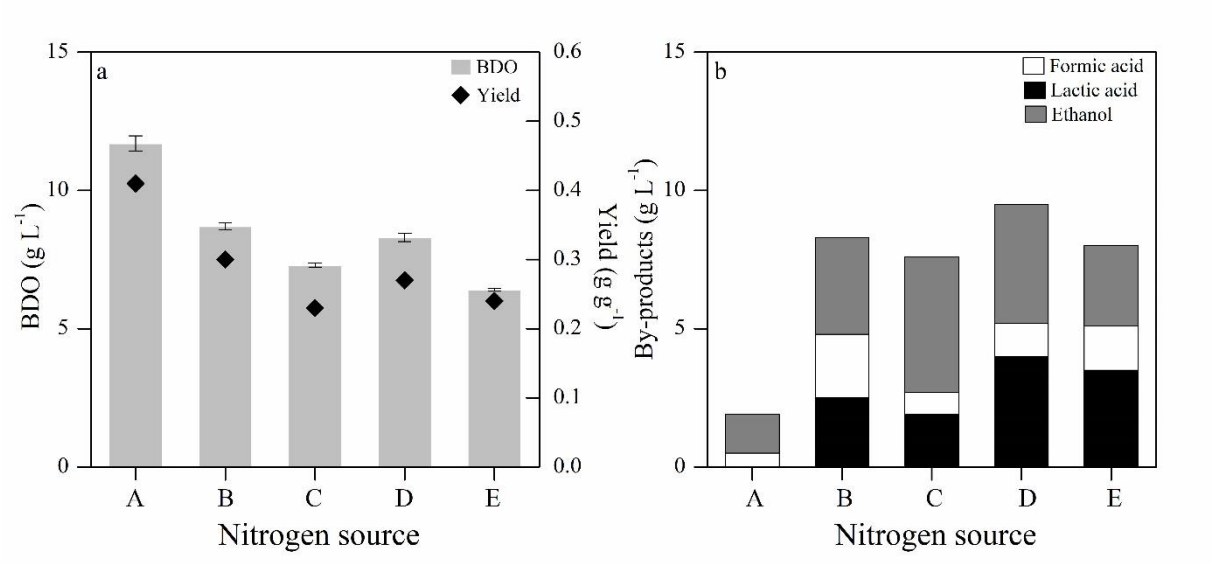


**Figure 6.1** Production of BDO and glycerol to BDO conversion yield (a) as well as by-product formation (b) achieved in shake flask cultures carried out with different nitrogen sources. (A)

7.2 g $L^{-1}$ $(NH_4)_2SO_4$, (B) 5 g $L^{-1}$ yeast extract, (C) 10 g $L^{-1}$ CSL, (D) mixture of 10 g $L^{-1}$ CSL and 5 g $L^{-1}$ yeast extract and (E) mixture of 7.2 g $L^{-1}$ $(NH_4)_2SO_4$ and 2.5 g $L^{-1}$ yeast extract

The highest BDO concentration (11.7 g $L^{-1}$) and conversion yield (0.41 g $g^{-1}$) were achieved using ammonium sulphate as the sole nitrogen source. The final BDO concentration was significantly lower (around 8 g $L^{-1}$) when either yeast extract or CSL were used as nitrogen source. Figure 6.1b shows that organic nitrogen sources favour the accumulation of organic acids and ethanol leading to lower glycerol to BDO conversion yield than the one achieved with ammonium sulphate. Using an inorganic nitrogen source rather than yeast extract would result in lower operating costs for fermentation media formulation. This is a significant advantage for industrial BDO production using the newly isolated strain of *K. michiganensis*. Thus, ammonium sulphate was chosen as nitrogen source in all subsequent fermentations.

### 6.3.2 Effect of temperature

The efficiency of bioprocess development is associated with the operating temperature during fermentation that affects enzymatic activity and cellular metabolism (Celińska and Grajek, 2009). The optimum temperature for bacterial growth and BDO production is usually in the range of 30-37 ˚C depending on the microbial strain and substrate used. Maina et al. (2019) reported that 33.9 ˚C was the optimum temperature for enhanced BDO production efficiency, leading to high productivity of 3.95 g $L^{-1}$ $h^{-1}$, by the bacterial strain *Enterobacter ludwigii* during fed-batch cultures with very high polarity cane sugar as carbon source. The effect of cultivation temperature on BDO production was investigated in shake flask cultures carried out within the range of 25-45 ˚C with pure glycerol as carbon source (Figure 6.2). The initial glycerol concentration was around 30 g $L^{-1}$.

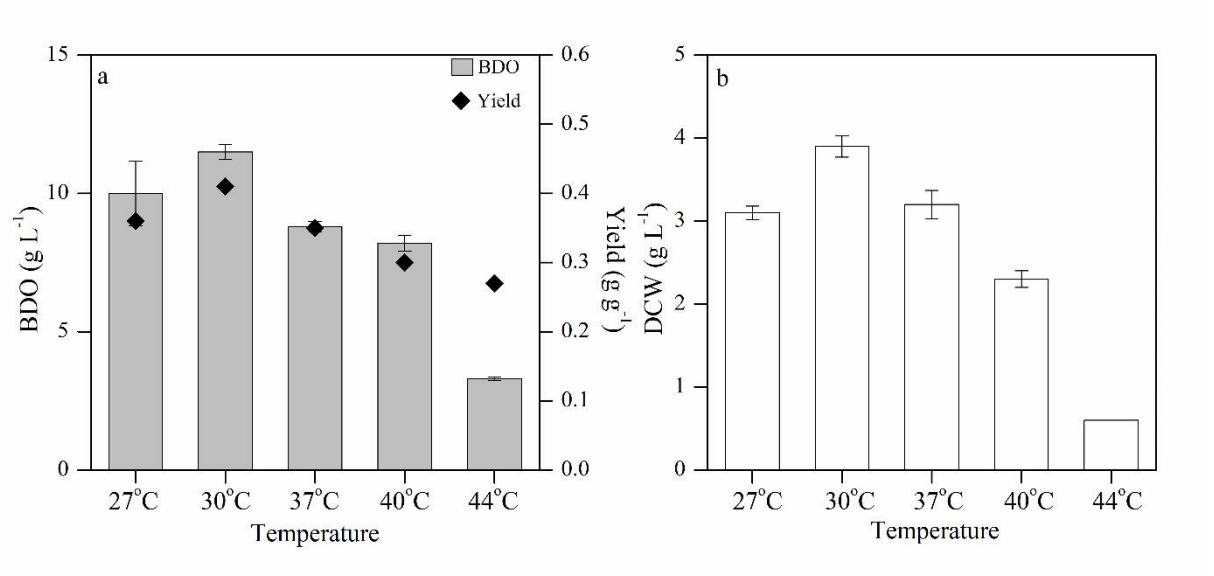


**Figure 6.2** BDO concentration and glycerol to BDO conversion yield (a) as well as DCW (b) achieved in shake flask cultures carried out on glycerol at different temperatures

The optimal temperature for bacterial growth, BDO production and glycerol to BDO conversion yield was 30 ˚C. At the optimal temperature, *K. michiganensis* produced 11.5 g $L^{-1}$ of BDO concentration with conversion yield of 0.41 g $g^{-1}$ and productivity of 0.47 g $L^{-1}$ $h^{-1}$. The DCW reached a maximum value of 3.9 g $L^{-1}$ at 30 ˚C, while increasing temperature from 30 ˚C to 44 ˚C led to gradual reduction in bacterial cell growth. Temperatures of 37 ˚C and 40 ˚C resulted in similar production of BDO, while higher temperature of 44 ˚C inhibited bacterial growth and hence BDO production. Considering the final BDO production efficiency and bacterial growth, all subsequent fermentations have been conducted at 30 ˚C.

### 6.3.3 Evaluation of different types of pretreated crude glycerol streams

The efficiency of *K. michiganensis* to convert crude glycerol for BDO production was evaluated using different types of crude glycerol generated from biodiesel production plant including 100-So, 90-So:10-Tf and 65-So:35-Tf. Pretreted crude glycerol streams were used in all shake flask fermentations. Initial concentration of crude glycerol was set at 30 g $L^{-1}$ in order to prevent substrate inhibition. *K. michiganensis* was capable of assimilating different types of crude glycerol for the synthesis of BDO (Table 6.3). Almost similar production of BDO (around 11 g $L^{-1}$) was produced by using pure, 100-So and 90-So:10-Tf pretreated crude glycerol streams. However, the use of 65-So:35-Tf pretreated crude glycerol stream as carbon source led to reduced BDO concentration (5.7 g $L^{-1}$). The final DCW (3.9 g $L^{-1}$) was the same in the fermentations carried out with pure glycerol and crude glycerol derived from biodiesel

production process using only soybean oil. The DCW was reduced with increasing animal fat content during biodiesel production. The maximum specific growth rate ($\mu_{max}$) was also affected by increasing animal fat usage during biodiesel production leading to around 64% lower value when 35% tallow fat was used for biodiesel production. The use of tallow fat influenced also the conversion yield and productivity achieved during fermentation. The glycerol to BDO conversion yield was reduced from 0.41 g $g^{-1}$ in the case of pure glycerol to 0.32 g $g^{-1}$ in the case of crude glycerols produced with either content of animal fat in biodiesel production processes. The high content of animal fat usage during biodiesel production led to significantly reduced productivities during fermentation of crude glycerol 90-So:35-Tf. When pretreated raw glycerol (90-So:10-Tf) derived from 90% soybean oil and 10% animal fats was used, the highest ethanol production (3.9 g $L^{-1}$) was observed. This set of experiments demonstrate that the type of feedstock used in biodiesel production processes result in crude glycerol streams that influence significantly the BDO production efficiency and bacterial growth during fermentation.

**Table 6.3** Effect of pretreated crude glycerols on BDO production by *K. michiganensis*

| Glycerol | $\mu_{max}$ ($h^{-1}$) | DCW (g $L^{-1}$) | BDO (g $L^{-1}$) | Yield (g $g^{-1}$) | Productivity (g $L^{-1}$ $h^{-1}$) | Ethanol (g $L^{-1}$) |
|---|---|---|---|---|---|---|
| pure | 0.28 | 3.9 ± 0.13 | 11.7 ± 0.27 | 0.41 ± 0.00 | 0.37 ± 0.00 | 1.3 ± 0.00 |
| 100-So | 0.29 | 3.9 ± 0.13 | 11.2 ± 0.29 | 0.37 ± 0.00 | 0.35 ± 0.02 | 1.4 ± 0.00 |
| 90-So:10-Tf | 0.27 | 2.3 ± 0.07 | 10.7 ± 0.20 | 0.32 ± 0.00 | 0.33 ± 0.01 | 3.9 ± 0.22 |
| 65-So:35-Tf | 0.18 | 1.3 ± 0.38 | 5.7 ± 1.08 | 0.32 ± 0.01 | 0.10 ± 0.02 | 0.3 ± 0.00 |

The origin of vegetable oil and animal fat feedstocks used in biodiesel production processes alters the composition of free fatty acids, unreacted mono-, di- and tri-acylglycerols, and methyl esters present in crude glycerol leading to variable levels of inhibition to bacterial growth and metabolite production. Furthermore, other impurities and salts (e.g. potassium, sodium) contained in crude glycerols also affect significantly the fermentation efficiency for the production of various metabolites. In the case of PHB production, the intracellular biopolymer content in fed-batch cultures of *Cupriavidus necator* JMP 134 was reduced from 70% when pure glycerol was used to 48% when a crude glycerol stream (80% purity, w $w^{-1}$) containing NaCl (5.5%, w $w^{-1}$) was used (Mothes et al., 2007). The intracellular PHB content of around 55% was achieved with a crude glycerol stream of 90% purity containing 1.6% $K_2SO_4$ (Mothes et al., 2007). Crude glycerols containing potassium salts are significantly more inhibitory on *C. necator* growth and PHB production than crude glycerols containing sodium salts (Garcia et

al., 2013; Mothes et al., 2007). Increasing concentrations of monovalent salts in fermentation media induce cell membrane swelling due to reduction in van der Waals forces in the lipid membrane (Samul et al., 2014). As a consequence of cell membrane swelling, the energetic barrier in membrane lipid layer is negatively influenced causing alterations in cellular metabolism.

Saturated and unsaturated free fatty acids (e.g. stearic, oleic and linoleic acids) cause variable level of inhibition on bacterial growth and metabolite production. Cell membranes contain fatty acids as structural components and they are incorporated into the acyl chains and alk-l-enyl chains of cellular lipids (Samul et al., 2014). The saturated C18:0 fatty acid, stearic acid, aligns with the fatty acid tails of the membrane. The monounsaturated C18:1 fatty acid, oleic acid, prevents the diffusion of nutrients and metabolites through the membrane. The high degree of saturation of the polyunsaturated C18:2 fatty acid, linoleic acid, causes limited production of some metabolic products. Substrate diffusion through the cellular membrane is negatively influenced by polyunsaturated fatty acid (Samul et al., 2014). The crude glycerol derived from soybean oil based biodiesel production processes is less inhibitory towards 1,3-propanediol production than crude glycerol generated via transesterification of waste vegetable oils (Moon et al., 2010). Furusawa and Koyama (2004) reported that bacterial growth is mainly inhibited by the presence of unsaturated fatty acids rather than the presence of saturated free fatty acids. Rehman et al. (2008) reported that *Clostridium butyricum* was significantly inhibited by the unsaturated fatty acids linoleic and oleic acid present in crude glycerol derived from sunflower oil based biodiesel production process. Oleic acid at concentration of 2% completely inhibits microbial growth of *C. butyricum* and 1,3-propanediol production due to the presence of double bond (Chatzifragkou et al., 2010).

The crude glycerols used in this study contain sodium salts and higher content of unsaturated than saturated fatty acids. Their effect on *K. michiganensis* growth and BDO production should be evaluated in fed-batch bioreactor cultures where crude glycerol is added in the broth during fermentation because the crude glycerol concentration used in shake flask cultures was relatively low.

### 6.3.4 Effect of initial glycerol concentration

In order to investigate the effect of initial glycerol concentration on BDO production and bacterial growth, shake flask fermentations were carried out at varying initial glycerol concentration (35.7, 62.0, 75.2, 115.9 and 135.9 g $L^{-1}$). The crude glycerol 90-So:10-Tf was used because it is not as inhibitory to bacterial growth and BDO production as the crude glycerol streams produced from transesterification of animal fat. Figure 6.3 shows the results obtained during shake flask cultures carried out with different initial glycerol concentrations. *K. michiganensis* showed tolerance to increasing crude glycerol concentrations. The final BDO concentration increased with increasing initial glycerol concentration. The highest BDO concentration (49.1 g $L^{-1}$) was achieved when an initial glycerol concentration of 135.9 g $L^{-1}$ was used corresponding to a glycerol to BDO conversion yield of 0.39 g $g^{-1}$. Yield (0.40 g $g^{-1}$) and productivity (0.41 g $L^{-1}$ $h^{-1}$) reached the highest values at an initial glycerol concentration of 35.7 g $L^{-1}$. It should be stressed though that similar glycerol to BDO conversion yields were observed in all cases. Negligible glycerol concentrations remained at the end of fermentation even at high initial glycerol concentration. The final DCW was reduced with increasing glycerol concentration, which was also observed in the case of maximum specific growth rate. The total concentration of metabolic by-products (ethanol, formic acid and lactic acid) was increased with increasing initial glycerol concentration from 35.7 g $L^{-1}$ to 75.2 g $L^{-1}$. Formic acid production was not observed at initial glycerol concentrations of 115.9 and 135.9 g $L^{-1}$ leading to lower total by-product formation at the end of fermentation.

Initial glycerol concentration in the range of 50 to 100 g $L^{-1}$ have been previously evaluated for BDO production by different strains. Huang et al. (2012) evaluated the effect of glycerol concentration on BDO production by *Klebsiella* sp.1, *Klebsiella* sp.2 and *Serratia* sp strains. Initial concentration of 75 g $L^{-1}$ resulted in enhanced values of conversion yield and productivity for all strains. Relatively high BDO yield (0.35 g $g^{-1}$) and concentration (16.8 g $L^{-1}$) has been obtained by *B. amyloliquefaciens* strain using initial glycerol concentration of 80 g $L^{-1}$ (Yang et al., 2013). Yield and productivity were reduced by applying higher glycerol concentrations. The influence of initial glycerol concentration (within the range of 30 to 90 g $L^{-1}$) on BDO production by *R. planticola* and *R. terrigena* was studied by Ripoll et al. (2016). High values of yield and productivity were obtained by increasing initial glycerol concentration. The highest BDO concentration, yield and productivity were achieved at initial glycerol concentration of 90 g $L^{-1}$.

Taking into account the results presented above, it could be concluded that *K. michiganensis* can produce BDO at high efficiency even at high initial crude glycerol concentrations when only soybean oil is used for biodiesel production. Thus, this is a promising bacterial strain for industrially efficient BDO production.

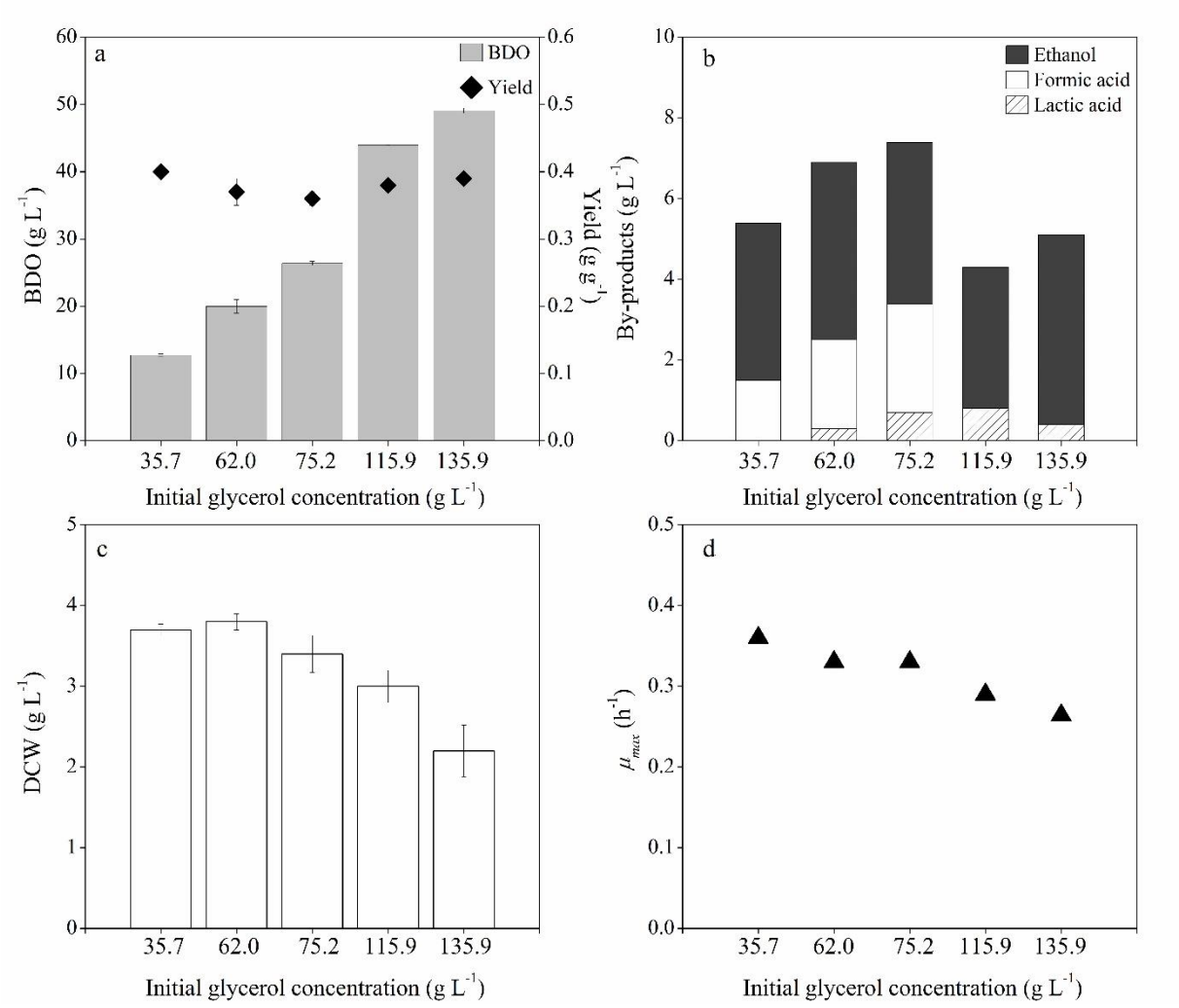


**Figure 6.3** Effect of initial glycerol concentration on final BDO concentration, glycerol to BDO conversion yield (a), by-product formation (b), DCW (c) and maximum specific growth rate (d) during shake flask fermentations with *K. michiganensis*. The crude glycerol stream used was 90-So:10-Tf.

## 6.4 BDO production in fed-batch fermentations using different crude glycerols

Fed-batch bioreactor fermentations were subsequently carried out in order to evaluate the BDO production efficiency by *K. michiganensis*. In shake flask fermentations, it was verified that synthetic media are suitable for bacterial growth boosting BDO production efficiency. Thus, synthetic media were used in bioreactor cultures. The temperature used was 30 °C as it was also verified in shake flask fermentations. An important parameter influencing BDO production in bioreactor cultures is oxygen supply. Thus, fed-batch fermentations were carried out in this study at varying volumetric oxygen mass transfer coefficients ($k_La$) in order to assess the effect of oxygen transfer on BDO production efficiency. The optimal level of aeration depends on the microbial strain and the substrate used. Under high oxygen supply, growth is favored at the expense of BDO, while oxygen-limited conditions lead to higher yield but relatively lower productivity (Celinska and Grajek, 2009).

Previous studies have also highlighted the effect of $k_La$ on BDO production efficiency (Fages et al., 1986; Silveira et al., 1993). Various oxygen supply control methodologies have been reported for enhanced BDO production efficiency. High combined BDO and acetoin production efficiency has been achieved by Zeng et al. (1991a) using *E. aerogenes* cultivated on glucose employing an oxygen uptake rate control strategy from 100 to 30 mmol $L^{-1}$ $h^{-1}$. Zhang et al. (2010a) employed respiratory quotient to control oxygen supply. Oxygen uptake rate, respiratory quotient and $k_La$ have been proposed as accurate and suitable parameters to enhance the production efficiency of aerobic bioprocesses (Zhou et al., 2018).

Figure 6.4 presents the production of BDO during fed-batch fermentations carried out at different types of crude glycerol and $k_La$ values. When pure glycerol was used as substrate (Figure 6.4a), the highest BDO concentration (107.9 g $L^{-1}$), yield (0.44 g $g^{-1}$) and productivity (0.90 g $L^{-1}$ $h^{-1}$) were achieved with agitating cascade corresponding to $k_La$ values in the range of 46-62 $h^{-1}$. Maintaining constant $k_La$ value (62 $h^{-1}$) during fermentation with pure glycerol led to a final BDO concentration of 93.5 g $L^{-1}$ with lower yield (0.31 g $g^{-1}$) and productivity (0.71 g $L^{-1}$ $h^{-1}$) than the fermentation carried out with agitation cascade. When crude glycerols (Figure 6.4b, c and d) were used, it is obvious that the BDO production efficiency is lower than the one achieved with pure glycerol. In the case of crude glycerol streams, the highest BDO concentration (87.6 g $L^{-1}$) was reached when 100-So crude glycerol was used at constant $k_La$ value of 62 $h^{-1}$ during fermentation. The $k_La$ value of 62 $h^{-1}$ was used with all glycerol purities demonstrating that the BDO concentration was reduced with increasing tallow fat usage during

biodiesel production. The lowest BDO concentration (54.2 g $L^{-1}$) was produced in the fermentation carried out with pretreated 65-So:35-Tf crude glycerol at $k_La$ value of 62 $h^{-1}$. In all types of glycerol, productivity was increased with increasing $k_La$ values up to approximately 77 $h^{-1}$, while low oxygen supply ($k_La$ value of 62 $h^{-1}$) led to low productivities. The highest productivity of 1.38 g $L^{-1}$ $h^{-1}$ with reasonable BDO concentration (76.1 g $L^{-1}$) and yield (0.41 g $g^{-1}$) were reached when 100-So glycerol and $k_La$ value of 77 $h^{-1}$ were used. The highest glycerol to BDO conversion yield (0.46 g $g^{-1}$) has been achieved with 100-So crude glycerol using a $k_La$ value of 62 $h^{-1}$. Very low yields (around 0.28 g $g^{-1}$) were achieved when the 65-So:35-Tf crude glycerol was used indicating the inhibitory effect of impurities produced during tallow fat transesterification. Generally, in all types of crude glycerol, the production of BDO and conversion yield were increased at low $k_La$ values, while maximum values of productivity were reached with increasing $k_La$. Regarding the stereoisomer ratio in the total BDO concentration, a mixture of meso-BDO and L-BDO was produced by *K. michiganensis* with the former to be in the range of 50-55%.

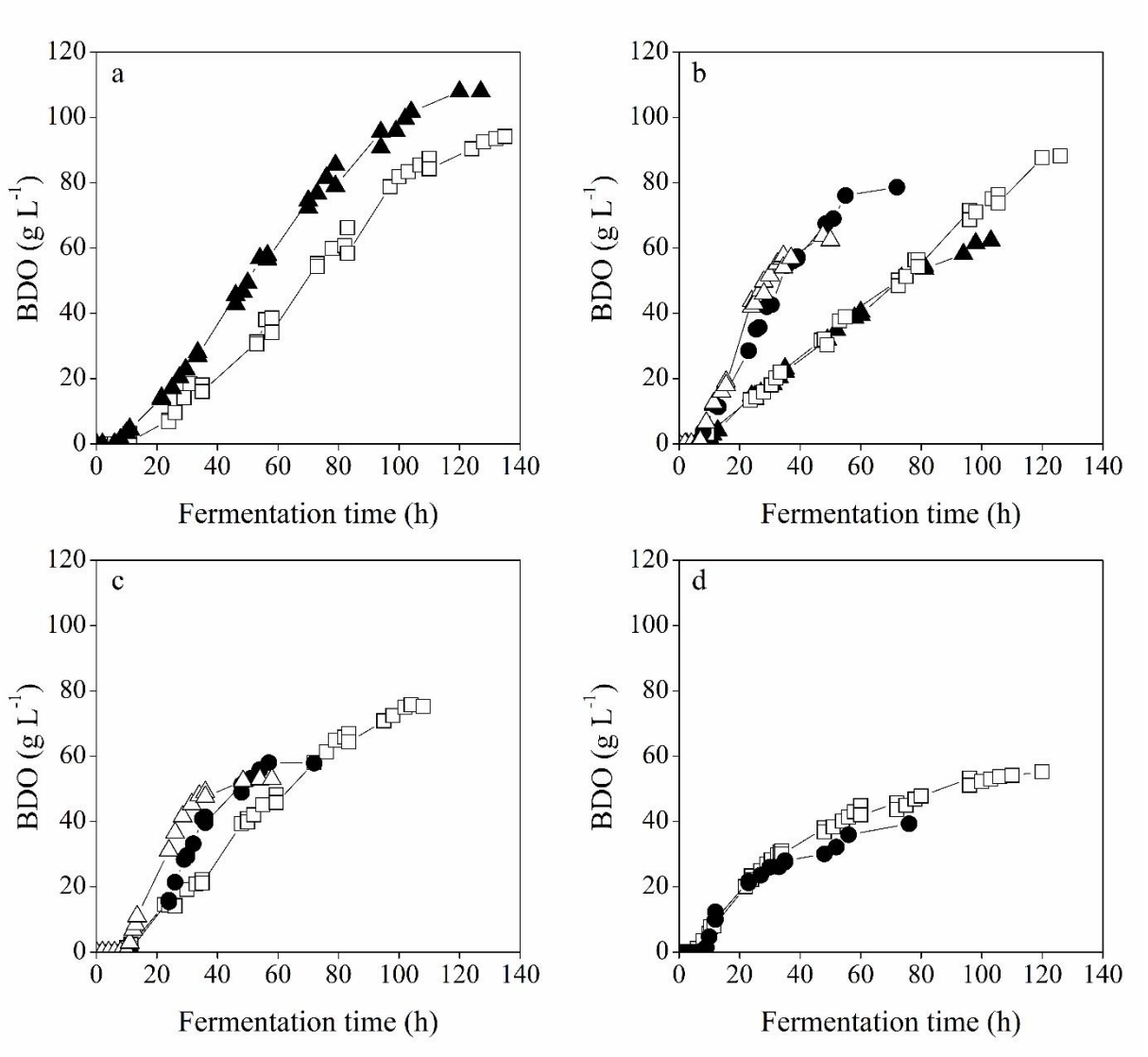


**Figure 6.4** BDO production in fed-batch fermentations of *K. michiganensis* cultivated on pure glycerol (a) and crude glycerols 100-So (b), 90-So:10-Tf (c) and 65-So:35-Tf (d) using $k_La$ values of 46-62 $h^{-1}$ (▲), 62 $h^{-1}$ (□), 77 $h^{-1}$ (●), and 83 $h^{-1}$ (∆)

Figures 6.5 and 6.6 present the metabolic by-products produced during fed-batch fermentations carried out with all types of crude glycerol. The main metabolic by-products produced were lactic acid and acetic acid. Ethanol and formic acid were also detected in some fermentations (Table 6.3). Oxygen supply along with the type of glycerol affect the production trend and the final concentration of organic acids (Figures 6.5 and 6.6).

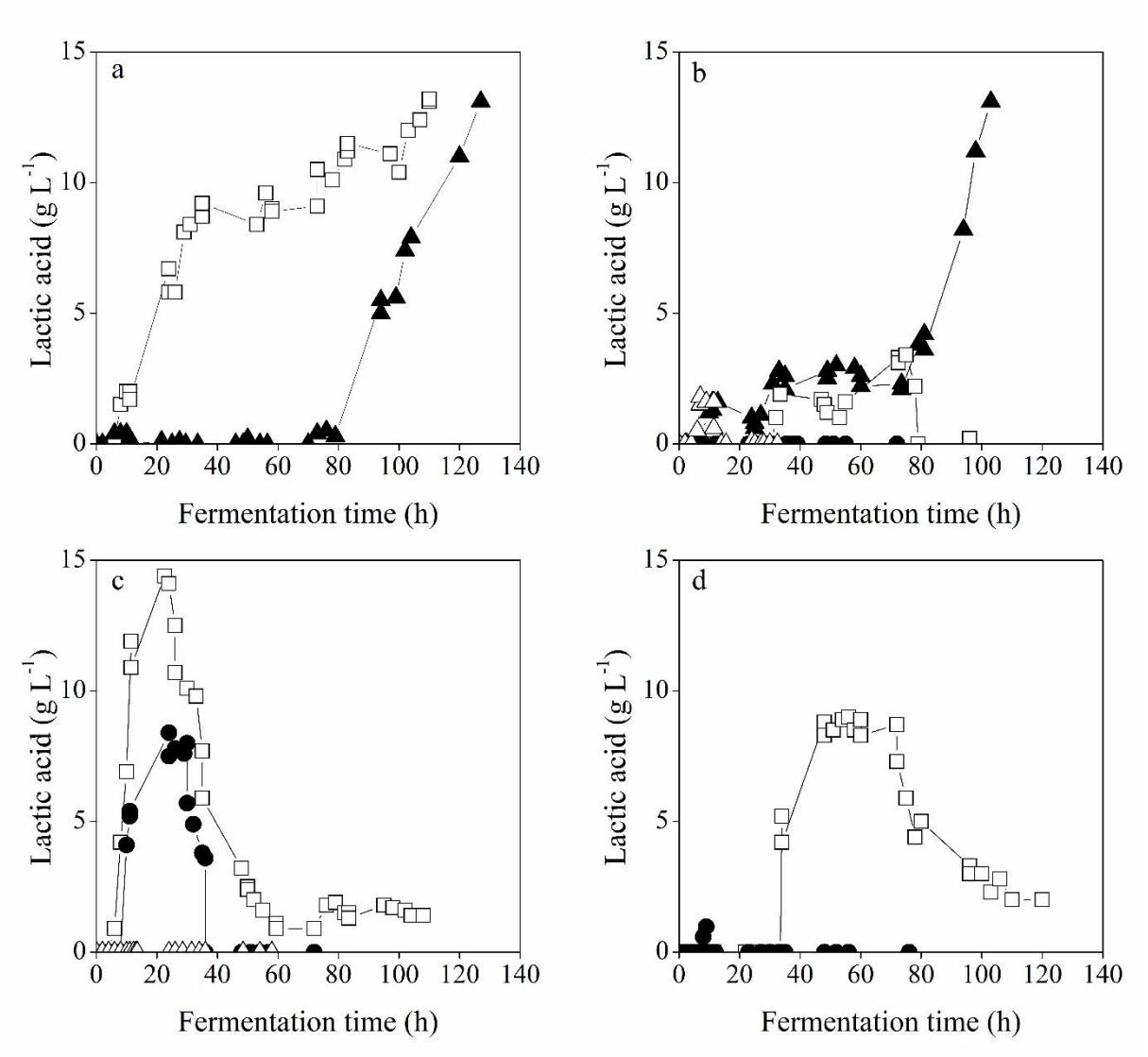


**Figure 6.5** Lactic acid production in fed-batch fermentations of *K. michiganensis* cultivated on pure glycerol (a) and crude glycerols 100-So (b), 90-So:10-Tf (c) and 65-So:35-Tf (d) using $k_La$ values of 46-62 $h^{-1}$ (▲), 62 $h^{-1}$ (□), 77 $h^{-1}$ (●), and 83 $h^{-1}$ (∆)

Lactic acid was the main metabolic by-product produced in fed-batch fermentations carried out with pure glycerol. The highest lactic acid concentration (13.1 g $L^{-1}$) was observed at constant $k_La$ value of 62 $h^{-1}$ when pure glycerol was used (Table 6.3). The utilisation of 100-So crude glycerol at $k_La$ values ranging from 46 to 62 $h^{-1}$ resulted in accumulation of lactic acid especially after 70 h, while at constant $k_La$ values low concentrations of lactic acid were observed. In all glycerol fermentations carried out at $k_La$ value of 62 $h^{-1}$, lactic acid was produced during fermentation and it was subsequently consumed completely exept for the fermentation carried out with pure glycerol where lactic acid production occurred throughout fermentation (Figure

6.5).

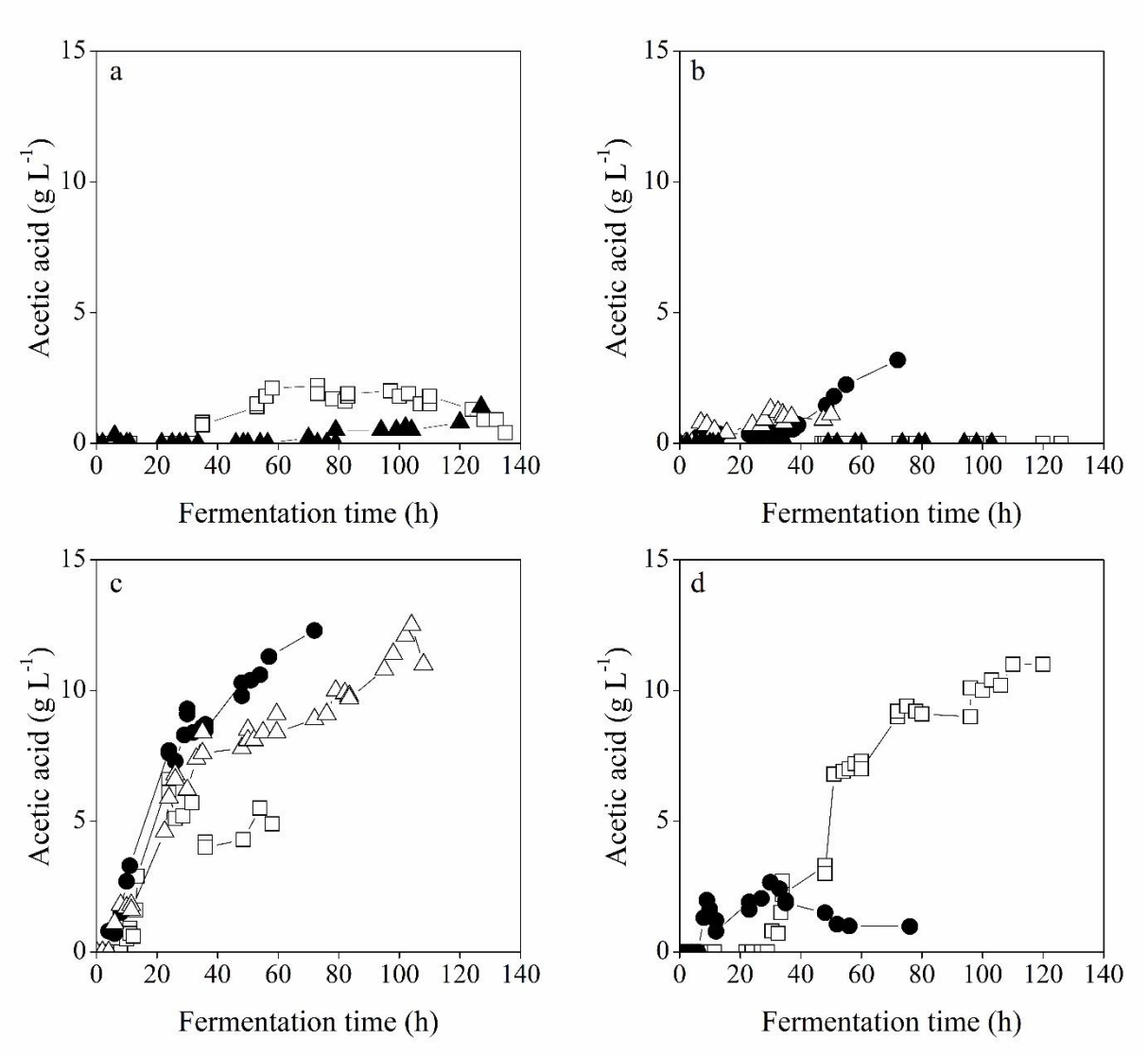


**Figure 6.6** Acetic acid production in fed-batch fermentations of *K. michiganensis* cultivated on pure glycerol (a) and crude glycerols 100-So (b), 90-So:10-Tf (c) and 65-So:35-Tf (d) using $k_La$ values of 46-62 $h^{-1}$ (▲), 62 $h^{-1}$ (□), 77 $h^{-1}$ (●), and 83 $h^{-1}$ (△)

In the case of pure glycerol fermentation, agitation cascade resulted in lactic acid production after 80 h. Acetic acid was mainly produced as by-product in fermentation with crude glycerol derived from biodiesel production processes using a mixture of soybean oil and animal fats (Figure 6.6). The highest acetic acid concentration (12.1 g $L^{-1}$) was produced with 90-So:10-Tf crude glycerol at $k_La$ value of 62 $h^{-1}$. The highest total by-product formation (around 19.2 g $L^{-1}$) was observed with 90-So:10-Tf crude glycerol at $k_La$ value of 62 $h^{-1}$. The fermentation carried

out with 100-So crude glycerol at $k_La$ value of 62 $h^{-1}$ leads to the lowest total by-product formation (2.9 g $L^{-1}$) that explains the high glycerol to BDO conversion yield (0.46 g $g^{-1}$) achieved.

Figure 6.7 presents the profiles of DCW observed during all fed-batch fermentations. In the case of pure glycerol, bacterial growth was favoured at contant $k_La$ value resulting in $\mu_{max}$ of 0.38 $h^{-1}$ (Table 6.3).

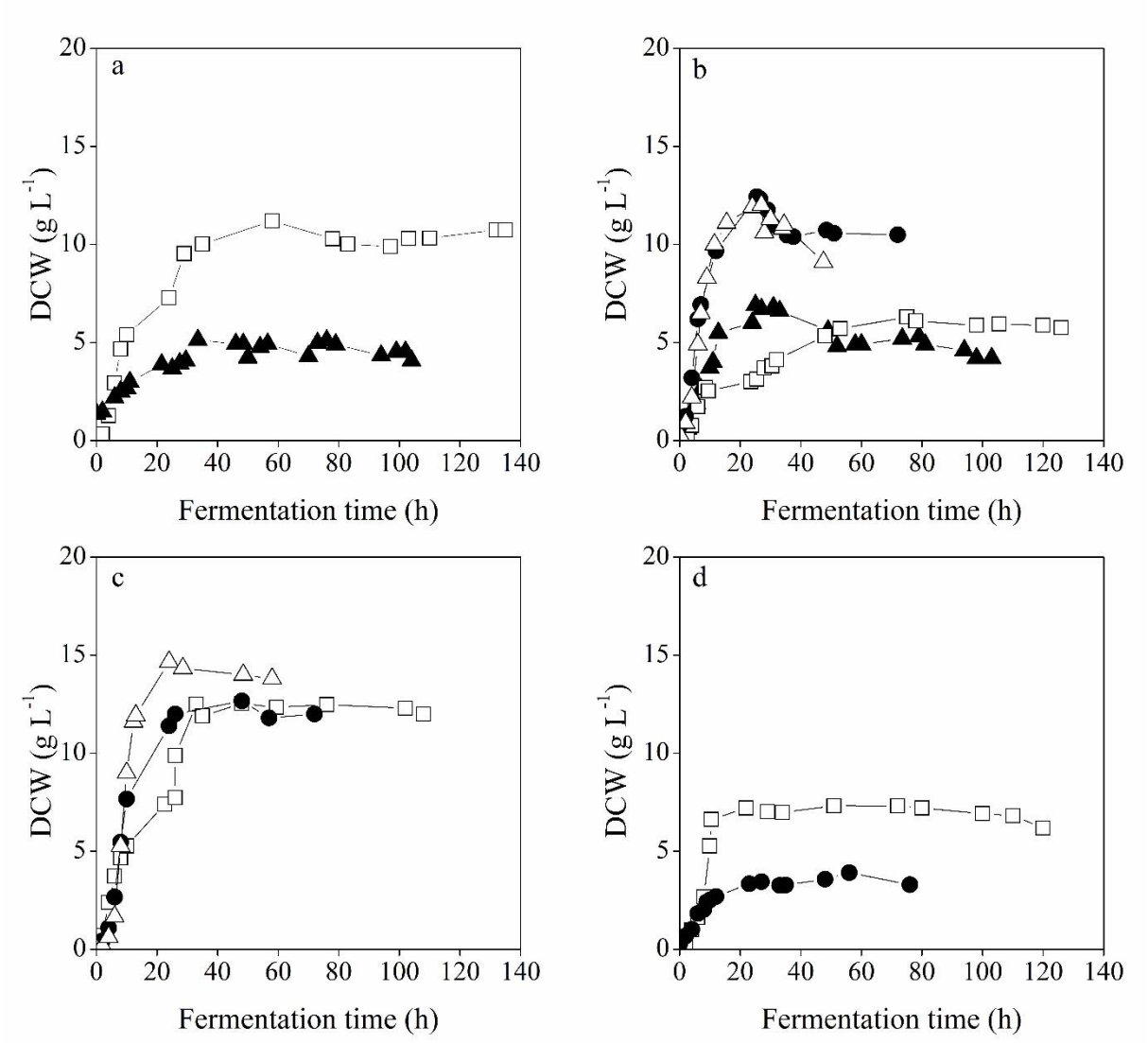


**Figure 6.7** Bacterial growth in fed-batch fermentations of *K. michiganensis* cultivated on pure glycerol (a) and crude glycerols 100-So (b), 90-So:10-Tf (c) and 65-So:35-Tf (d) using $k_La$ values of 46-62 $h^{-1}$ (▲), 62 $h^{-1}$ (□), 77 $h^{-1}$ (●), 83 $h^{-1}$ (Δ)

Increased bacterial growth was observed by increasing the oxygen supply ($k_La$ values of 77 $h^{-1}$ and 83 $h^{-1}$) with 100-So and 90-So:10-Tf glycerol. In the case of 100-So crude glycerol, the highest maximum specific growth rate (0.41 $h^{-1}$) was achieved at the highest $k_La$ value (83 $h^{-1}$) used. The use of 90-So:10-Tf crude glycerol resulted in the highest final DCW (around 15 g $L^{-1}$) and maximum specific growth rates (0.44 – 0.48 $h^{-1}$) among all types of crude glycerol used. The use of 65-So:35-Tf crude glycerol inhibited growth resulting in the low DCW and $\mu_{max}$ values (Figure 6.7 and Table 6.3). The increasing DCW and $\mu_{max}$ with 90-So:10-Tf crude glycerol could be explained by the fact that in some cases crude glycerol may boost bacterial growth. In some studies, the presence of nutrients in crude glycerol has exhibited possitive effect on bacterial growth (Cho et al., 2015). Moreover, the production of net NADH consuming by-products decreased because of the additional NADH consumption for cell growth.

Figures 6.8-6.11 present the profile of glycerol consumption during fed-batch cultures carried out with pure and crude glycerols. The initial glycerol concentration in all fermentations was in the range of 30-50 g $L^{-1}$ and pulse feeding with a concentrated feeding solution of crude glycerol was conducted when the glycerol concentration was around 5 g $L^{-1}$. At low oxygen supply, glycerol consumption rate was relatively low, while at increasing $k_La$ values glycerol was consumed faster. Specifically, at $k_La$ values in the range of 46-62 $h^{-1}$ and 62 $h^{-1}$, glycerol consumption rate was around 2.2 g $L^{-1}$ $h^{-1}$ in all glycerols used. At $k_La$ value of 77 $h^{-1}$, glycerol consumption rate was increased to 4.3 and 4.6 g $L^{-1}$ $h^{-1}$ when 100-So and 90-So:10-Tf crude glycerols were used, respectively. At $k_La$ value of 83 $h^{-1}$, glycerol consumption rate was increased further (aroung 5.3 g $L^{-1}$ $h^{-1}$). The use of 65-So:35-Tf crude glycerol inhibited growth and hence glycerol consumption rate remained at low levels.

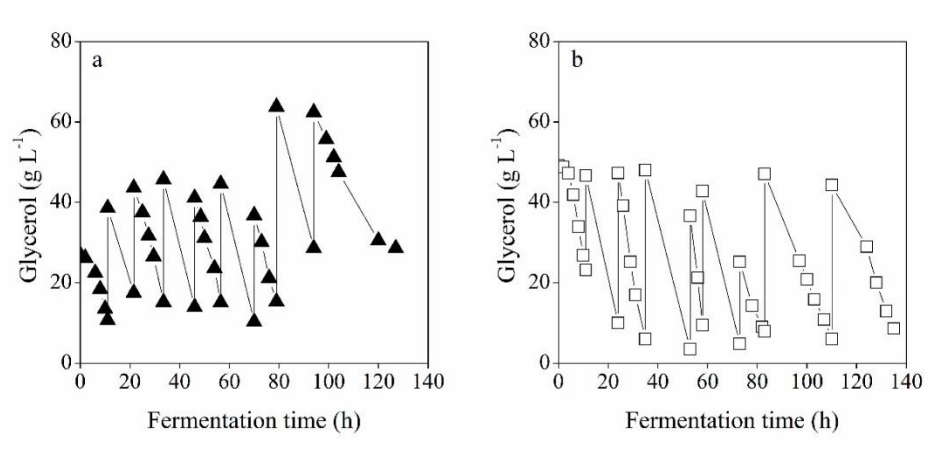


**Figure 6.8** Glycerol consumption in fed-batch fermentations of *K. michiganensis* cultivated on pure glycerol at $k_La$ value in the range of 46-62 $h^{-1}$(a) and at $k_La$ value of 62 $h^{-1}$ (b)

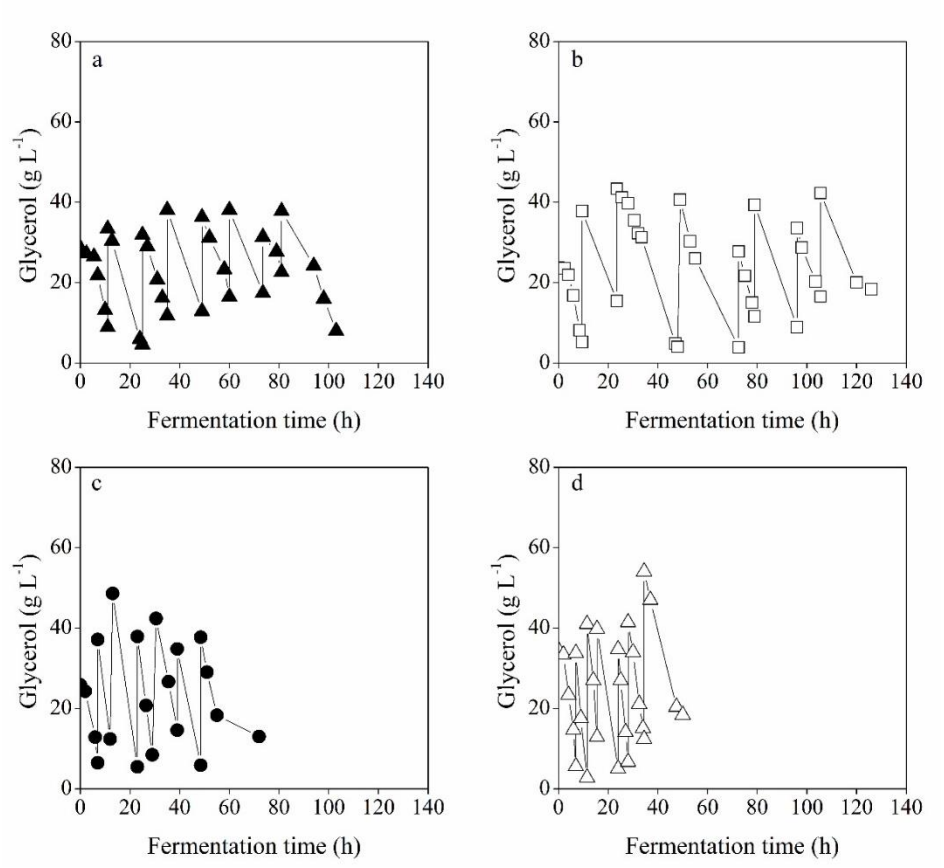


**Figure 6.9** Glycerol consumption in fed-batch fermentations of *K. michiganensis* cultivated on crude glycerol 100-So at $k_La$ value in the range of 46-62 $h^{-1}$(a), $k_La$ value of 62 $h^{-1}$ (b) $k_La$ value of 77 $h^{-1}$ (c) and $k_La$ value of 83 $h^{-1}$ (d)

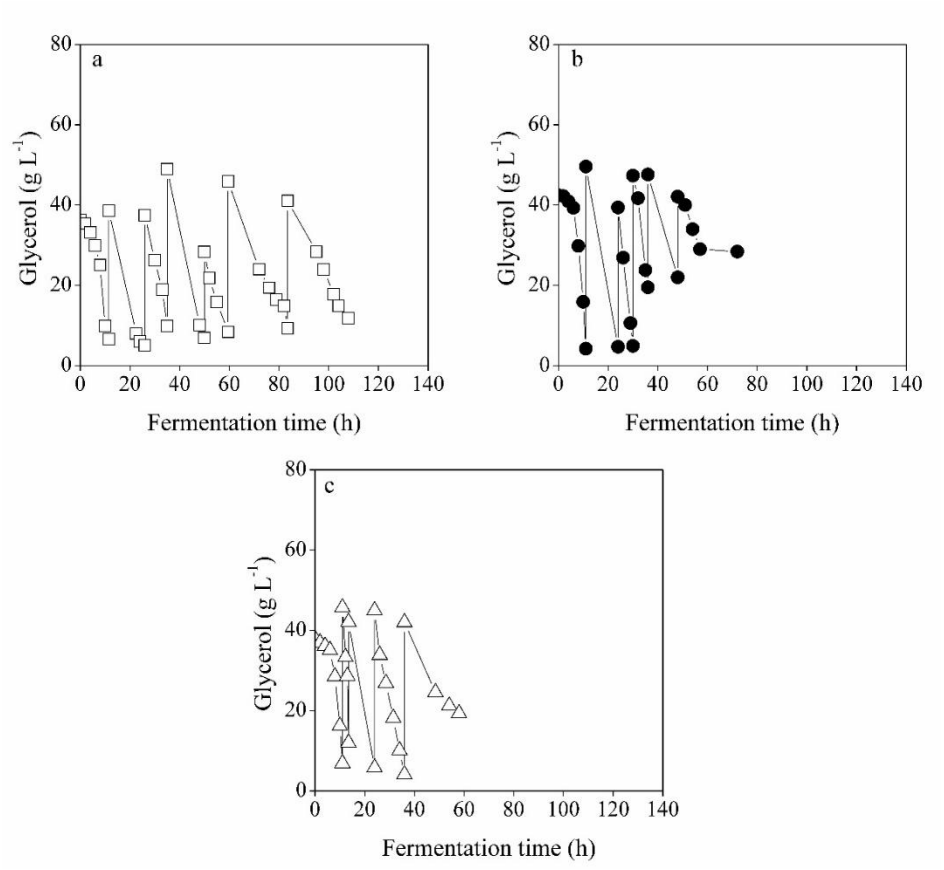


**Figure 6.10** Glycerol consumption in fed-batch fermentations of *K. michiganensis* cultivated on crude glycerol 90-So:10-Tf at $k_La$ value of 62 $h^{-1}$ (a) $k_La$ value of 77 $h^{-1}$ (b) and $k_La$ value of 83 $h^{-1}$ (c)

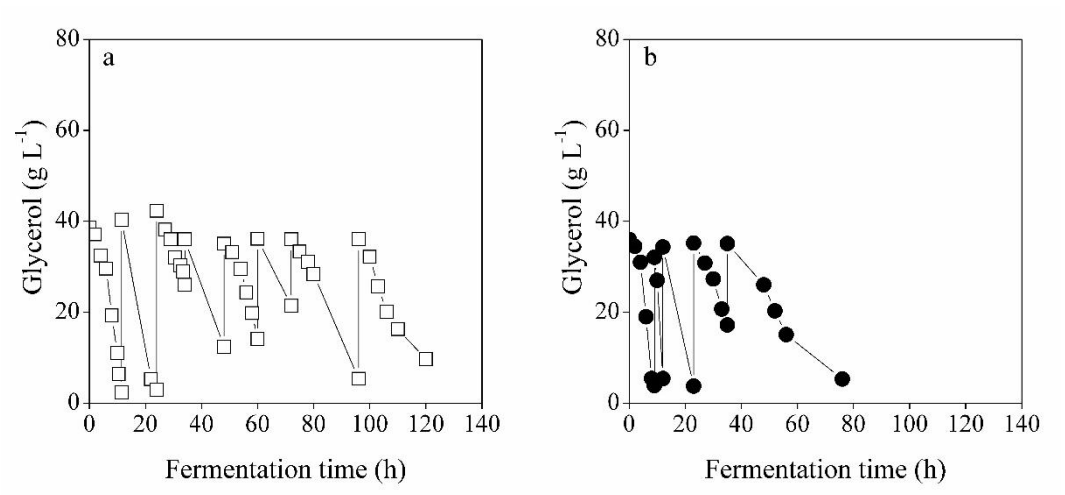


**Figure 6.11** Glycerol consumption in fed-batch fermentations of *K. michiganensis* cultivated on crude glycerol 65-So:35-Tf at $k_La$ value of 62 $h^{-1}$ (a) and $k_La$ value of 77 $h^{-1}$ (b)

# Chapter 6

The presence of impurities in crude glycerol, the origin of the feedstock used in biodiesel production along with oxygen supply affect significantlly bacterial growth, BDO production, yield and productivity. Table 6.4 summarises the final results of *K. michiganensis* fed-batch fermentations conducted with different types of crude glycerol and $k_La$ values. Future study should focus on the impact of impurities present in glycerol on BDO biosynthesis so as to increase further BDO production.

**Table 6.4** Fermentation efficiency of *K. michiganensis* using different types of crude glycerol in fed-batch cultures carried out with different $k_La$ values

| $k_La$ ($h^{-1}$) | $\mu_{max}$ ($h^{-1}$) | DCW ($g\ L^{-1}$) | BDO ($g\ L^{-1}$) | Yield ($g\ g^{-1}$) | Productivity ($g\ L^{-1}\ h^{-1}$) | Lactic acid ($g\ L^{-1}$) | Formic acid ($g\ L^{-1}$) | Acetic acid ($g\ L^{-1}$) | Ethanol ($g\ L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | *Pure* | | | | |
| 46-62 | 0.33 | 6.6 | 107.9 | 0.44 | 0.90 | 11.0 | 0 | 0.8 | 0 |
| 62 | 0.38 | 10.2 | 93.5 | 0.31 | 0.71 | 13.1 | 1.6 | 0 | 0 |
| | | | | | 100-So | | | | |
| 46-62 | 0.29 | 5.5 | 61.4 | 0.37 | 0.63 | 11.2 | 3.1 | 0 | 1.6 |
| 62 | 0.36 | 6.3 | 87.6 | 0.46 | 0.73 | 0 | 2.9 | 0 | 0 |
| 77 | 0.40 | 11.0 | 76.1 | 0.41 | 1.38 | 0 | 3.7 | 2.2 | 0 |
| 83 | 0.41 | 11.1 | 63.7 | 0.30 | 1.34 | 0 | 4.8 | 1.0 | 1.1 |
| | | | | | 90-So:10-Tf | | | | |
| 62 | 0.44 | 10.6 | 75.0 | 0.35 | 0.74 | 1.6 | 5.5 | 12.1 | 0 |
| 77 | 0.45 | 10.9 | 58.0 | 0.28 | 1.02 | 0 | 0 | 11.3 | 4.1 |
| 83 | 0.48 | 12.3 | 52.4 | 0.36 | 1.08 | 0 | 0 | 4.3 | 0 |
| | | | | | 65-So:35-Tf | | | | |
| 62 | 0.38 | 6.6 | 54.2 | 0.27 | 0.49 | 2.0 | 0 | 11.0 | 2.0 |
| 77 | 0.30 | 3.2 | 35.9 | 0.28 | 0.64 | 0 | 0 | 1.0 | 0 |

Table 6.5 presents the fermentation efficiency reported by different literature-cited publications on BDO production using glycerol as carbon source. Significant improvements have been achieved for enhanced BDO production from glycerol by optimizing operating conditions, employing fed-batch operation mode and developing mutant strains. Petrov and Petrova (2010) developed a forced pH fluctuation process by consecutive raising of ΔpH value at 1 every 12 h

for BDO production using pure glycerol resulting in the highest reported conversion yield. The BDO concentration reached 70 g $L^{-1}$ with a conversion yield of 0.53 g $g^{-1}$ and productivity of 0.47 g $L^{-1}$ $h^{-1}$ (Petrov and Petrova, 2010). High BDO production efficiency using biodiesel-derived glycerol by wild type strain was reported by Yang et al. (2013). *Bacillus amyloliquefaciens* strain was reported to produce 102.3 g $L^{-1}$ of BDO concentration without by-product formation in fed-batch fermentation mode using crude glycerol stream (88% purity, w $w^{-1}$) containing chlorides (4%, w $w^{-1}$) and supplemented with molasses as co-substrate (Yang, 2013). The most efficient BDO producers are metabolically engineered strains developed by simultaneous expression of the genes involved in BDO production and inactivation of genes involved in a competitive pathway. Kim et al. (2017) demonstrated BDO production from crude glycerol using a *Raoultella ornithinolytica* strain with overexpression of *bud*ABC genes. Under optimized conditions, 65.4 g $L^{-1}$ of BDO concentration was produced using crude glycerol (68.3% purity, w $w^{-1}$) containing sodium (10,137.5 mg $kg^{-1}$). The implementation of pre-treated crude glycerol (70.2% purity, w $w^{-1}$) increased BDO production and yield by 19.4% and 7.7%, respectively (Kim, 2017). An adapted mutant *Klebsiella variicola* SW3 strain displayed high tolerance in crude glycerol (up to 200 g $L^{-1}$) for BDO production (Rahman, 2017). In fed-batch fermentation, *K. variicola* SW3 reached 64.9 g $L^{-1}$ of BDO concentration from crude glycerol (50% purity w $w^{-1}$) showing that the overexpression of GDH significantly increased *bud*B and *bud*C genes (Rahman, 2017). The highest BDO concentration (131.5 g $L^{-1}$) from crude glycerol (81.7% purity w $w^{-1}$) with 2.4% of sodium have been achieved by a metabolically engineered *Klebsiella oxytoca* strain. Deletion of *pdu*C and *ldh*A genes improved BDO production and reduced by-product formation, namely PDO and lactic acid (Cho, 2015). A mixture of L-BDO and meso-BDO in ratio of 0.92:1 was produced by *K. oxytoca* M3 (Cho, 2015). The results presented in Table 6.4 show that the BDO production efficiency achieved by the newly isolated strain of *K. michiganensis* is among the highest achieved in the international literature with wild-type strains.

## 6.5 Conclusions

In this chapter, the evaluation of different glycerols derived from vegetable oil and animal fat feedstocks was studied for BDO production by *K. michiganensis*. Initially, media composition, initial glycerol concentration and nitrogen sources were evaluated in shake flask fermentations. Minimal media and inorganic nitrogen source enhanced BDO production. Initial substrate concentration influences BDO production and metabolites profile. Relatively high BDO

concentration (49.1 g $L^{-1}$) was achieved when an initial glycerol concentration of 135.9 g $L^{-1}$ was used, whereas yield and productivity reached the highest values (0.40 g $g^{-1}$ and 0.41 g $L^{-1}$ $h^{-1}$, respectively) at an initial glycerol concentration of 35.7 g $L^{-1}$. The influence of oxygen supply was evaluated in fed-batch cultures using various $k_La$ values. In all types of crude glycerol, the production of BDO and conversion yield were increased at low $k_La$ values, while maximum values of productivity were reached with increasing $k_La$. The highest BDO production (87.6 g $L^{-1}$) achieved at low $k_La$ values (62 $h^{-1}$) when glycerol generated from soybean oil biodiesel process was used. High values of productivity (around 1.3 g $L^{-1}$ $h^{-1}$) were achieved at high oxygen supply ($k_La$ values of 77 and 83 $h^{-1}$). Crude glycerols generated from soybean oil and tallow fats biodiesel production resulted in lower BDO production efficiency.

**Table 6.5** BDO production using glycerol as substrate

| Strain | Type of glycerol | BDO ($g\ L^{-1}$) | Yield ($g\ g^{-1}$) | Productivity ($g\ L^{-1}\ h^{-1}$) | By-products | Fermentation mode | References |
|---|---|---|---|---|---|---|---|
| *Klebsiella pneumoniae* G31 | Pure | 70.0 | 0.53 | 0.47 | PDO (10.9) | Fed-batch (initial pH:8, ΔpH:1) | Petrov et al., 2010 |
| | | 54.5 | 0.48 | 0.57 | PDO (17.2) | Fed-batch (initial pH:8, ΔpH:3) | |
| *Klebsiella oxytoca* M1 | Pure | 59.4 | 0.31 | 0.52 | Lactic acid (18.6), PDO (8.9), Ethanol (4) | Fed-batch | Cho et al., 2015a |
| | crude 81.7% w $w^{-1}$ | 73.8 | 0.42 | 0.68 | Lactic acid (9.8), PDO (3.7), Ethanol (1.9) | | |
| *Klebsiella oxytoca* M3 (↑budC) | Pure | 115.0 | 0.39 | 1.01 | PDO (6.9), Ethanol (9.3) | | |
| | crude 81.7% w $w^{-1}$ | 131.5 | 0.44 | 0.84 | Ethanol (1.7) | | |
| *Bacillus amyloliquefaciens* | crude 88% w $w^{-1}$ | 102.3 | 0.44 | 1.16 | | Fed-batch | Yang et al., 2015 |
| *Raoultella ornithinolytica* B6 (↑*bud*ABC) | Pure | 89.5 | 0.41 | 0.75 | Acetoin (9.1) | Fed-batch | Kim et al., 2017 |
| | crude 68.3% w $w^{-1}$ | 65.4 | 0.39 | 0.76 | Acetoin (11.5) | | |
| | pre-treated crude 70.23% w $w^{-1}$ | 78.1 | 0.42 | 0.62 | Acetoin (8.8) | | |
| *Klebsiella variicola* SRP3 | pure | 59.3 | 0.59 | | Acetoin (8.1), PDO (3.1) | Fed-batch (shake flasks) | Rahman et al., 2017 |
| | crude 50% w $w^{-1}$ | 43.8 | 0.57 | | Acetoin (5.7), PDO (4.8) | | |
| *Klebsiella variicola* SW3 (↑GDH) | pure | 82.5 | 0.62 | | Acetoin (5.9), PDO (2.4) | | |
| | crude 50% w $w^{-1}$ | 64.9 | 0.63 | | Acetoin (6.3), PDO (2.9) | | |

# Chapter 7

# Bioprocess development for D-butanediol and acetoin production using very high polarity cane sugar and sugarcane molasses by a *Bacillus amyloliquefaciens*

# Chapter 7

## 7.1 Introduction

Highly efficient 2,3-butanediol (BDO) producer strains are classified as pathogenic or opportunistic pathogens (genera of risk group 2). *Bacillus* species, like *B. licheniformis*, *B. amyloliquefaciens* and *B. subtilis* are microorganisms with high biotechnological interest for BDO and acetoin fermentation due to their GRAS status. *Bacillus* generate mainly D-BDO and low amounts of meso-BDO, while acetoin is produced as precursor of BDO fermentation. The existence of various 2,3-butanediol dehydrogenases (BDHs) differing in their stereo specificities in native BDO producers leads to mixed formation of acetoin and BDO stereoisomers (Jia et al., 2017; He et al., 2018). Culture conditions including pH, temperature and oxygen supply as long as substrate employed influence the ratio between acetoin and D-BDO. Additionally, fermentation media and type of nitrogen source affect their biosynthesis (Häßler et al., 2012; Okonkwo et al., 2017b).

One key aspects in the fermentation process is the development of a cost-effective medium which lead to maximum product yield. The valorisation of by-product streams, instead of commercial substrates, could provide an effective approach for BDO production (Kallbach et al., 2017; Li et al., 2013; Qiu et al., 2016). Molasses, a co-product from sugar industry containing high levels of fermentative sugars, inorganic salts, proteins and vitamins, can be potentially utilised as carbon source for BDO and acetoin production (Dai et al., 2015; Deshmukh et al., 2015; Sikora et al., 2015). High concentrations could be toxic or inhibitory to cells hence a critical initial concentration should be evaluated for fermentation process.

The main objective of this study was the evaluation of the newly isolated GRAS strain *B. amyloliquefaciens* for the production of D-BDO and acetoin. The potential development of a bioprocess for the production of either D-BDO or acetoin has been also investigated in batch and fed-batch cultures. Fermentation conditions were evaluated in shake flask and batch bioreactor fermentations using very high polarity (VHP) cane sugar and sugarcane molasses. Fed-batch fermentations were subsequently carried out using VHP cane sugar and sugarcane molasses with different feeding solutions to evaluate the high potential of this strain for the production of either D-BDO or acetoin, especially when molasses was used.

## 7.2 Characterization of VHP cane sugar and sugarcane molasses

The VHP cane sugar has a sucrose content of 99.0% (w $w^{-1}$). Sugarcane molasses contains about (w $w^{-1}$) 43.5% sucrose, 7.3% glucose, 6.3% fructose, 3.2% protein (expressed as total Kjeldahl nitrogen × 6.25), 29.7% moisture and 70.4% solids. The elemental composition of VHP cane sugar and sugarcane molasses is presented in Table 7.1.

**Table 7.1** Elemental composition of VHP cane sugar and sugarcane molasses

| Elements | VHP cane sugar (ppm) | Sugarcane molasses (ppm) |
|---|---|---|
| Ca | 0.034 | 6.67 |
| S | 0.027 | 5.23 |
| Mg | 0.014 | 4.70 |
| K | 0.052 | 1.75 |
| P | 0.002 | 0.21 |
| Fe | 0.001 | 0.12 |
| Mn | <0.0005 | 0.03 |
| Zn | 0.0002 | 0.003 |
| Co | <0.0005 | <0.0005 |

## 7.3 Effect of fermentative parameters on BDO production

### 7.3.1 Evaluation of the initial VHP cane sugar concentration

The effect of substrate concentration on the synthesis of BDO and acetoin was investigated by varying initial VHP cane sugar concentration from 30 to 220 g $L^{-1}$. The final concentration of both D-BDO and acetoin increased from 11.3 to 83.3 g $L^{-1}$with increasing VHP cane sugar concentration (Table 7.2). The highest total sugar to total metabolite conversion yield (0.46 g $g^{-1}$) and productivity (0.73 g $L^{-1}$ $h^{-1}$) were achieved when the initial cane sugar concentration was 90.9 g $L^{-1}$. Higher initial VHP cane sugar concentration resulted in gradually reducing values of yield and productivity. DCW reached the highest value (7.6 g $L^{-1}$) at initial VHP cane sugar concentration of 60.9 g $L^{-1}$, while it was gradually reduced with increasing initial sugar concentration. Generally, strains belonging to the *Bacillus* genus tolerate high initial sugar concentrations. The highest yield and productivity achieved by *B. amyloliquefaciens* were observed at initial glucose concentration of 120 g $L^{-1}$ (Yang et al., 2011). The optimum range of glucose for *B. licheniformis* was 54 – 125 g $L^{-1}$ (Li et al., 2013).

The ratio of D-BDO to acetoin was also affected by the initial VHP cane sugar concentration (Table 7.2). A decreasing trend of D-BDO to acetoin ratio was observed with increasing sugar concentration. In particular, the percentage of D-BDO as related to the total BDO and acetoin production was reduced from 94.9% to 56.2% with increasing VHP cane sugar concentration (31.1 - 223.4 g $L^{-1}$). This phenomenon could be explained by the increasing accumulation of D-BDO that leads to conversion of D-BDO to acetoin in order to ameliorate the toxic effect of D-BDO on bacterial cells. Okonkwo et al. (2017a) reported that D-BDO concentrations higher than 48 g $L^{-1}$ cause inhibition to *P. polymyxa* cell growth and conversion to acetoin is observed to alleviate toxicity levels. Considering the yield, productivity and DCW achieved in this set of experiments, subsequent fermentations were carried out with initial VHP cane sugar concentration within the range 60 - 90 g $L^{-1}$.

**Table 7.2** Effect of initial VHP cane sugar concentration of BDO production

| Initial VHP cane sugar conc. (g $L^{-1}$) | DCW (g $L^{-1}$) | BDO[1]-acetoin (g $L^{-1}$) | Yield[2] BDO[1]-acetoin (g $g^{-1}$) | Productivity BDO[1]-acetoin (g $L^{-1}$ $h^{-1}$) | D-BDO[3] (%) |
|---|---|---|---|---|---|
| 31.1 ± 0.19 | 3.3 ± 0.20 | 11.3 ± 0.48 | 0.36 ± 0.02 | 0.47 ± 0.02 | 94.9 |
| 60.9 ± 0.23 | 7.6 ± 0.30 | 27.9 ± 0.12 | 0.46 ± 0.00 | 0.65 ± 0.00 | 77.4 |
| 90.9 ± 1.83 | 7.0 ± 0.05 | 41.5 ± 0.24 | 0.46 ± 0.01 | 0.73 ± 0.00 | 75.9 |
| 111.7 ± 1.33 | 6.0 ± 0.06 | 50.1 ± 0.03 | 0.45 ± 0.01 | 0.42 ± 0.00 | 66.7 |
| 152.6 ± 1.45 | 5.6 ± 0.14 | 62.0 ± 2.54 | 0.41 ± 0.01 | 0.43 ± 0.02 | 67.3 |
| 166.8 ± 0.51 | 5.3 ± 0.19 | 72.7 ± 0.14 | 0.44 ± 0.00 | 0.43 ± 0.00 | 68.5 |
| 193.4 ± 0.86 | 4.9 ± 0.19 | 80.9 ± 1.40 | 0.42 ± 0.01 | 0.40 ± 0.01 | 64.7 |
| 223.4 ± 0.76 | 4.4 ± 0.15 | 83.3 ± 2.06 | 0.37 ± 0.00 | 0.41 ± 0.01 | 56.2 |

[1] D-BDO

[2] Yield was calculated based on the initial VHP cane sugar concentration

[3] Percentage of D-BDO as related to total D-BDO and acetoin produced

### 7.3.2 Effect on nitrogen source

Based on previous literature-cited studies, complex organic nitrogen sources are beneficial for cell growth and production of BDO and acetoin (Li et al., 2013; Tian et al., 2016). Yeast extract is typically used as nitrogen source for BDO by *Bacillus* species, while corn steep liquor (CSL) has been evaluated as alternative organic nitrogen source due to its relatively low cost.

Figure 7.1 presents D-BDO and acetoin production as well as yield and productivity for both D-BDO and acetoin production achieved during shake flask fermentations carried out with different organic nitrogen sources. Yeast extract resulted in the highest fermentation efficiency than peptone and CSL. In particular, 15 g $L^{-1}$ of yeast extract concentration resulted in 27.4 g $L^{-1}$ of BDO and acetoin concentration with conversion yield of 0.45 g $g^{-1}$. Lower yeast extract concentration led to decreased accumulation of BDO and acetoin and conversion yield. Moreover, the use of yeast extract as nitrogen source led to increased ratio of D-BDO to acetoin, while CSL favor the production of acetoin. Thus, 15 g $L^{-1}$ yeast extract concentration was used in all subsequent fermentations carried out in this study.

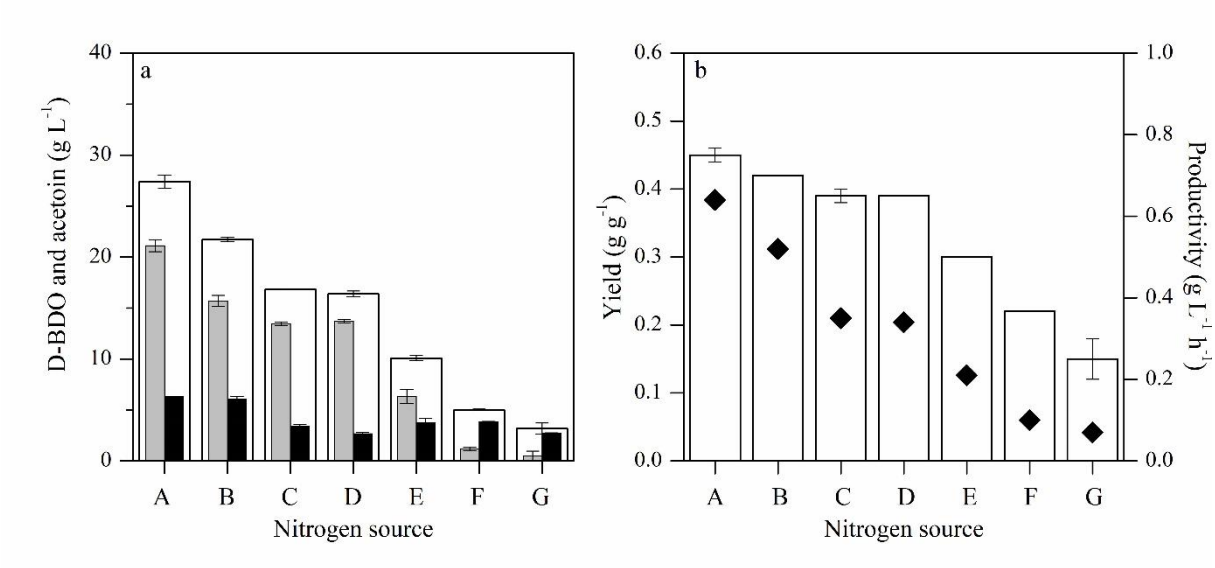


**Figure 7.1** Shake flask fermentations carried out with different organic nitrogen sources at 30 ˚C, initial pH of 6.8 and inoculum size of 10% (v $v^{-1}$). Figure 1a: Total D-BDO and acetoin (white column), D-BDO (grey column) and acetoin (black column). Figure 1b: total consumed sugar to D-BDO and acetoin conversion yield (white column) and productivity for both metabolic products (♦). The letters in the x-axes stand for: (A) 15 g $L^{-1}$ yeast extract, (B) 10 g $L^{-1}$ yeast extract, (C) 5 g $L^{-1}$ yeast extract, (D) 10 g $L^{-1}$ peptone, (E) 5 g $L^{-1}$ peptone, (F) 10 g $L^{-1}$ CSL and (G) 5 g $L^{-1}$ CSL. The error bars indicate the standard deviations of two parallel replicates.

### 7.3.3 Effect of temperature on BDO and acetoin production

Temperature is another important parameter that affects BDO and acetoin formation. The optimum temperature for BDO production by *Bacilliaceae* strains is usually in the range of 34 to 37 ˚C (Perego et al., 2003; Yang et al., 2011). The optimal temperature for D-BDO and

acetoin production by *B. amyloliquefaciens* was identified in shake flask cultures carried out at different temperatures (Figure 7.2). The combined D-BDO and acetoin concentration, yield and productivity were gradually increased when the temperature was increased from 27 to 40 ˚C (Figure 2). The highest D-BDO and acetoin concentration (43.8 g $L^{-1}$) with conversion yield of 0.50 g $g^{-1}$ was obtained at 40 ˚C. The lowest metabolite production was observed at 27 ˚C. Based on the results presented in Figure 7.2, all subsequent fermentations were carried out at 40 ˚C.

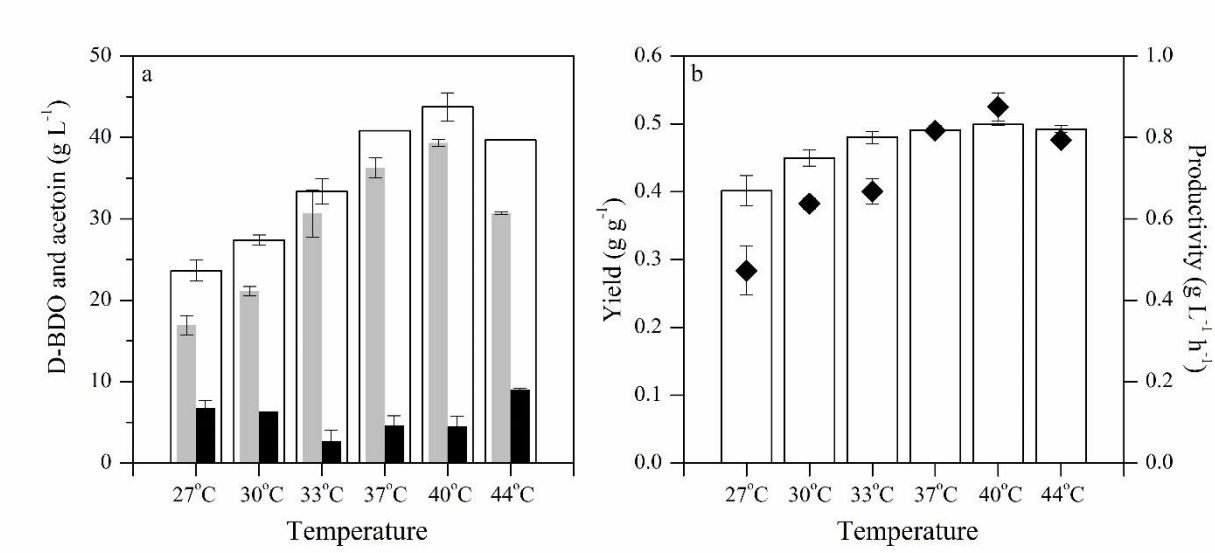


**Figure 7.2** Effect of temperature on the fermentation efficiency of *B. amyloliquefaciens* for the production of D-BDO and acetoin. Figure 2a: Total D-BDO and acetoin (white column), D-BDO (grey column) and acetoin (black column). Figure 2b: total consumed sugar to D-BDO and acetoin conversion yield (white column) and productivity for both metabolic products (♦).

### 7.3.4 Effect of inoculum size

Inoculum size has been reported to improve BDO productivity and yield (Perego et al., 2003; Okonkwo et al., 2017b). To determine the optimum inoculum size, a set of shake flask fermentations was carried out with different inoculum sizes (Figure 7.3). Similar D-BDO and acetoin concentrations (around 40 g $L^{-1}$) were achieved with inoculum sizes in the range of 5 – 20% (v $v^{-1}$). The highest yield was achieved at inoculum size of 15% and for this reason this inoculum size was used in all subsequent fermentations.

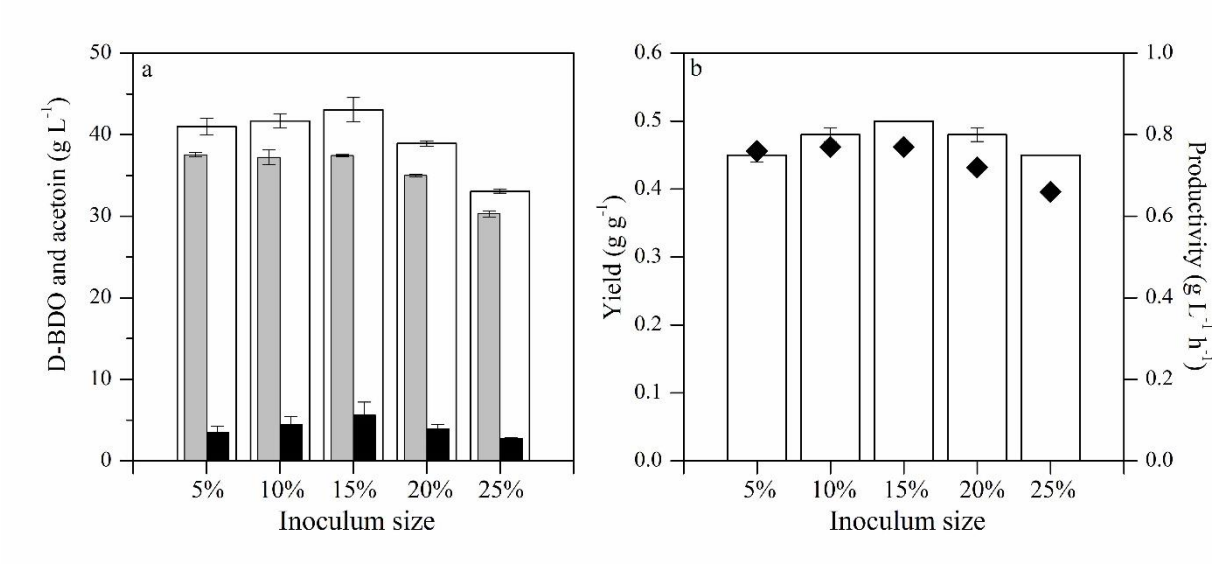


**Figure 7.3** Effect of inoculum size on the fermentation efficiency of *B. amyloliquefaciens* for the production of D-BDO and acetoin. Figure 3a: Total D-BDO and acetoin (white column), D-BDO (grey column) and acetoin (black column). Figure 3b: total consumed sugar to D-BDO and acetoin conversion yield (white column) and productivity for both metabolic products (♦).

### 7.3.5 Evaluation of oxygen supply on BDO synthesis

The effect of oxygen supply on D-BDO and acetoin production was investigated in batch bioreactor cultures carried out at various $k_La$ values ranging from 17 to 132 $h^{-1}$ (Figure 7.4). At $k_La$ values within the range of 17 – 49 $h^{-1}$, D-BDO and lactic acid were the main metabolic products. The highest D-BDO concentration (28 g $L^{-1}$) was achieved at 30 $h^{-1}$, while the highest sugar to D-BDO conversion yield (0.43 g $g^{-1}$) was achieved at $k_La$ value of 49 $h^{-1}$. Similar productivities (0.93 g $L^{-1}$ $h^{-1}$) for D-BDO were achieved at $k_La$ values of 36 $h^{-1}$ and 49 $h^{-1}$. When $k_La$ values higher than 49 $h^{-1}$ were used, the production of D-BDO was reduced and acetoin gradually became the predominant metabolic product. It was also observed that lactic acid and acetic acid production was not observed at $k_La$ values higher than 76 $h^{-1}$. Acetoin reached 25.6 g $L^{-1}$ at $k_La$ value of 104 $h^{-1}$ with sugar to acetoin conversion yield of 0.40 g $g^{-1}$ and productivity of 1.42 g $L^{-1}$ $h^{-1}$. The main conclusion drawn from this set of fermentations is that higher oxygen supply increased acetoin production and reduced D-BDO as well as organic acid formation.

The results of this study are consistent with previous literature-cited studies (Zhang et al., 2013b; Fu et al., 2016; Tian et al., 2016). Häßler et al. (2012) reported that high yield of BDO and low acetoin and by-product accumulation can be achieved at $k_La$ value of 30 $h^{-1}$. The effect of agitation on BDO and acetoin production via *B. amyloliquefaciens* fermentation was studied

by Zhang et al. (2013). Under constant agitation rate of 300 rpm, a BDO concentration of 58.1 g $L^{-1}$ was achieved, while an acetoin concentration of 51.2 g $L^{-1}$ was reached by manipulating the carbon flux distribution via a two-stage agitation speed control strategy. Generally, microorganisms employed for BDO production via fermentation can obtain energy by both respiration and fermentation pathways. The distribution of metabolites depends on the relative activities of each pathway. Minimizing the oxygen supply enhances BDO yield due to limitation of the respiration pathway, however growth is lower in limited oxygen availability and hence productivity is reduced (Yang et al., 2011; Yang et al., 2015). Under high oxygen supply conditions, more NADH is oxidized to $NAD^+$ through oxidative phosphorylation, thereby limiting available NADH for the reduction of acetoin to BDO which leads to accumulation of acetoin (Zhang et al., 2013b). Moreover, the key enzymes are influenced by the availability of oxygen. Under not fully aerobic conditions, pyruvate is converted into lactate, formate and $\alpha$-acetolactate that leads to BDO production, while under aerobic conditions $\alpha$-acetolactate synthase (ALS), the enzyme that leads to BDO formation, is inhibited (Ji et al., 2011).

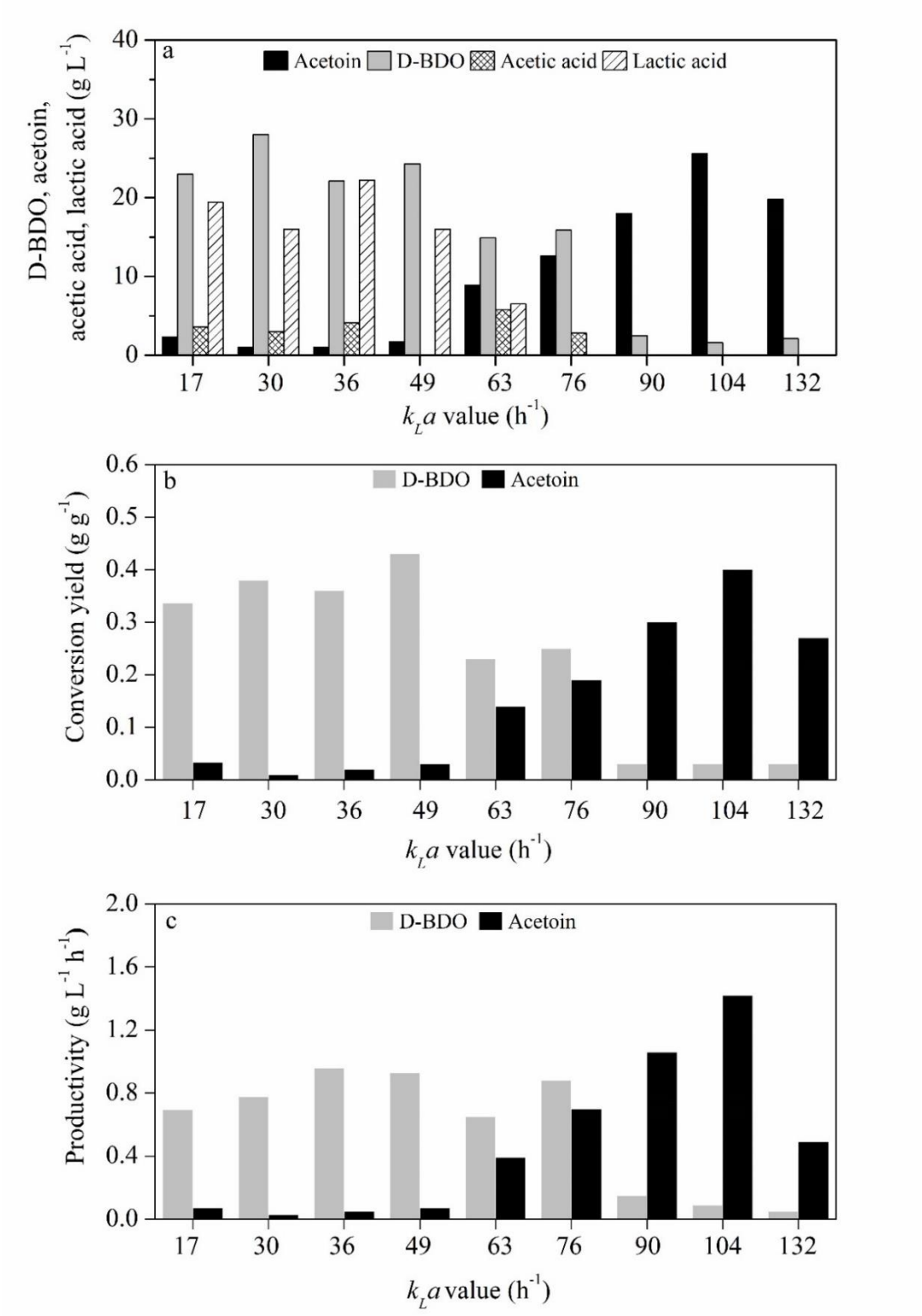


**Figure 7.4** Effect of $k_La$ value during batch bioreactor fermentations on (a) metabolites formation, (b) sugar yield to either D-BDO or acetoin conversion yield and (c) productivity of either D-BDO or acetoin

### 7.3.6 Effect of pH on BDO production

The effect of pH on D-BDO production was investigated in batch bioreactor cultures at $k_La$ value of 49 $h^{-1}$ using VHP can sugar (Table 7.3). The highest D-BDO concentration (32.3 g $L^{-1}$) was observed at pH value of 6.0. Lactic acid production was mainly observed at pH value of 6.8 leading to reduced D-BDO yield. The highest DCW was observed at pH value of 6.0, while lower values were observed at pH values of 5.5 (5.7 g $L^{-1}$) and 6.4 (6.3 g $L^{-1}$).

**Table 7.3** Effect of pH on D-BDO production in batch bioreactor cultures carried out at $k_La$ value of 49 $h^{-1}$

| pH | DCW (g $L^{-1}$) | D-BDO (g $L^{-1}$) | Yield D-BDO (g $g^{-1}$) | Productivity D-BDO (g $L^{-1}$ $h^{-1}$) | Acetoin (g $L^{-1}$) | Lactic acid (g $L^{-1}$) | Acetic acid (g $L^{-1}$) |
|---|---|---|---|---|---|---|---|
| 5.5 | 5.7 | 24.9 | 0.37 | 1.00 | 2.9 | 0.0 | 0.0 |
| 6.0 | 7.8 | 32.3 | 0.47 | 1.08 | 5.5 | 0.0 | 0.0 |
| 6.4 | 6.3 | 27.5 | 0.44 | 0.90 | 8.5 | 0.0 | 0.4 |
| 6.8 | 6.4 | 24.3 | 0.43 | 0.93 | 1.7 | 16.0 | 0.0 |

In the case of acetoin production in batch bioreactor cultures carried out at $k_La$ value of 104 $h^{-1}$, the highest acetoin concentration (27.3 g $L^{-1}$) and yield (0.40 g $g^{-1}$) were also achieved at pH value of 6 (Table 7.4). Based on the results presented in Tables 7.3 and 7.4, the pH value of 6.0 was used in all subsequent fermentations for D-BDO and acetoin production.

**Table 7.4** Effect of pH on acetoin production in batch bioreactor cultures carried out at $k_La$ value of 104 $h^{-1}$

| pH | DCW (g $L^{-1}$) | Acetoin (g $L^{-1}$) | Yield (g $g^{-1}$) | Productivity (g $L^{-1}$ $h^{-1}$) | D-BDO (g $L^{-1}$) |
|---|---|---|---|---|---|
| 5.5 | 5.2 | 24.3 | 0.34 | 0.90 | 1.4 |
| 6.0 | 6.0 | 27.3 | 0.40 | 1.24 | 0.9 |
| 6.4 | 5.7 | 24.4 | 0.36 | 1.18 | 1.5 |
| 6.8 | 5.1 | 25.6 | 0.35 | 1.22 | 1.6 |

The optimal pH varies depending on the strain used. Yang et al. (2011) reported the effect of pH on BDO production by *B. amyloliquefaciens* with pH values lower than 5.5 resulting in significant reduction in BDO production efficiency, while the highest BDO concentration, yield

and productivity were obtained at pH value of 6.5 (Lee et al., 2017). Garg and Jain (1995) reported that alkaline conditions favor organic acid production, whereas under acidic conditions BDO production is favored. It has been reported that pH values higher than 6 cause a decline in the activity of *α*-acetolactate synthase in BDO production by *K. pneumonia* (Stormer et al., 1986). Voloch et al. (1985) showed that pH values within the range of 5 to 6 is optimum for BDO production by *K. oxytoca*, while pH value of 6 is beneficial for *E. aerogenes* (Converti et al., 2003; Perego et al., 2000).

### 7.4 Evaluation of sugarcane molasses as substrate

Shake flask fermentations were carried out in order to evaluate the effect of different initial sugarcane molasses concentration (63 – 296 g $L^{-1}$) on combined BDO and acetoin production (Table 7.5). The bacterial strain *B. amyloliquefaciens* demonstrated high tolerance to increased initial molasses concentrations. Increasing initial molasses concentration up to 269 g $L^{-1}$ resulted in enhanced BDO and acetoin concentrations (up to 74.8 g $L^{-1}$) and corresponding yield (up to 0.49 g $g^{-1}$). However, employing high initial molasses concentration led to reduced productivity. Initial molasses concentration between 63 to 125 g $L^{-1}$ led to lower acetoin formation and higher percentage of D-BDO (77.1 – 84%) in the metabolite mixture. Higher concentrations of acetoin were obtained at molasses concentration higher than 159 g $L^{-1}$, though in all cases D-BDO was higher than 70.2%.

**Table 7.5** Effect of initial sugarcane molasses concentration on combined D-BDO and acetoin production in shake flask cultures

| Initial sugarcane molasses (g $L^{-1}$) | Initial sugars (g $L^{-1}$) | BDO[1]-acetoin (g $L^{-1}$) | Yield[2] BDO[1]-acetoin (g $g^{-1}$) | Productivity (g $L^{-1}$ $h^{-1}$) | D-BDO[3] (%) |
|---|---|---|---|---|---|
| 63 | 35.9 ± 0.70 | 13.7 ± 0.26 | 0.38 ± 0.01 | 0.57 ± 0.01 | 84.0 |
| 83 | 47.4 ± 2.20 | 19.0 ± 1.06 | 0.40 ± 0.00 | 0.56 ± 0.03 | 80.5 |
| 125 | 71.3 ± 0.20 | 31.5 ± 0.05 | 0.44 ± 0.00 | 0.58 ± 0.02 | 77.1 |
| 158 | 90.1 ± 1.20 | 40.8 ± 0.44 | 0.45 ± 0.00 | 0.57 ± 0.01 | 74.9 |
| 189 | 108.1 ± 0.20 | 49.4 ± 0.92 | 0.46 ± 0.01 | 0.41 ± 0.01 | 72.5 |
| 213 | 121.5 ± 2.90 | 58.4 ± 1.11 | 0.48 ± 0.02 | 0.41 ± 0.01 | 75.1 |
| 269 | 153.5 ± 0.90 | 74.8 ± 0.01 | 0.49 ± 0.00 | 0.45 ± 0.00 | 74.3 |
| 278 | 158.9 ± 0.76 | 71.9 ± 0.84 | 0.45 ± 0.01 | 0.43 ± 0.00 | 74.2 |
| 296 | 168.8 ± 1.10 | 73.8 ± 0.69 | 0.44 ± 0.00 | 0.35 ± 0.00 | 70.2 |

[1] D-BDO

[2] Yield was calculated based on initial total sugar concentration

[3] Percentage of D-BDO as related to total D-BDO and acetoin produced

Shake flask fermentations carried out with VHP cane sugar (Table 7.2) and molasses (Table 7.5) show that high concentrations of the latter enhances D-BDO concentration and yield. For instance, an initial VHP cane sugar concentration of 152.6 g $L^{-1}$ led to D-BDO and acetoin concentration of 62 g $L^{-1}$ with yield of 0.41 g $g^{-1}$, whereas an initial molasses concentration of 269 g $L^{-1}$ (initial total sugars of 153.5 g $L^{-1}$) led to 17% higher D-BDO and acetoin concentration and 16.3% higher yield. The presence of metal ions and vitamins in molasses may improve BDO and acetoin production in shake flask cultures.

## 7.5 Fed-batch fermentations

In order to increase further the production of BDO and acetoin, fed-batch fermentations were conducted at optimal fermentation conditions identified in previous experiments. Two fed-batch fermentations were conducted with VHP cane sugar as carbon source at the beginning of fermentation using as feeding solutions either a mixture of glucose and fructose (Figure 7.5) or VHP cane sugar (Figure 7.6). Two fed-batch fermentations were carried out with molasses at the beginning of fermentation using as feeding solutions either VHP cane sugar (Figure 7.7) or diluted sugarcane molasses (Figure 7.8).

A similar total BDO and acetoin concentration (127 g $L^{-1}$) was achieved in fed-batch fermentations carried out with VHP cane sugar using either mixture of glucose and fructose (Figures 7.5) or VHP cane sugar (Figure 7.6) as feeding solution. In both cases, conversion yield and productivity of BDO and acetoin were 0.50 g $g^{-1}$ and 1.04 g $L^{-1}$ $h^{-1}$ respectively. However, as shown in Figures 5a and 5b, the trend and proportion of D-BDO, acetoin and meso-BDO during the two fermentations varied. Both fermentations can be divided into four phases. Until 22 h, D-BDO was produced as the predominant metabolic product reaching a concentration of around 24 g $L^{-1}$. In the second phase (22 – 70 h), acetoin accumulation was observed in both fermentations. During this second phase, in the fermentation carried out with mixture of glucose and fructose as feeding solution, D-BDO production coincided with acetoin production. However, when VHP cane sugar was used as feeding solution, D-BDO production stopped during the second phase and acetoin concentration was increased from 4.4 g $L^{-1}$ to 46.5 g $L^{-1}$. In the third phase (70 – 100 h), acetoin concentration was gradually decreased with simultaneous production of D-BDO and meso-BDO. In the fourth phase, meso-BDO and D-

BDO production stopped, while acetoin concentration was increased. The final ratio of D-BDO, acetoin and meso-BDO were 0.54:0.19:0.27 and 0.39:0.44:0.17 in the fed-batch fermentations carried out with mixture of glucose and fructose or VHP cane sugar, respectively.

Fed-batch fermentation carried out with molasses and VHP cane sugar as feeding solution resulted in 101.9 g $L^{-1}$ of BDO and acetoin concentration with yield and productivity of 0.42 g $g^{-1}$ and 0.83 g $L^{-1}$ $h^{-1}$, respectively (Figure 7.7). The trend of D-BDO and meso-BDO production was similar with the fermentation that was initiated and supplemented with VHP cane sugar (Figure 7.6). However, when molasses was used at the beginning of fermentation, acetoin production (37.4 g $L^{-1}$) was lower. The ratio of D-BDO, acetoin and meso-BDO in the fermentation presented in Figure 7.6 was 0.42:0.37:0.21.

When molasses was used as feeding medium, total BDO and acetoin production of 67.9 g $L^{-1}$, with D-BDO being the predominant metabolite (72.3% purity), was achieved at 78 h with high yield (0.50 g $g^{-1}$) and productivity (0.87 g $L^{-1}$ $h^{-1}$) (Figure 7.8). The distribution of BDO isomers and acetoin varied during fermentation. D-BDO (48.7 g $L^{-1}$) was predominantly produced until 59 h with yield of 0.4 g $g^{-1}$ and productivity of 0.83 g $L^{-1}$ $h^{-1}$. The purity of D-BDO at 59 h was 87.8% with acetoin being also present (6.7 g $L^{-1}$). After 59 h, D-BDO production remained low, but acetoin production followed an increasing trend to 16 g $L^{-1}$ at 78 h of fermentation. In the final stage (78 – 130 h), D-BDO was converted into acetoin (55.4 g $L^{-1}$) and meso-BDO (4.1 g $L^{-1}$) with the remaining D-BDO being 1.6 g $L^{-1}$. In this way, sugarcane molasses could be used as fermentation medium for the production of either D-BDO or acetoin. Further optimisation is required in order to improve the production efficiency of either metabolite.

Based on the above results, it can be concluded that the substrate used significantly affects the final concentrations and the distribution of acetoin and BDO isomers during fermentation. The elemental composition of VHP cane sugar and sugarcane molasses may alter the activities of the enzymes associated with BDO and acetoin biosynthesis. For instance, significantly higher D-BDO concentration was produced in the fermentation where molasses was used throughout fermentation leading to accumulation of minerals, such as Ca, S and Mg, in the fermentation medium. It has been reported that the enzymes involved in BDO and acetoin biosynthesis are the key factors for the mixed formation of acetoin and BDO stereoisomers. Several enzymes involved in acetoin and BDO isomers formation have been characterized. The regulation and activity of each enzyme is depended on several factors including pH, temperature, substrate and metal ions. The presence of ions could act as inhibitors of specific enzymatic activities. The

BDO dehydrogenases activity from *Corynebacterium crenatum* was increased by $Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$ and $Mn^{2+}$ ions, besides $Cu^{2+}$ that inhibited BDH activity (Zhao et al., 2015). Yu et al. (2015_ reported and characterized the D-BDH from *Rhodococcus erythropolis*. The cation $K^{+}$ enhanced the activity, the cations $Na^{+}$ and $Mg^{2+}$ and $Co^{2+}$ slightly decreased the activity, while the enzyme activity was inhibited by $Ag^{+}$, $Cu^{2+}$ and $Fe^{2+}$. In the case of *Serratia marcescens*, the conversion of 3S-acetoin into meso-BDO could be strongly inhibited by $Fe^{2+}$ and $Fe^{3+}$, while $Mn^{2+}$ could increase meso-BDO production (Zhang et al., 2014).

Two fed-batch fermentations were carried out with VHP cane sugar and sugarcane molasses at $k_La$ value of 104 $h^{-1}$ to evaluate the production of acetoin. However, an increase of viscosity was observed that resulted in low acetoin concentration (32 g $L^{-1}$), yield (0.23 g $g^{-1}$) and productivity (0.67 g $L^{-1}$ $h^{-1}$) when VHP cane sugar was used. Similar results were observed when molasses was used as carbon source. This may be attributed to exopolysaccharides formation that leads to osmotic stress. Further study should be carried out in order to minimise EPS and enhance acetoin production.

Fed-batch fermentations showed that the newly isolated *B. amyloliquefaciens* strain used in this study may reach high BDO and acetoin concentration at high yield resulting from the absence of by-product formation. Table 7.6 presents the fermentation efficiency achieved by various *B. amyloliquefaciens* strains cultivated in different substrates. Yang et al. reported the highest BDO concentration (92.3 g $L^{-1}$) achieved by a *B. amyloliquefaciens* strain isolated from soil when glucose-based media were used in fed-batch cultures (Yang et al., 2011). Luo et al. (2014) reported the highest acetoin concentration (71.5 g $L^{-1}$) achieved by an acetoin tolerant mutant of *B. amyloliquefaciens* cultivated in glucose-based media. These two studies do not mention what is the proportion of D-BDO and meso-BDO in the final BDO concentration. The results achieved in this study are the highest reported by wild-type *B. amyloliquefaciens* strains for the production of either D-BDO or acetoin using only molasses as initial carbon source and feeding medium (Table 7.6). Further optimisation of fermentation conditions is expected to enhance fermentation efficiency.

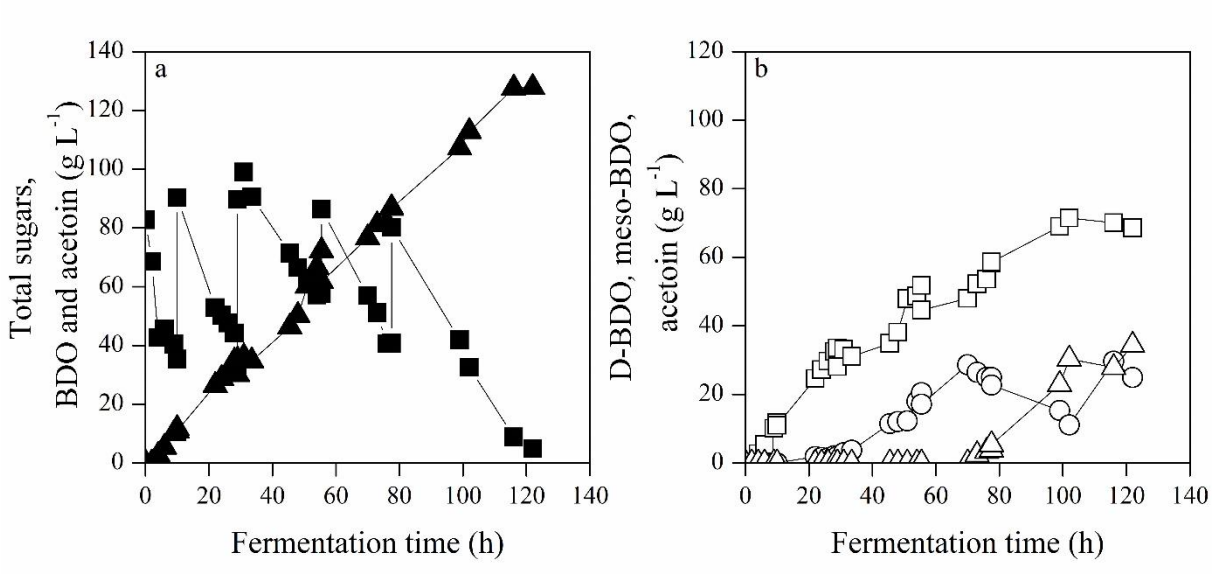


**Figure 7.5** Sugar consumption (■), BDO and acetoin (▲) production(a) and profile change of D-BDO (□), meso-BDO (∆) and acetoin (○) (b) during fed-batch fermentation with VHP cane sugar and mixture of glucose and fructose as feeding solution

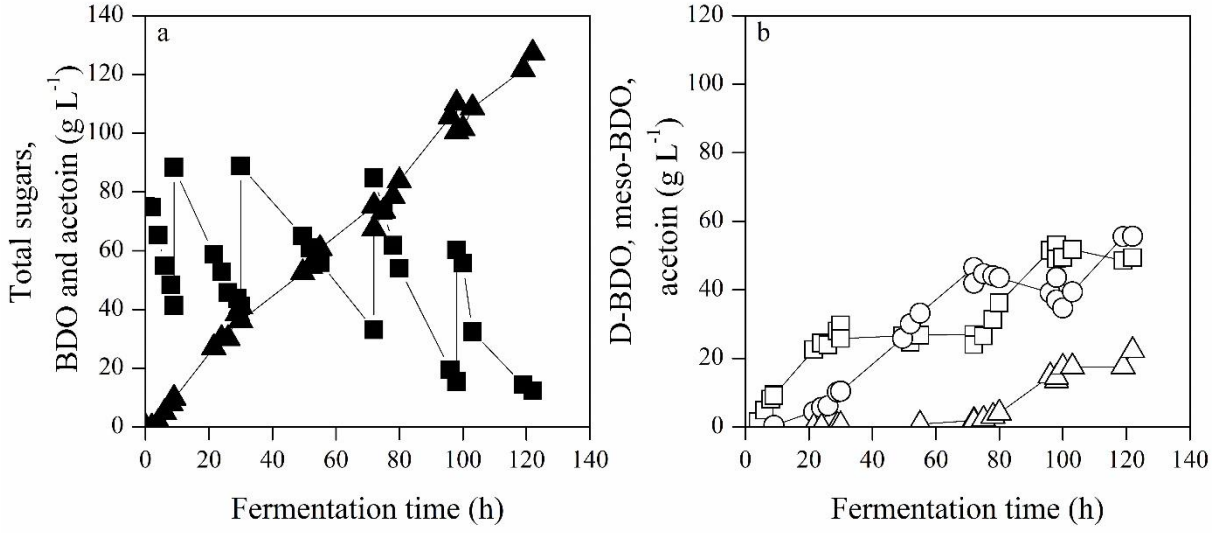


**Figure 7.6** Sugars consumption (■) and total BDO (▲) production(a) and profile change of D-BDO (□), meso-BDO (∆) and acetoin (○) (b) during fed-batch fermentation with VHP cane sugar as sole carbon source

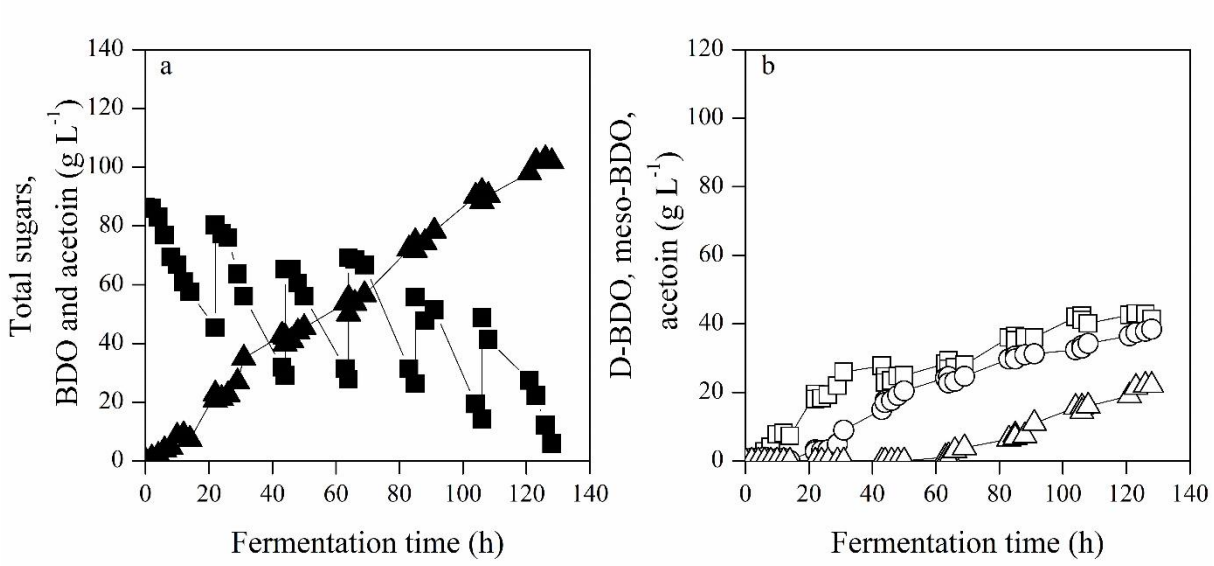


**Figure 7.7** Sugars consumption (■) and total BDO (▲) production (a) and profile change of D-BDO (□), meso-BDO (∆) and acetoin (○) (b) during fed-batch fermentation with sugarcane molasses and VHP cane sugar as feeding solution

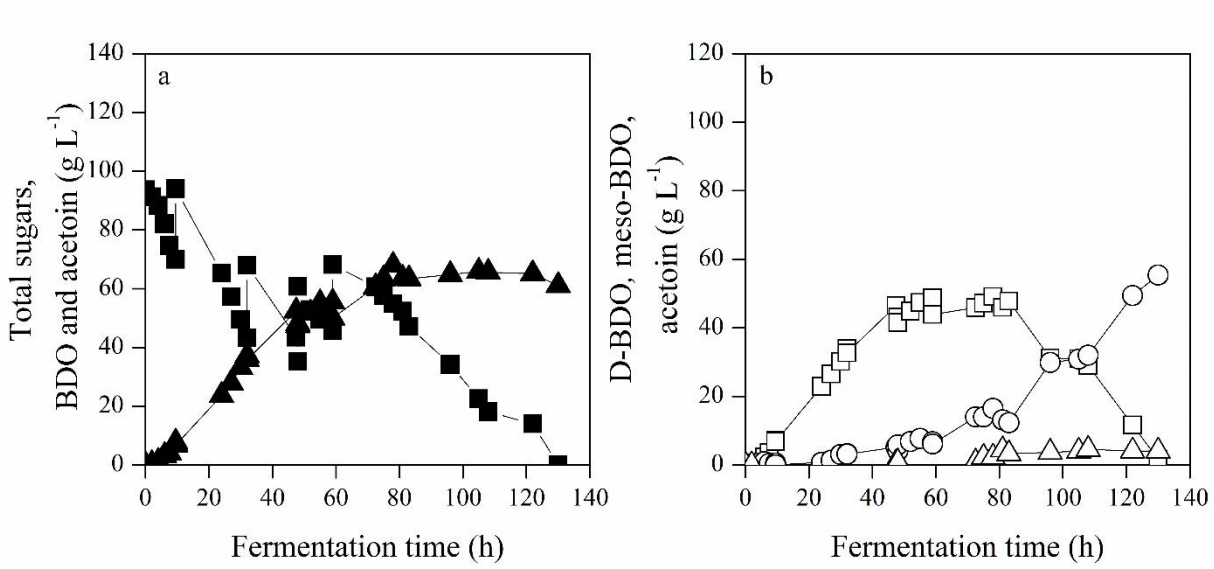


**Figure 7.8** Sugars consumption (■) and total BDO (▲) production(a) and profile change of D-BDO (□), meso-BDO (∆) and acetoin (○) (b) during fed-batch fermentation with sugarcane molasses as sole carbon source

**Table 7.6** BDO and acetoin production by *B. amyloliquefaciens* strains

| Strain | Substrate | Nitrogen source | Oxygen supply | Time (h) | BDO (g $L^{-1}$) | Acetoin (g $L^{-1}$) | Fermentation mode | Ref. |
|---|---|---|---|---|---|---|---|---|
| FMME044 | Glucose | Yeast extract 10 g $L^{-1}$, peptone 10g $L^{-1}$ | Two stage agitation rates (350 rpm until 24 h and then 500 rpm) | 24 | 53.7 | 5.0 | Batch | Zhang et al., 2013b |
| | | | | 48 | ~4 | 51.2 | | |
| isolated from soil | Glucose | CSL 10 g $L^{-1}$, soybean meal 10 g $L^{-1}$ | 300 rpm and 0.3 vvm | 96 | 92.3 | ~22 | Fed-batch | Yang et al., 2011 |
| | | | | 120 | ~69 | 42.0 | | |
| E-11 (acetoin tolerant mutant) | Glucose | Yeast extract 12.5 g $L^{-1}$, peptone 12.5 g $L^{-1}$ | Two stage agitation rates (350 rpm 0-32 h and then switched to 500 rpm) | 32 | 52 | 30.0 | Fed-batch | Luo et al., 2014 |
| | | | | 44 | 11 | 71.5 | | |
| B10-127 | Glucose | CSL 31.9 g $L^{-1}$, soybean meal 22 g $L^{-1}$, ammonium citrate 5.58 g $L^{-1}$ | 350 rpm and 0.66 vvm | 36 | 61.4 | 10.0 | Batch (20-L bioreactor) | Yang et al., 2012 |
| | | | | 44 | ~44 | ~29 | | |
| WX-02 *ΔgldA* | *Miscanthus floridulus* hydrolysate | CSL 33 g $L^{-1}$, $(NH_4)_2SO_4$ 9 g $L^{-1}$ | Two stage agitation rates (400 rpm and 1 vvm until 24 h and then adjusted to 200 rpm and 0.5vvm ) | 192 | 48.5 | 10.8 | Fed-batch | Gao et al., 2018 |
| B10-127 | Crude glycerol (88%, w $w^{-1}$) 80% and molasses 15% | CSL 30 g $L^{-1}$, soybean meal 20 g $L^{-1}$ | / | | 83.3 | | Fed-batch | Yang et al., 2013 |
| 18025 | Sugarcane molasses | Yeast extract 15 g $L^{-1}$ | 400 rpm and 1 vvm ($k_La$ value of 49 $h^{-1}$) | 59 | 48.7 | 6.7 | Fed-batch | This study |
| | | | | 130 | 5.7 | 55.4 | | |

# Chapter 7

## 7.6 Conclusions

The GRAS *B. amyloliquefaciens* strain can efficiently produce D-BDO and acetoin using VHP cane sugar and sugarcane molasses. *B. amyloliquefaciens* has tolerance towards high concentrations of VHP cane sugar and sugarcane molasses. Batch cultures showed that manipulation of $k_La$ values could divert bacterial metabolism towards either D-BDO or acetoin production. At $k_La$ values within the range of 17 – 49 $h^{-1}$, D-BDO and lactic acid were the main metabolic products. $k_La$ values higher than 49 $h^{-1}$ led to gradually increase production of acetoin. At $k_La$ value of 104 $h^{-1}$, acetoin was produced as the main metabolic product with high values of sugar to acetoin conversion yield and productivity.

High BDO and acetoin production was achieved in fed-batch fermentations using VHP cane sugar and sugarcane molasses. The distribution of BDO and acetoin was affected by the carbon source along with feeding solution used. Fed-batch cultures with sugarcane molasses could lead to either D-BDO or acetoin production with negligible by-product formation.

## Chapter 8

# Bakery waste valorisation for D-BDO and acetoin production by *Bacillus amyloliquefaciens*

# Chapter 8

## 8.1 Introduction

Food waste is organic waste discharged from food processing plants and the retail market (Kiran et al., 2015; Lin et al., 2014). A significant amount of bakery waste is generated during the production process and after consumption. Approximately 5% of total bakery products produced are lost or wasted (Chandrasekaran, 2012). The major conventional waste management and valorisation strategies for food wastes include the production of animal feed, fertilizer and biogas through anaerobic digestion (Lin et al., 2014). Alternatively, food wastes could be a valuable resource for the extraction of value added products and the production of chemicals and fuels (Maina et al., 2017).

Bakery wastes, rich in macromolecules, mainly starch and protein, as well as several macronutrients, could be hydrolysed for the formulation of fermentative feedstock that could be further used for the production of value added products. Under this concept, the main objective of this chapter was the valorisation of bakery wastes for the production of a nutrient rich feedstock for the production of D-2,3-butanediol (D-BDO) and acetoin by *Bacillus amyloliquefaciens*. The scaling up of the batch process was also investigated using the volumetric oxygen mass transfer coefficient ($k_La$) as control parameter.

## 8.2 Characterization of bakery wastes

Two different types of bakery wastes were used, namely sugar bakery (Sbw) and bread waste (Bw). Sbw contained different types of bread (sweet bread, whole wheat bread, rye bread, pretzel, and croissant), while Bw contained mainly whole wheat, whole gran rye and five grain (wheat, rye, barley, oats and maize) breads. Both wastes were obtained from a local bread market after the end of their shelf-life. The composition of bakery waste is presented in Table 8.1.

## 8.3 Enzymatic hydrolysis of bakery wastes

Sbw and Bw were initially used in enzymatic hydrolysis using commercial enzyme preparations. The glucose production trend during enzymatic hydrolysis using Sbw and Bw in 10 L reactor is presented in Figure 8.1a. High hydrolytic performance was observed at the beginning of hydrolysis leading to 86.6 g $L^{-1}$ and 123.8 g $L^{-1}$ of glucose concentration from Sbw and Bw, respectively. Glucose concentration was further increased until 18 h of enzymatic reaction. The final hydrolysate of Sbw and Bw contained glucose concentrations of 106 g $L^{-1}$

and 148.6 g $L^{-1}$, respectively. The Sbw contained an initial glucose concentration of 14 g $L^{-1}$, which means that around 92 g $L^{-1}$ glucose concentration were produced during enzymatic hydrolysis. The initial Sbw concentration on dry basis was 240 g $L^{-1}$ corresponding to a hydrolysis yield of higher than 97% considering that the maximum glucose that can be produced from this suspension is 94.5 g $L^{-1}$. The initial Bw concentration on dry basis was 260 g $L^{-1}$ corresponding to a hydrolysis yield of higher than 97% considering that the maximum glucose that can be produced from this suspension is 152.7 g $L^{-1}$. Optimisation of enzyme dosage requirement for hydrolysis was not carried out as the main objective of this study was to evaluate the production of D-BDO and acetoin from bakery wastes.

**Table 8.1** Composition of Sbw and Bw

| Component (w $w^{-1}$, %) | Sbw | Bw |
|---|---|---|
| Moisture | 5.90 | 7.90 |
| Ash (db) | 1.50 | 3.90 |
| Starch (db) | 35.8 | 53.4 |
| Lipids (db) | 3.50 | 3.66 |
| Protein (db) | 9.50 | 12.5 |
| Cellulose (db) | 0.70 | 2.06 |
| Hemicellulose (db) | 2.53 | 9.01 |

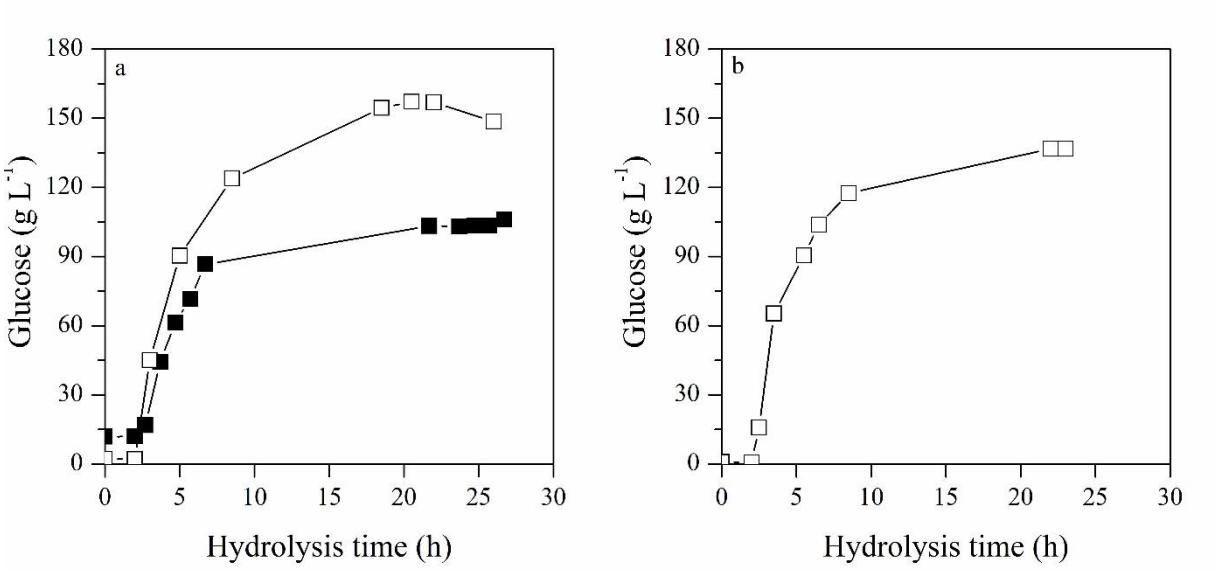


**Figure 8.1** Enzymatic hydrolysis of Sbw (■) and Bw (□) suspensions in 10 L reactor (a) and 50 L reactor (b)

Enzymatic hydrolysis of 245 g $L^{-1}$ Bw suspension was subsequently carried out in 50 L reactor. The glucose production trend during enzymatic hydrolysis in the 50 L reactor is presented in Figure 8.1b. Glucose production of 103.8 g $L^{-1}$ was achieved at 6.5 h and it was further increased to 137 g $L^{-1}$ after 22 h. The starch to glucose conversion yield was slightly lower (around 95%) than the one achieved in 10 L reactor.

The composition of the bakery waste used in this study affects the composition of Sbw and Bw hydrolysates (Table 8.2). The Bw hydrolysate contains higher concentration of $Cl^-$, $Na^+$ and $K^+$ than Sbw hydrolysate. Mineral composition may affect bacterial growth and metabolite production during fermentation.

**Table 8.2** Composition (ppm) of Sbw and Bw hydrolysates

| Hydrolysate | $PO_4^{3}$ | $Cl^-$ | $SO_4^{2-}$ | $Na^+$ | $K^+$ | $Mg^{2+}$ | $Ca^{2+}$ | $NH_4^+$ |
|---|---|---|---|---|---|---|---|---|
| Sbw (10 L) | 112 | 174 | 563 | 441 | 266 | 37.3 | 112 | 2.4 |
| Bw (10 L) | 295 | 2863 | 843.0 | 2344 | 950.6 | 239 | 57.5 | 5.5 |
| Bw (50 L) | 288 | 2277 | 969.1 | 1986 | 824.6 | 232 | 55.1 | 6.2 |

## 8.4 Batch fermentations for BDO production using glucose-based media

The Sbw and Bw hydrolysates having high concentrations of glucose and nutrients were initially investigated for D-BDO production by *B. amyloliquefaciens* in batch bioreactor fermentations. Commercial glucose was also used as carbon source. Sbw and Bw hydrolysates produced in 10 L reactor were centrifuged to remove suspended solids and sterilised. The sugar concentration of bakery hydrolysates was around 120 g $L^{-1}$ for both Sbw and Bw. The Bw hydrolysate contained only glucose, while the Sbw hydrolysate contained also low concentrations of sucrose and fructose. Figure 8.2 presents the production of total BDO and acetoin, D-BDO and acetoin along with glucose consumption during batch bioreactor fermentations. It is observed that both hydrolysates could be effectively used for the production of D-BDO and acetoin. In all fermentations presented in Figure 8.2, D-BDO production was mainly observed until approximately 30 h when complete glucose consumption was accomplished. The highest D-BDO concentrations achieved in all fermentations was around 40 g $L^{-1}$. When commercial glucose was used, the D-BDO yield and productivity achieved were 0.39 g $g^{-1}$ and 1.54 g $L^{-1}$ $h^{-1}$, respectively. Utilisation of hydrolysates led to similar results with high concentration of D-BDO. The highest D-BDO concentration (42.1 g $L^{-1}$) with conversion

yield of 0.46 g $g^{-1}$ and productivity of 1.89 g $L^{-1}$ $h^{-1}$ was achieved in the case of Sbw hydrolysate. The highest productivity (2.31 g $L^{-1}$ $h^{-1}$) for D-BDO was achieved in the fermentation carried out with Bw hydrolysate.

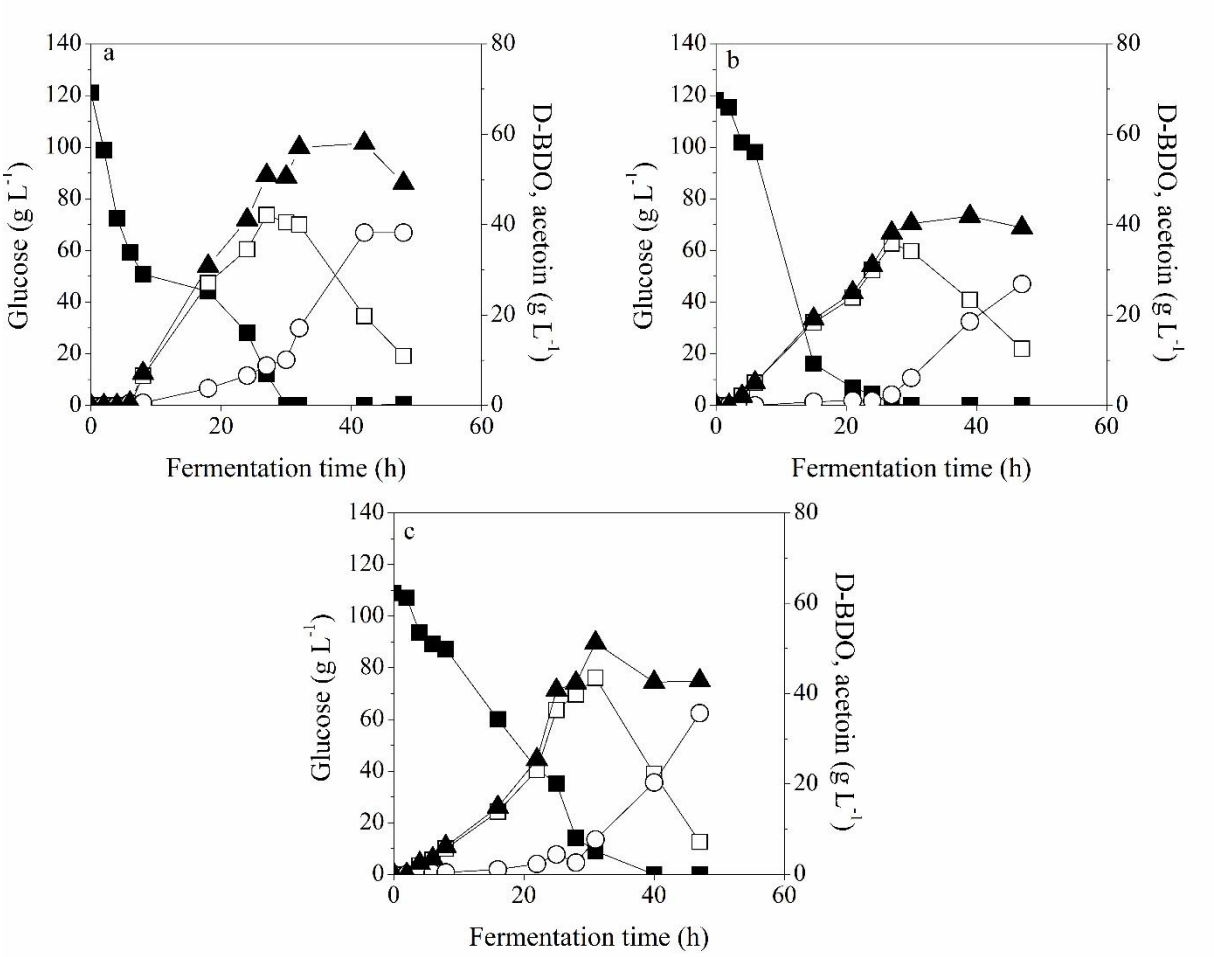


**Figure 8.2** BDO and acetoin (▲), D-BDO (□), and acetoin (○) production as well as glucose (■) consumption in batch fermentations carried out with Sbw hydrolysate (a), Bw hydrolysate (b) and commercial glucose (c) using the bacterial strain *B. amyloliquefaciens*

In all fermentations, acetoin production was relatively low until D-BDO reached the highest concentration. Higher acetoin concentration during the D-BDO production phase was observed in the case of Sdw hydrolysate fermentation, while in the other two fermentations acetoin production was very low. When glucose was completely consumed, D-BDO was converted into acetoin. The highest acetoin concentration (around 39 g $L^{-1}$) was achieved with Sbw hydrolysate. The lowest acetoin concentration (28 g $L^{-1}$) was produced with Bw hydrolysate. The conversion of D-BDO into acetoin, when glucose has been depleted, can be explained by the bacterial metabolism. In particular, glucose is metabolized during fermentation via

glycolysis to provide NADH, while when glucose is depleted the NADH required by cell metabolism is produced via conversion of BDO into acetoin (Zhang et al., 2013b; Li et al., 2017). This phenomenon has been reported previously with *Serratia marcesncens*, *B. subtilis* and *B. licheniformis* strains cultivated on sugar based fermentation media (Sun et al., 2012a; Tian et al., 2016; Li et al., 2017).

### 8.5 Evaluation of oxygen supply in batch cultures carried out with Bw hydrolysate

The effect of different agitation rates with constant aeration rate (1 vvm) corresponding to $k_La$ values within the range of 26-203 $h^{-1}$ on D-BDO and acetoin production was investigated in batch fermentations in 5 L bench-top bioreactor (Figure 8.3). In this set of experiments, filtrated Bw hydrolysate was used as carbon source with initial glucose concentration of 110-130 g $L^{-1}$. At the lowest $k_La$ value of 26 $h^{-1}$, glucose consumption rate was low (Figure 8.3a) leading to low final D-BDO concentration (23.7 g $L^{-1}$) and no acetoin production throughout fermentation. When $k_La$ value of 64 $h^{-1}$ was used, glucose consumption rate was faster with complete glucose consumption observed at 45 h (Figure 8.3a). The highest final D-BDO concentration (55.2 g $L^{-1}$) with a yield of 0.42 g $g^{-1}$ was observed at $k_La$ value of 64 $h^{-1}$. In this $k_La$ value, acetoin production commenced after 30 h (Figure 8.3d) and reached a final concentration of around 10 g $L^{-1}$ at the end of fermentation when the highest D-BDO concentration was observed. D-BDO and acetoin concentration were produced simultaneously after 30 h as it is indicated by their total concentration trend presented in Figure 8.3b.

At $k_La$ values of 113 $h^{-1}$ and 157 $h^{-1}$, glucose consumption rate increased further and different production profiles for D-BDO and acetoin were observed as compared to lower $k_La$ values. D-BDO was produced until complete glucose consumption followed by conversion of D-BDO into acetoin as indicated by the constant total D-BDO and acetoin concentration presented in Figure 8.3b after 25-30 h fermentation. Furthermore, higher D-BDO productivities were observed at these $k_La$ values as compared to lower ones. Around 40 g $L^{-1}$ of D-BDO concentration was observed in both cultures when complete glucose consumption was achieved corresponding to productivities of around 1.7 g $L^{-1}$ $h^{-1}$. In both cases, D-BDO production was accompanied with acetoin accumulation, while after glucose depletion D-BDO was converted into acetoin with final concentrations around 50 g $L^{-1}$ when a $k_La$ value of 157 $h^{-1}$ was used. At the highest $k_La$ value (203 $h^{-1}$) used, acetoin was the predominant metabolic product with a final concentration of 47.4 g $L^{-1}$ and productivity of 2.16 g $L^{-1}$ $h^{-1}$ (Figure 8.3d). A low D-BDO

concentration (6.5 g $L^{-1}$) was produced at 8 h that remained constant until complete glucose consumption followed by a reducing trend thereafter.

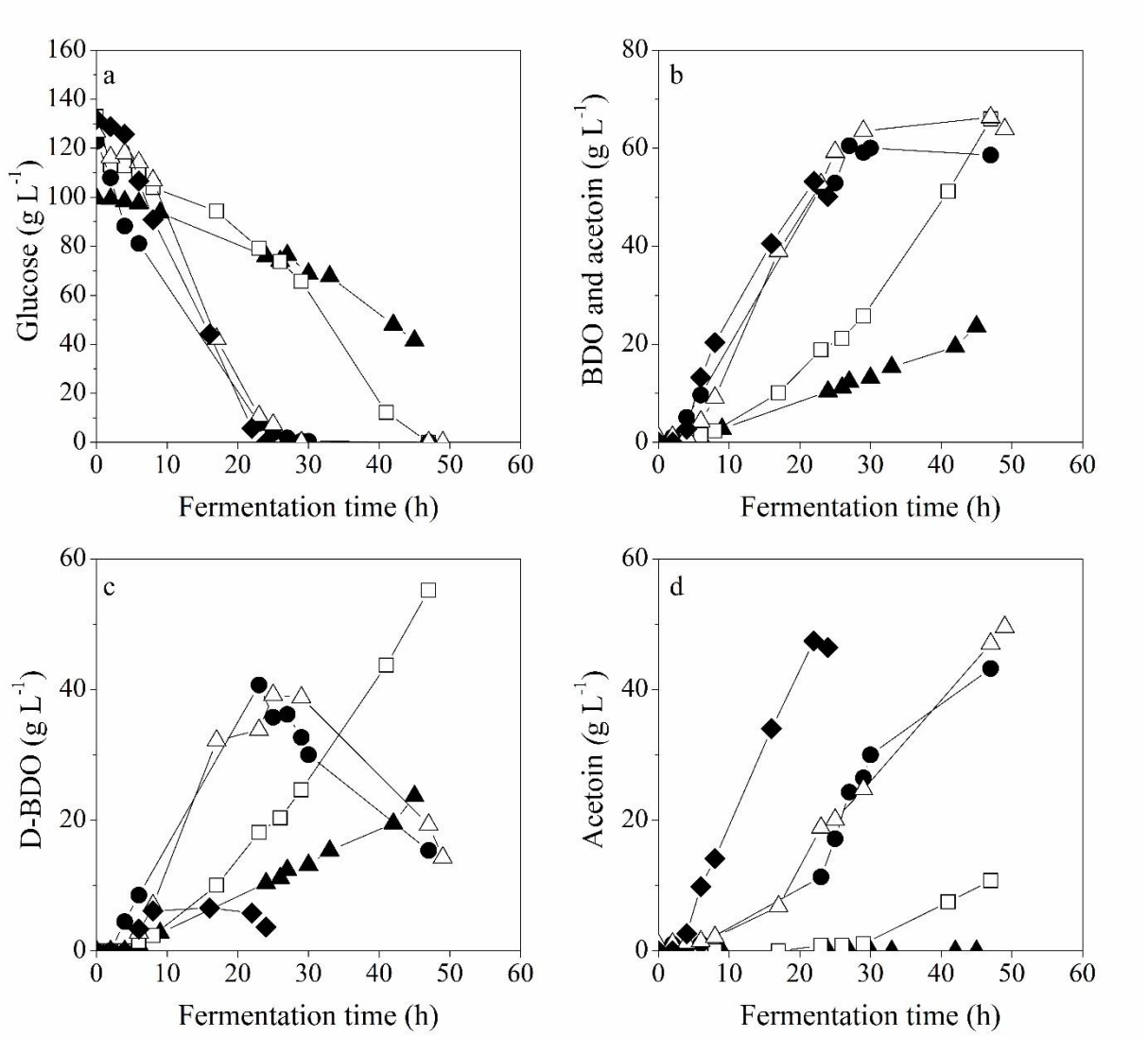


**Figure 8.3** Glucose consumption (a) as well as BDO and acetoin (b), D-BDO (c) and acetoin (d) production during batch fermentations carried out in 5 L bioreactor under different $k_La$ value of 26 $h^{-1}$ (▲), 64 $h^{-1}$ (□), 113 $h^{-1}$ (●), 157 $h^{-1}$ (Δ) and 203 $h^{-1}$ (♦)

The results presented in Figure 8.3 demonstrate that manipulation of $k_La$ during fermentation can be used to control metabolic shift in *B. amyloliquefaciens* cells towards either D-BDO or acetoin production. Four aeration regimes can be observed. Very low oxygen supply favour D-BDO production at low productivities. Low oxygen supply increase D-BDO production efficiency with acetoin production commencing when D-BDO concentrations higher than 45 g $L^{-1}$ are reached. Intermediate oxygen supply lead to moderate D-BDO production followed by conversion of D-BDO into acetoin after complete glucose consumption. Finally, at high oxygen

supply, predominantly only acetoin production is observed at high productivity (2.16 g $L^{-1}$ $h^{-1}$). Producing high-value natural acetoin from a food waste stream at such high productivity using a GRAS strain increases significantly the industrial implementation potential of this process.

## 8.6 Batch fermentation in 50 L bioreactor

Microbial production of BDO, let alone D-BDO, has rarely been studied in large scale fermentations (Yang et al., 2012; Ge et al., 2016). Oxygen supply, the most critical factor for efficient D-BDO or acetoin production, should be maintained in the same level between different bioreactors in order to provide comparable results. The volumetric oxygen mass transfer coefficient ($k_La$) is considered as a quite suitable scale-up parameter for fermentation processes (Zhou et al., 2017). Many methods have been proposed for $k_La$ estimation including chemical, physical and dynamic methods (Tobajas et al., 2000; Garcia-Ochoa et al., 2010). In this study, $k_La$ was estimated based on the method reported by Maina et al. (2019). Table 8.3 shows the $k_La$ values for 5 L, 6.7 L and 50 L bioreactor obtained using aeration response experiments under different agitation rates with constant aeration rate of 1 vvm.

**Table 8.3** $k_La$ values in different bioreactors at the same aeration rate of 1 vvm and variable agitation speeds

| **5 L** | | **6.7 L** | | **50 L** | |
|---|---|---|---|---|---|
| **Agitation rate (rpm)** | **$k_La$ ($h^{-1}$)** | **Agitation rate (rpm)** | **$k_La$ ($h^{-1}$)** | **Agitation rate (rpm)** | **$k_La$ ($h^{-1}$)** |
| 200 | 26 | 200 | 42 | 200 | 61 |
| 300 | 64 | 400 | 95 | 300 | 116 |
| 600 | 203 | 600 | 152 | 400 | 231 |

In order to ensure similar oxygen supply conditions with fermentation conducted in 5 L bioreactor at $k_La$ value of 113 $h^{-1}$, agitation rate of 300 rpm was used in the batch fermentation conducted in 50 L bioreactor corresponding to $k_La$ value of 116 $h^{-1}$. Since it was not possible to conduct all fermentations presented in Figure 8.3 in the 50 L bioreactor, it was decided to carry out the fermentation with an intermediate $k_La$ value in order to evaluate the replicability of experimental results between bioreactors of 10 times scale difference. The results of batch fermentations conducted in 5 L and 50 L bioreactors are presented in Figure 8.4. Similar rates of glucose consumption as well as D-BDO and acetoin production were observed in both fermentations. Complete glucose consumption was observed 5 h earlier in the fermentation

carried out in the 5 L bioreactor. Simultaneous D-BDO and acetoin production was observed until complete glucose consumption, while after glucose depletion D-BDO conversion into acetoin was observed. In the fermentations carried out in 50 L bioreactor almost all D-BDO was converted into acetoin at 46 h. The highest D-BDO productivity achieved in 50 L bioreactor was 1.64 g $L^{-1}$ $h^{-1}$, which is similar to the productivity achieved in 5 L bioreactor. The glucose to D-BDO conversion yields achieved at this fermentation time were also similar. Thus, the experimental results demonstrate that similar $k_La$ values could be used for replicating the results achieved in bench-top bioreactor.

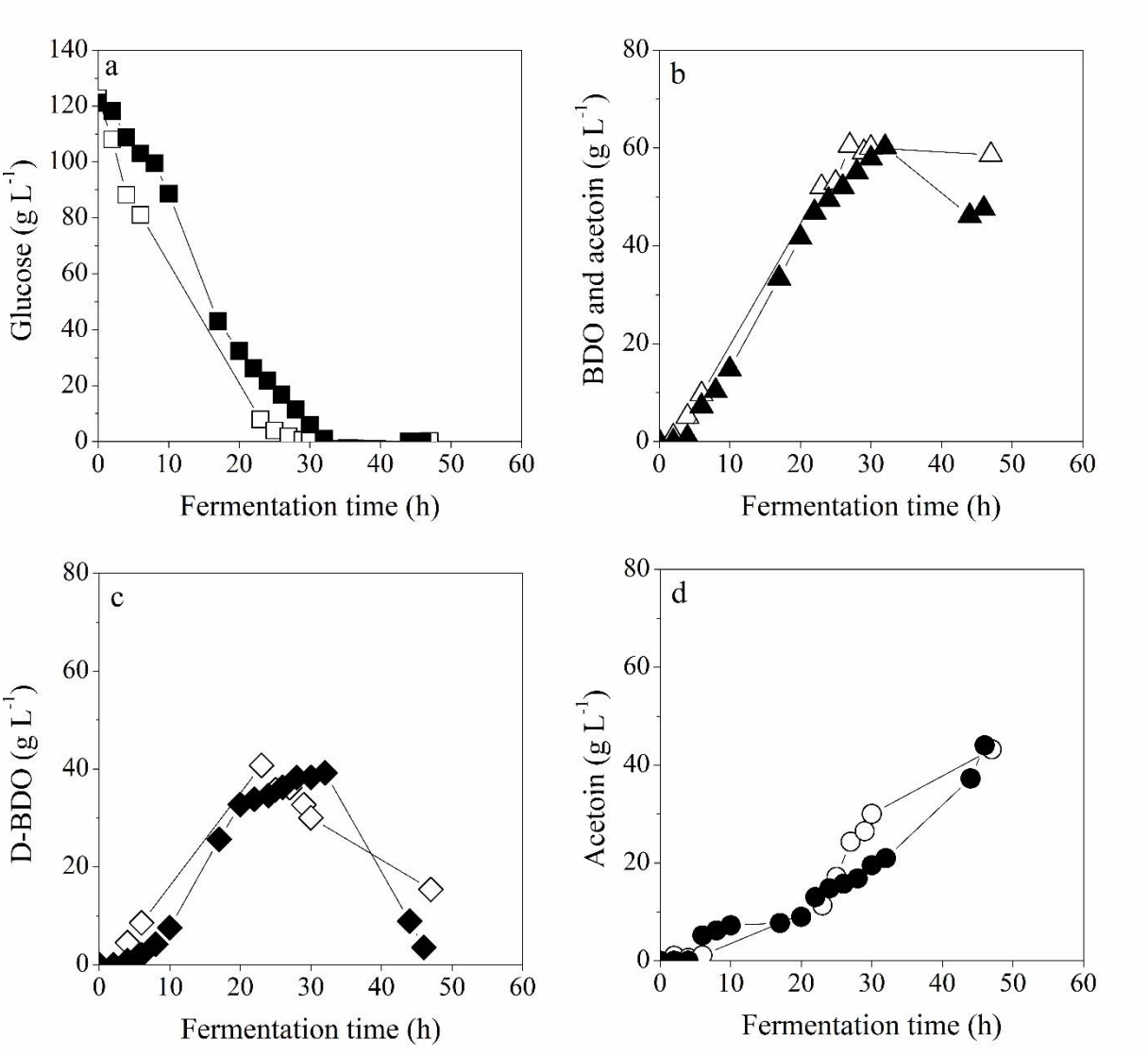


**Figure 8.4** Comparison of glucose consumption (a) as well as BDO and acetoin (b) D-BDO (c) and acetoin (d) production during batch fermentations conducted in 5 L (unfilled symbols) and 50 L (filled symbols) bioreactors with the bacterial strain *B. amyloliquefaciens* cultivated on Bw hydrolysate

## 8.7 Fed-batch fermentation

The potential to enhance further the production of D-BDO and acetoin was investigated in a fed-batch fermentation (Figure 8.5). This fermentation was carried out in a 6.7 L bioreactor with working volume of 3 L and $k_La$ value of 110 $h^{-1}$. A total BDO and acetoin concentration of 103.9 g $L^{-1}$ was produced at 120 h corresponding to yield and productivity of 0.39 g $g^{-1}$ and 0.87 g $L^{-1}$ $h^{-1}$, respectively. At this phase though, D-BDO, meso-BDO and acetoin were contained in the fermentation broth (Figure 8.5b). D-BDO (35.6 g $L^{-1}$) was mainly produced until 29 h at productivity of 1.23 g $L^{-1}$ $h^{-1}$. After this point and until 71 h, the productivity of D-BDO was decreased to 0.39 g $L^{-1}$ $h^{-1}$ and low acetoin production was also observed. When D-BDO reached the maximum concentration of around 53 g $L^{-1}$, D-BDO production stopped and acetoin and meso-BDO production took place. At the end of fermentation meso-BDO was produced reaching a concentration of 13.7 g $L^{-1}$.

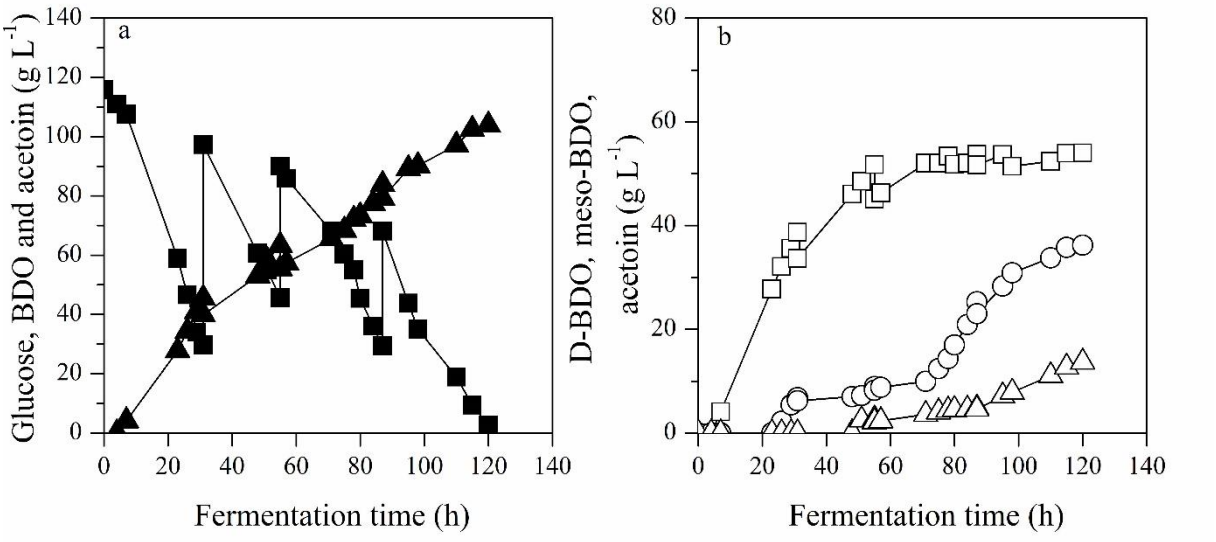


**Figure 8.5** Glucose consumption (■) as well as BDO and acetoin (▲) production (a) along with D-BDO (□), meso-BDO (Δ) and acetoin (○) production (b) during fed-batch fermentation of *B. amyloliquefaciens* in 6.7 L bioreactor conducted with Bw hydrolysate

The results presented in Figure 8.5 indicate that fed-batch fermentation may lead to higher total BDO and acetoin concentration, but this mixture contains D-BDO, meso-BDO and acetoin. It will be highly beneficial from an industrial point of view to develop a bioprocess that leads to either D-BDO or acetoin production at high purity and efficiency at the end of fermentation. The supply of glucose and the manipulation of $k_La$ values during fermentation play a significant

role in the final proportion of metabolic products. For this reason, future research should focus on the optimization of fed-batch fermentations.

Table 8.4 presents the fermentation efficiency reported by different literature-cited publications on BDO and acetoin production using wild-type *Bacillus* strains in various substrates. In general, glucose is mainly used as carbon source whereas high concentrations of organic nitrogen sources are required. A mixture of BDO and acetoin is produced by native *Bacillus* strains. It is important to notice that BDO is produced as the beginning of fermentation, while acetoin is accumulated after carbon source depletion at the end of fermentation (Zhang et al., 2013; Tian et al., 2016). High BDO production (53.7 g $L^{-1}$) has been achieved by *B. amiloliquefaciens*, whereas the highest acetoin production of 55.3 g $L^{-1}$ has been reported by *P. polymyxa* in fed-batch culture (Zhang et al., 2012; Zhang et al., 2013). The utilisation of corn stover hydrolysate has been used as alternative low-cost substrate resulting in high BDO production (74 g $L^{-1}$) without acetoin accumulation (Li et al., 2014b). Sugarcane molasses has been also evaluated by Dai et al. (2015). BDO (90.8 g $L^{-1}$) and acetoin (8.7 g $L^{-1}$) production has been achieved by *B. substilis*. Moreover, inulin, a storage polysaccharide in many plants including *Vernonia herbacea* and *Helianthus tuberosus*, has been valosized as low cost substrate resulting in high production of meso- and D-BDO in a simultaneous saccharifiaction and fermentation process (Li et al., 2014a).

The results of this study indicate that *B. amyloliquefaciens* can be used for D-BDO and acetoin production using bakery waste hydrolysates. Fed-batch fermentation led to production of a mixture of D-BDO and acetoin. Further study for production of either optical pure D-BDO or acetoin is needed.

**Table 8.4** BDO and acetoin production by wild-type *Bacillus* strains

| Microorganism | Carbon source | Time (h) | BDO (g $L^{-1}$) | Acetoin (g $L^{-1}$) | Agitation - Aeration | Nitrogen source (g $L^{-1}$) | Fermentation mode | Ref. |
|---|---|---|---|---|---|---|---|---|
| *B. liqueniformis* MEL09 | Glucose | 36 | 12.0 | 41.26 | | Yeast extract 12, peptone 1 $(NH_4)SO_4$ 8 | Batch (sf) | Liu et al., 2011 |
| *B. subtilis* JNA 3-10 | Glucose | 144 | 15.8 | 42.2 | | Beef extract 5, CSL 6, urea 2 | Batch (sf) | Zhang et al., 2011 |
| *Paenibacillus polymyxa* CS107 | Glucose | 42 | 11.0 | 55.3 | 500 rpm and 0.05 vvm | Yeast extract 15.93 | Fed-batch | Zhang et al., 2012 |
| *B. amyloliquefaciens* | Glucose | 24 | 53.7 | <5 | 350 rpm for 24 h and then 500 rpm | Yeast extract 10, peptone 10 | Batch | Zhang et al., 2013b |
| | | 40 | <5 | 51.2 | | | | |
| *B. licheniformis* ATTV 14580 | Inulin | 30 | 103 (meso- and D- 1:1) | 2.0 | 400 rpm for 10 h and then 200 rpm | Yeast extract 5.8 CSL 14.7 | Fed-batch - SSF | Li et al., 2014a |
| *B. licheniformis* strain X10 | Corn stover hydrolysate | 36 | 74.0 | | 400 rpm and 1 vvm | Yeast extract 5, CSLP, 14.5 | Fed-batch | Li et al., 2014b |
| *B. amyloliquefaciens* FMME044 | Glucose | 18 | 24.0 | 33.0 | 450 rpm | Yeast extract 12.5, peptone 12.5 | Batch | Luo et al., 2014 |
| | | 46 | 11.1 | 47.9 | | | | |
| *B. subtilis* CICC10025 | Sugarcane molasses | 60 | 90.8 | 8.7 | 300 rpm and 1 vvm | Urea 8.7, CSL 2 | Fed-batch | Dai et al., 2015 |
| *B. subtilis* SF4-3 | Glucose | 36 | 21.0 | 21.2 | 500 for 6 h and then 300 rpm | Yeast extract 10, CSD 5, urea 2 | Batch | Tian et al., 2016 |
| | | 60 | 14.2 | 33.4 | | | | |
| *B. amyloliquefaciens* | Bw hydrolysate | 47 | 55.2 | 10.8 | $k_La$: 64 $h^{-1}$ | Yeast extract 15 | Batch | This study |
| | | 22 | 5.8 | 47.4 | $k_La$: 203 $h^{-1}$ | | | |
| | | 55 | 54.4 (D- 95%) | 8.9 | $k_La$: 110 $h^{-1}$ | | Fed-batch | |
| | | 120 | 67.7 (D- 79.8%) | 36.2 | | | | |

# Chapter 8

## 8.8 Conclusion

The results presented in this chapter demonstrate that the newly isolated GRAS strain *B. amyloliquefaciens* can be cultivated on bakery waste hydrolysates for the production of D-BDO and acetoin.

The glucose-based hydrolysates were evaluated in batch bioreactor cultures conducted in bench-top (5 L) and pilot (50 L) bioreactor scale. Evaluation of different $k_La$ values in the bench top bioreactor showed that using different $k_La$ values during fermentation leads to different trend and production efficiency for D-BDO and acetoin.

Four metabolite production regimes were identified depending on the $k_La$ value range. Very low $k_La$ value of 26 $h^{-1}$ divert bacterial metabolism towards D-BDO production with low productivities. At $k_La$ values around 64 $h^{-1}$, the highest D-BDO production efficiency is attained with low acetoin production that is observed when D-BDO concentration reaches 45 g $L^{-1}$. At $k_La$ values of 113 $h^{-1}$ and 157 $h^{-1}$, moderate D-BDO production is observed with D-BDO conversion into acetoin after complete glucose consumption. At high $k_La$ value of 203 $h^{-1}$, bacterial metabolism is diverted towards acetoin production at high productivity (2.16 g $L^{-1}$ $h^{-1}$) and very low D-BDO production at the end of fermentation. Conducting a fermentation in 50 L bioreactor scale with a $k_La$ value of 116 $h^{-1}$ showed that good replicability can be achieved with fermentation results obtained in 5 L bioreactor scale.

Fed-batch fermentation showed that glucose supply and $k_La$ manipulation during fermentation should be optimised in order to boost production efficiency and product purity of either D-BDO or acetoin during fermentation. The fed-batch fermentation conducted in this chapter at $k_La$ value of 110 $h^{-1}$ in a 6.7 L bioreactor showed that continuous feeding of glucose does not lead to conversion of D-BDO into acetoin, while glucose is still present in the fermentation broth. Besides this, low meso-BDO production is also observed.

D-BDO and acetoin can be used as both platform chemicals and high value additives. For instance, D-BDO can be used as antifreeze agent and as precursor for the production of high value chiral chemicals. Likewise, natural acetoin is used as additive in flavour and fragrances. For this reason, there is high commercial interest on the development of a robust fermentation process leading to either D-BDO or acetoin production. Future research should focus on the optimisation of glucose supply and $k_La$ regimes during fed-batch fermentation.

## Chapter 9

# Conclusions and future perspectives

# Chapter 9

The development of bioprocesses for the production of bio-based chemicals through the utilisation of low cost crude renewable feedstocks is an attractive alternative to the use of petroleum based feedstocks. Among the different chemicals, PDO and BDO are versatile molecules that act as building blocks for the production of chemicals and polymers. The global market of PDO and BDO covers a range of industries including food, pharmaceuticals, plastics, coating and paint industries and as precursors for the production of numerous chemicals. Studies on the microbial production of these chemicals allow us to assess each platform molecule for commercial exploitation. Information regarding current production capacities and future needs, available wild-type or engineered microorganisms, carbon and nitrogen requirements, knowledge of metabolic pathways and fermentation efficiency considering final concentration, yield and productivity are required for the assessment.

This thesis focused on the evaluation of different feedstocks for PDO and BDO production by wild-type microorganisms. The state of the art in the international literature on microbial PDO and BDO production is reviewed in Chapter 2. The fermentation parameters affecting their biosynthesis along with the various strategies for efficient production including strain improvement, substrate alternatives and process development are highlighted. The availability of industrial side streams and food wastes is also underlined with respect to the current production capacities, utilisation strategies and conversion technologies emerging from the bio-economy sector.

In the first part of the experimental work, SBC and crude glycerol generated from biodiesel production plants using soybean seeds were evaluated via an integrated approach for PDO production by two wild-type *Citrobacter freundii* strains (Chapter 5). *C. freundii* VK-19 exhibited higher tolerance against crude glycerol impurities than *C. freundii* FMCC-8, while SBC hydrolysate provided adequate nutrients for fermentative PDO production by both strains. The inhibitory effect of metabolites showed that lactic acid, acetic acid and formic acid caused the highest inhibition effect on the growth of *C. freundii* VK-19 and *C. freundii* FMCC-8. *C. freundii* strains were more tolerant to succinic acid and PDO even in high concentrations. The type of salts presents in crude glycerol affects cell growth and PDO production. The presence of sodium salts in crude glycerol proved to be a restraining factor for microbial growth resulting in lower PDO production during fermentation than in the case that the crude glycerol contains potassium salts.

# Chapter 9

In the second part of this study, industrial side streams and food wastes were evaluated for BDO production by two wild-type microorganisms aiming at producing different BDO stereoisomers. Crude glycerol generated from biodiesel plants was applied for meso- and L-BDO production by a newly isolated *K. michiganensis* strain (Chapter 6). Different types of crude glycerol and oxygen supply were investigated with the objective of increasing BDO production efficiency. The strain exhibits high tolerance to high concentrations of crude glycerol. Oxygen supply proved to be the most critical factor affecting BDO production efficiency. The performance of fed-batch fermentations showed that high BDO production efficiency can be achieved with this wild-type microorganism as compared to literature-cited studies. Results from this study demonstrated that *K. michiganensis* has great potential to produce BDO from crude glycerol.

In Chapter 7, co-products from sugar mills were evaluated as fermentation feedstocks for D-BDO and acetoin production by the GRAS *B. amyloliquefaciens* strain. The fermentation parameters influencing BDO synthesis were studied. High BDO production was achieved under the optimum fermentation conditions using either VHP cane sugar or sugarcane molasses. Low oxygen supply favoured BDO synthesis, whereas increased agitation speeds led to acetoin accumulation in the fermentation broth. Fed-batch fermentations showed that distribution of BDO stereoisomers and acetoin is highly affected by the elemental composition of the substrate. Besides sugar mill co-products, bakery wastes were investigated as potential substrate for D-BDO and acetoin production by *B. amyloliquefaciens* (Chapter 8). Bakery waste hydrolysates were produced via enzymatic hydrolysis and evaluated in microbial fermentation under different agitation rates for D-BDO and acetoin production. Scale-up of the fermentation process was applied using the volumetric oxygen mass transfer coefficient ($k_La$) as the scale-up parameter. Bakery hydrolysate demonstrated to be a suitable substrate for D-BDO and acetoin synthesis. The results indicated that the metabolic shift towards D-BDO or acetoin production is closely dependent on oxygen supply. D-BDO production efficiency was improved at lower $k_La$ values, while acetoin production was favoured at higher $k_La$ values. Batch bioreactor fermentations were successfully scaled up from 5 L to 50 L resulting in similar fermentation profiles.

The research on PDO production indicates that reducing by-product formation generated during fermentation is particularly important in order to enhance PDO formation. The fermentation efficiency is also influenced by the catalysts used during biodiesel production. Evaluation of

fermentation strategies such as continuous and immobilized systems and/or metabolic engineering approaches is essential in order to achieve efficient PDO production by *Citrobacter* strains. Efficiency and simplified methods for removal of inhibitory compounds from crude glycerol should be evaluated in future research.

The results presented in this study regarding BDO production by *K. michiganensis* suggest that crude glycerol is a promising feedstock for BDO synthesis. Even though the yield and productivity achieved from crude glycerol are among the highest obtained from crude glycerol, the final BDO concentration, yield and productivity should be further improved. A promising approach that is currently suggested by some researchers is the two-stage pH and dissolved oxygen control strategy (Yang et al., 2015). Overexpression and/or deletion of genes related to BDO and by-products accumulation could effectively improve BDO production. Future research should focus on the evaluation of the techno-economic viability and the environmental impact of the proposed processes for the production of BDO in comparison to the current production routes.

The study on D-BDO and acetoin synthesis by the GRAS *B. amyloliquefaciens* strain serves as the basis for further optimisation studies. Low-cost industrial side streams and food supply chain wastes provide potential feedstocks for efficient D-BDO and acetoin synthesis with potentially low production cost. The usage of low-cost supplements instead of yeast extract is also essential for industrial production of D-BDO and acetoin by *B. amyloliquefaciens*. Understanding the mechanism for the production of BDO stereoisomers and acetoin is necessary for efficient production of optically pure D-BDO or acetoin. Innovative strategies including fermentation combined with electrochemical conversion could be applied for the conversion of acetoin into D-BDO and vice versa.

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