ΓΕΩΠΟΝΙΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ

ΣΧΟΛΗ ΤΡΟΦΙΜΩΝ ΒΙΟΤΕΧΝΟΛΟΓΙΑΣ ΚΑΙ ΑΝΑΠΤΥΞΗΣ

ΤΜΗΜΑ ΕΠΙΣΤΗΜΗΣ ΤΡΟΦΙΜΩΝ ΚΑΙ ΔΙΑΤΡΟΦΗΣ ΤΟΥ ΑΝΘΡΩΠΟΥ

ΕΡΓΑΣΤΗΡΙΟ ΜΙΚΡΟΒΙΟΛΟΓΙΑΣ ΚΑΙ ΒΙΟΤΕΧΝΟΛΟΓΙΑΣ ΤΡΟΦΙΜΩΝ

Μελέτη της βιοτεχνολογικής παραγωγής 2,3-βουτανοδιόλης κατά την αύξηση επιλεγμένων βακτηριακών στελεχών σε σακχαρούχα ανανεώσιμα υποστρώματα

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

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Study of the biotechnological production of 2,3-butanediol during growth of selected bacterial strains on sugar-based renewable substrates

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Palaiogeorgou Anastasia-Marina

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SUPERVISOR

Seraphim Papanikolaou

7-MEMBER COMMITTEE:

Seraphim Papanikolaou, Associate Professor, Agricultural University of Athens, Greece

Apostolos Koutinas, Associate Professor, Agricultural University of Athens, Greece

George Aggelis, Professor, University of Patras, Greece

Nikolaos G. Stoforos, Professor, Agricultural University of Athens, Greece

Eleftherios H. Drosinos, Professor, Agricultural University of Athens, Greece

Dimitris Sarris, Assistant Professor, University of the Aegean, Greece

Afroditi Chatzifragkou, Assistant Professor, University of Reading, U.K.

«Το μαχαίρι»

Όπως αργεί τ' ατσάλι να γίνει κοφτερό και χρήσιμο μαχαίρι
έτσι αργούν κ' οι λέξεις ν' ακονιστούν σε λόγο.
Στο μεταξύ
όσο δουλεύεις στον τροχό
πρόσεχε μην παρασυρθείς
μην ξιπαστείς
απ' τη λαμπρή αλληλουχία των σπινθήρων.
Σκοπός σου εσένα το μαχαίρι.

[πηγή: Άρης Αλεξάνδρου, *Ποιήματα (1941-1974)*, Εκδόσεις Καστανιώτη, Αθήνα 1981, σ. 99]

Στους μικρούς ή μεγάλους μας καθημερινούς αγώνες,

σε αυτούς που πορευόμαστε πλαϊ-πλαϊ

Ευχαριστίες

Σε αυτό το γεμάτο εμπειρίες ταξίδι θα ήθελα να ευχαριστήσω όλους εκείνους που συνέβαλλαν, ο καθένας με τον τρόπο του, στο να ολοκληρωθεί. Αρχικά, οφείλω ένα μεγάλο ευχαριστώ στον κ. Παπανικολάου Σεραφείμ για την εμπιστοσύνη που μου έδειξε όλα αυτά τα χρόνια, τις πολύτιμες συμβουλές του και την ουσιαστική καθοδήγησή του τόσο στο εργαστηριακό κομμάτι όσο και στη συγγραφή της Διδακτορικής Διατριβής. Επίσης θα ήθελα να ευχαριστήσω τον κ. Κουτίνα Απόστολο για την άψογη συνεργασία και την σημαντική καθοδήγησή του για την πραγματοποίηση ενός μεγάλου κομματιού της πειραματικής διαδικασίας.

Φυσικά οφείλω ένα τεράστιο ευχαριστώ στους συνοδοιπόρους μου στο εργαστήριο με τους οποίους μοιραστήκαμε αγωνίες, χαρές, απογοητεύσεις, κούραση και όνειρα για το μέλλον. Έτσι, θα ήθελα να ευχαριστήσω τη Ροζανίνα Φιλιππούση, τον Βαγγέλη Ξενόπουλο, τη Sidoine Sadjeu, τη Λίνα Βάρφη, τη Λόριτ Καμπούτση, την Ειρήνη Καλλία και τη Μαίρη Αλεξανδρή γιατί πέρα από συνάδελφοι υπήρξαν και είναι φίλοι αληθινοί.

Τέλος, θα ήθελα να ευχαριστήσω την οικογένεια μου και τους αμέτρητους φίλους μου για την υποστήριξή τους και τη δύναμη που μου έδιναν όλα αυτά τα χρόνια.

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ABSTRACT

Aim of the study was to develop an optimized bioprocess for the efficient production of 2,3-butanediol (BDO) and acetoin (Ace). These metabolites have numerous applications and can be produced via microbial fermentations using commercial carbon sources and industrial wastes rich in carbohydrates. During the first part of this research, the potential of nine newly isolated strains, which belong to the family of Enterobacteriaceae, to produce BDO was assessed during initial batch experiments in Duran bottles using glucose and sucrose as carbon source. Although most of the strains were capable of converting both carbon sources into BDO in promising bioconversion yields and productivity rates, two of them were selected for further investigation. The implicated strains that were natural ones (viz. non-genetically modified) and food-derived, were *Klebsiella oxytoca* FMCC-197 and *Enterobacter* sp. FMCC-208. In particular, the strain *Klebsiella oxytoca* FMCC-197 totally consumed both carbon sources during the anaerobic batch fermentations, reaching a yield of over 0.40 g g^{-1} in both cases. The second strain, *Enterobacter* sp. FMCC-208, totally consumed both glucose and sucrose during the initial batch trials, reaching a bioconversion yield higher that 0.44 g g^{-1}.

The second part of the study focused on the investigation of different parameters (initial substrate concentration and threshold of substrate inhibition, assimilation of different carbon sources, incubation temperature, aeration) in order to optimize the process of BDO and Ace production using the strain *K. oxytoca* FMCC-197. Given that one of the main carbohydrates in various food wastes and residues is that of sucrose, this sugar was a principal carbon source used throughout the research. Batch cultures under anaerobic conditions and aerobic conditions were conducted using five different initial sucrose concentrations (30, 60, 90, 120 and 150 g l^{-1}). Both in the case of anaerobic and aerobic conditions, the strain was able to totally consume the substrate when 30, 60 and 90 g l^{-1} were used. However, in higher initial concentrations, part of the substrate remained unconsumed, lowering the final bioconversion yield. It should also be stressed that, interestingly and in contrast to the BDO production theory, higher production values, rates and conversion yields were noted in aerobic conditions. Another set of shake flask (aerobic) experiments was performed using various carbon sources (i.e. glucose, fructose, mannose, xylose, arabinose, galactose, sucrose and molasses) in order to evaluate the ability of the strain *K. oxytoca* FMCC-197 to produce BDO and Ace. The final bioconversion yield was for most sugars tested higher than 0.40 g g^{-1}. Moreover, shake flask experiments were conducted under different temperature values (25, 30, 34, 37, 40 and 42 °C), using sucrose as carbon source, in order to estimate the effect in growth, sugar consumption and BDO and Ace production. The strain was able to grow in a wide range of temperature values, however when temperature value was >37 °C, conversion yield and productivity rate were decreased.

From the evaluation of the results obtained and in order to maximize BDO production, fed-batch bioreactor experiments changing different parameters were performed. Two fed-batch bioreactor experiments were conducted at 30 °C under anaerobic and aerobic conditions using molasses and sucrose as carbon sources. The final product synthesis was increased in aerobic conditions and fed-batch bioreactor experiments that followed were performed using aeration. For instance, 31 g l^{-1} of BDO and 7 g l^{-1} of Ace were produced under anaerobic conditions while 101.1 g l^{-1} of BDO and 14.2 g l^{-1} of Ace were produced under aerobic conditions. On the other hand, a fed-batch bioreactor experiment carried out at 37 °C using molasses and sucrose as carbon sources, led to a remarkably lower productivity rate. The final product synthesis was decreased as 63.0 g l^{-1} of BDO and 8.8 g l^{-1} of Ace were produced. In another fed-batch experiment, molasses were used as the sole carbon source at 30 °C, in order to investigate any substrate inhibition. The final production was also reduced as 35.1 g l^{-1} of BDO and 8 g l^{-1} of Ace were produced and the productivity rate was remarkably lower.

Two fed-batch experiments in shake flasks were carried out using sucrose as the sole carbon source at 30 °C. In the first case the medium had been previously sterilized while in the second one the medium had been previously pasteurized. The final yield and productivity values were lower when the medium had been previously pasteurized. On the other hand, besides BDO production, trials were carried out on molasses employed as the sole carbon source in order to observe potential color removal observed simultaneously with the production of microbial metabolites. Indeed, anaerobic and aerobic cultures performed were accompanied by a non-negligible decolorization of the medium of *c.* 40% and 50%, respectively.

During the third part of the research, *Enterobacter* sp. FMCC-208 was selected in order to evaluate its ability to produce BDO and Ace, and different culture parameters were changed in order to perform optimization of the bioprocess. Preliminary tests in 1-l Duran bottles were performed using sucrose and molasses as carbon source in three different initial concentrations of total sugars (15, 30 and 60 g l^{-1}). The strain successfully converted the whole substrate into BDO and Ace in bioconversion yields higher that 0.38 g g^{-1}. As in the previous set of experiments with *K. oxytoca*, interestingly and in contrast to the theory of BDO production, the productivity rate was remarkably higher in the case of aerobic fermentations and the following batch experiments were all conducted in shake flasks.

Seven different sugar substrates frequently found in food-based residues and lignocellulosic hydrolysates (i.e. glucose, fructose, mannose, xylose, arabinose, galactose, sucrose) as well as cane molasses were used from the strain *Enterobacter* sp. FMCC-208, that in all cases was revealed capable of producing BDO and Ace in remarkable yields (>0.36 g g^{-1}) and productivity rates (>0.80 g l^{-1} h^{-1}), with the exception of xylose employed as the sole carbon source, that was identified as a

substrate that promoted lower productivity and conversion yield. From the results obtained during fermentations in different incubation temperatures (T=25, 30, 34, 37, 40 and 42 °C), the productivity rate was decreased when temperatures >37 °C were applied into the culture medium.

Bacterial growth was studied using a wide range of initial sucrose concentrations in order to identify the maximum value in which no substrate inhibition was observed. In particular, 10 different initial sucrose concentrations (viz. 5, 10, 15, 20, 40, 60, 80, 110, 130 and 150 g l^{-1}) were used in shake-flask experiments at T=37 °C. High values of specific growth rate (μ_{max}>0.70 h^{-1}) were obtained when the initial substrate concentration was lower than 40 g l^{-1}. In higher initial sucrose concentrations employed, μ_{max} values were decreased. In order to evaluate the optimum pH value for the bacterial growth and the production of BDO, four batch bioreactor experiments were carried out at constant pH values of 5.0, 6.0, 6.5 and 7.0. The strain *Enterobacter* sp. FMCC-208 gave better results at pH value of 6.5 and 7.0. In addition, the ability of the strain to grow in shake flasks using previously pasteurized media was evaluated, using sucrose as carbon source at 37 °C. Although other bacterial species were also observed in the culture broth, the final bioconversion yield and productivity rate were satisfying, reaching a value of 0.41 g g^{-1} and 1.22 g l^{-1} h^{-1} respectively.

From the results obtained through the previous sets of experiments using the strain *Enterobacter* sp. FMCC-208, different fed-batch experimental strategies were designed in order to enhance the final product synthesis. Two fed-batch bioreactor experiments were conducted at 30 °C using molasses and sucrose as carbon sources. The first one was conducted under anaerobic conditions and at the end of the fermentation 30.1 g l^{-1} of BDO and 5.0 g l^{-1} of Ace were produced reaching a yield of 0.39 g g^{-1}. The second one was conducted under aerobic conditions. Remarkable increase was noted at the final product accumulation into the medium. Therefore, 90.3 g l^{-1} of BDO and 10.0 g l^{-1} of Ace were produced while the conversion yield of products synthesized per unit of sugar consumed was also increased (0.43 g g^{-1}). Another fed-batch bioreactor experiment using the same combination of substrates (molasses and commercial sucrose) at 37 °C was performed and although the final production was lower, a remarkable increase in productivity rate was observed. Additionally, molasses was used as the sole carbon source in another fed-batch experiment at 37 °C in order to investigate any substrate inhibition due to the impurities of molasses. After 64 hours, 52 g l^{-1} of BDO and 8.7 g l^{-1} of Ace were produced and the bioconversion yield and the productivity were remarkably decreased. Two fed-batch experiments were also carried out in shake flasks, the one using previously sterilized medium and the other using pasteurized medium at 37 °C. Although the results were satisfying in both cases, the final yield and productivity rate were reduced in the second experiment. Finally, the decolorization of the medium was also investigated in anaerobic

and aerobic cultures on molasses employed as the sole carbon source. A color removal of 25% and 35% was observed respectively.

The present research, therefore, indicates that two new bacterial strains, isolated from food-stuffs, namely *K. oxytoca* FMCC-197 and *Enterobacter* sp. FMCC-208, could be regarded as possible candidates for BDO and Ace production in industrial scale, using various low-cost sugar-based substrates as microbial carbon sources.

Key words: 2,3-butanediol, acetoin, molasses, microbial fermentation, *Klebsiella oxytoca*, *Enterobacter* sp.

ΠΕΡΙΛΗΨΗ

Ο σκοπός της παρούσας διδακτορικής διατριβής ήταν η μελέτη της αξιοποίησης άγριων βακτηριακών στελεχών, απομονωμένων από διάφορα είδη τροφίμων και δυνητικώς μη-παθογόνων, σχετικά με τη χρήση τους ως μικροβιακά κυτταρικά εργαστήρια, για την παραγωγή 2,3-βουτανοδιόλης και ακετοΐνης. Η παραγωγή των δύο αυτών μεταβολιτών παρουσιάζει ιδιαίτερο ενδιαφέρον καθώς τα προϊόντα αυτά βρίσκουν πολυάριθμες εφαρμογές στη βιομηχανία μελανιών, αρωμάτων, πλαστικών, στη βιομηχανία των τροφίμων αλλά και στην φαρμακοβιομηχανία. Πολλοί μονοσακχαρίτες αλλά και βιομηχανικά απόβλητα πλούσια σε σάκχαρα, μετατρέπονται σε 2,3-βουτανοδιόλη και το παράγωγό αυτής, την ακετοΐνη, με τη χρήση μικροοργανισμών, μέσω του βιοσυνθετικού μονοπατιού παραγωγής 2,3-βουτανοδιόλης - οργανικών οξέων.

Στο πρώτο μέρος της παρούσας έρευνας, μελετήθηκε η ικανότητα εννέα άγριων βακτηριακών στελεχών που απομονώθηκαν από τρόφιμα, να αφομοιώνουν τη γλυκόζη και τη σακχαρόζη προς παραγωγή 2,3-βουτανοδιόλης και ακετοΐνης. Τα στελέχη που χρησιμοποιήθηκαν ήταν τα *Enterobacter ludwigii* FMCC-204, *Enterobacter aerogenes* FMCC-9, *Enterobacter aerogenes* FMCC-10, *Citrobacter freundii* FMCC-207, *Klebsiella oxytoca* FMCC-197, *Enterobacter* sp. FMCC-208, *Citrobacter freundii* FMCC-8, *Citrobacter farmeri* FMCC-5 και *Citrobacter farmeri* FMCC-7. Οι παραπάνω μικροοργανισμοί καλλιεργήθηκαν σε κλειστού τύπου ζυμώσεις οι οποίες πραγματοποιήθηκαν σε φιάλες Duran στους 30 °C. Πέραν των τριών τελευταίων στελεχών, τα υπόλοιπα έξι έδωσαν ικανοποιητικά αποτελέσματα αφού ήταν ικανά να καταναλώσουν τόσο τη γλυκόζη όσο και τη σακχαρόζη προς παραγωγή 2,3-βουτανοδιόλης. Από τα ειρημένα στελέχη, δύο, ήτοι τα *K. oxytoca* FMCC-197 και *Enterobacter* sp. FMCC-208, επελέγησαν για περαιτέρω έρευνα, αφού συνδύασαν ικανοποιητική απόδοση και παραγωγικότητα κατά την αύξησή τους και στις δύο πηγές ανωτέρω άνθρακα.

Στο δεύτερο μέρος της διατριβής, μελετήθηκε ο τρόπος επίδρασης διαφορετικών παραγόντων στην αύξηση του στελέχους *K. oxytoca* FMCC-197 αλλά και στην παραγωγή 2,3-βουτανοδιόλης και ακετοΐνης. Έτσι, μελετήθηκε η επίδραση της θερμοκρασίας αλλά και της αρχικής συγκέντρωσης του υποστρώματος στην αύξηση του μικροοργανισμού και την τελική συγκέντρωση προϊόντος. Πιο συγκεκριμένα, το ανωτέρω στέλεχος έδωσε ιδιαίτερα ικανοποιητικά αποτελέσματα σε ένα μεγάλο εύρος θερμοκρασιών κατά τη διάρκεια αερόβιων καλλιεργειών σε κωνικές φιάλες, χρησιμοποιώντας ως πηγή άνθρακα τη σακχαρόζη, ενώ σε θερμοκρασίες μεγαλύτερες από τους 37 °C, η τελική απόδοση προϊόντος αλλά και η παραγωγικότητα μειώθηκαν αισθητά. Σε μία επόμενη σειρά πειραμάτων χρησιμοποιήθηκαν διαφορετικές αρχικές συγκεντρώσεις σακχαρόζης και το στέλεχος *K. oxytoca* FMCC-197 έδειξε ιδιαίτερη ικανότητα να αναπτύσσεται ακόμα και σε συγκεντρώσεις υψηλότερες των 90 g l^{-1}. Ωστόσο, σε συγκεντρώσεις σακχάρου μεγαλύτερες των 120 g l^{-1}, παρατηρήθηκε το φαινόμενο της παρεμπόδισης εκ του

υποστρώματος, με μειωμένη απόδοση παραγωγής και μη-αμελητέο μέρος του υποστρώματος να παραμένει ακατανάλωτο κατά το τέλος της διεργασίας. Η μελέτη του συγκεκριμένου στελέχους συνεχίστηκε με την καλλιέργειά του σε διαφορετικές πηγές άνθρακα. Έτσι σε μία σειρά αερόβιων ζυμώσεων σε κωνικές φιάλες χρησιμοποιήθηκαν οι εξής πηγές άνθρακα: γλυκόζη, φρουκτόζη, μαννόζη, ξυλόζη, αραβινόζη, γαλακτόζη, σακχαρόζη και μελάσα. Τα αποτελέσματα των ζυμώσεων ήταν εξίσου ικανοποιητικά, αφού καταναλώθηκαν όλες οι πηγές άνθρακα αποδεικνύοντας ότι το στέλεχος *K. oxytoca* FMCC-197 μπορεί να χρησιμοποιηθεί σε μεγάλη κλίμακα για τη βιομετατροπή αποβλήτων πλούσιων στις παραπάνω πηγές άνθρακα προς 2,3-βουτανοδιόλη και ακετοΐνη. Επίσης κατά τη διάρκεια αναερόβιων και αερόβιων καλλιεργειών αξιολογήθηκε η ικανότητα αποχρωματισμού της μελάσας, όπου ξεπέρασε το 40% και στις δυο περιπτώσεις, χωρίς η μελέτη της διεργασίας του αποχρωματισμού να υφίσταται περαιτέρω αριστοποίηση.

Αξιολογώντας τα αποτελέσματα των παραπάνω ζυμώσεων και με στόχο την αύξηση της τελικής συγκέντρωσης του προϊόντος, πραγματοποιήθηκαν ημι-συνεχείς τροφοδοτούμενες καλλιέργειες σε βιοαντιδραστήρα κάτω από διαφορετικές συνθήκες ζύμωσης. Οι δύο πρώτες ημι-συνεχείς τροφοδοτούμενες καλλιέργειες πραγματοποιήθηκαν στους 30 °C κάτω από αναερόβιες ή αερόβιες συνθήκες, χρησιμοποιώντας ως πηγή άνθρακα μίγμα μελάσας και εμπορικής σακχαρόζης. Τω όντι, η συγκέντρωση του τελικού προϊόντος αυξήθηκε πάρα πολύ στις αερόβιες συνθήκες οι οποίες είχαν επιβληθεί, όπου παρήχθησαν 101.1 g l^{-1} 2,3-βουτανοδιόλης και 14.2 g l^{-1} ακετοΐνης. Οι τιμές αυτές είναι από τις πλέον ικανοποιητικές της διεθνούς βιβλιογραφίας στο εν λόγω επιστημονικό θέμα και για άγρια (μη-γενετικώς τροποποιημένα) βακτηριακά στελέχη χρησιμοποιούμενα ως κυτταρικά εργαστήρια. Στη συνέχεια πραγματοποιήθηκε ημι-συνεχής τροφοδοτούμενη καλλιέργεια σε βιοαντιδραστήρα στους 37 °C χρησιμοποιώντας μελάσα και σακχαρόζη ως πηγή άνθρακα, έτσι ώστε τα αποτελέσματα να συγκριθούν με τα αντίστοιχα που επετεύχθησαν στους 30 °C. Παρά το γεγονός ότι παρατηρήθηκε μικρή διαφορά στην τελική απόδοση, η παραγωγικότητα ήταν σημαντικά χαμηλότερη στους 37 °C. Τέλος, πραγματοποιήθηκε ημι-συνεχής τροφοδοτούμενη καλλιέργεια σε βιοαντιδραστήρα στους 30 °C χρησιμοποιώντας τη μελάσα ως τη μοναδική πηγή άνθρακα έτσι ώστε να μελετηθεί τυχόν παρεμπόδιση λόγω υποστρώματος, με την τελική συγκέντρωση προϊόντος και την παραγωγικότητα να είναι σημαντικά χαμηλότερες. Περαιτέρω υπήρξε ενδιαφέρον σχετικά με την πραγματοποίηση ζυμώσεων σε μη προηγουμένως αποστειρωθέν υπόστρωμα, γι' αυτό πραγματοποιήθηκαν δυο ημι-συνεχείς τροφοδοτούμενες ζυμώσεις σε κωνικές φιάλες, χρησιμοποιώντας στην πρώτη περίπτωση αποστειρωμένο θρεπτικό μέσο και στη δεύτερη περίπτωση μέσο που είχε υποστεί προηγούμενη παστερίωση. Η τελική παραγωγή προϊόντος ήταν ικανοποιητική ακόμα και στην περίπτωση τους προηγουμένως παστεριωμένου θρεπτικού μέσου.

Στο τρίτο μέρος της διδακτορικής διατριβής, πραγματοποιήθηκε αντίστοιχη μελέτη για το στέλεχος *Enterobacter* sp. FMCC-208 όπου προσδιορίστηκε η επίδραση διαφορετικών παραγόντων της ζύμωσης στην αύξηση και την παραγωγή 2,3-βουτανοδιόλης και ακετοΐνης. Έτσι, μελετήθηκε η επίδραση της θερμοκρασίας, της τιμής του pH αλλά και της αρχικής συγκέντρωσης του υποστρώματος. Το στέλεχος παρουσίασε σημαντική ικανότητα παραγωγής σε ένα μεγάλο εύρος θερμοκρασιών, αν και σε τιμές μεγαλύτερες από 37 °C, η παραγωγικότητα και η τελική απόδοση μειώθηκαν σημαντικά. Επίσης, καλύτερα αποτελέσματα σημειώθηκαν σε τιμές pH 6.5-7.0, ενώ δεν παρατηρήθηκε καθόλου αύξηση στην τιμή pH=5.0. Όσον αφορά στην παρεμπόδιση εκ του υποστρώματος, αυτή παρατηρήθηκε σε αρχική συγκέντρωση σακχαρόζης μεγαλύτερη από 90 g l^{-1}, ενώ το στέλεχος κατανάλωσε τη συνολική πηγή άνθρακα στις μικρότερες αρχικές συγκεντρώσεις. Η μελέτη του συγκεκριμένου στελέχους συνεχίστηκε όπως και για το προηγούμενο στέλεχος (*K. oxytoca* FMCC-197) με την καλλιέργειά του χρησιμοποιώντας διαφορετικές πηγές άνθρακα (γλυκόζη, φρουκτόζη, μαννόζη, ξυλόζη, αραβινόζη, γαλακτόζη, σακχαρόζη και μελάσα). Σε όλες τις περιπτώσεις καταναλώθηκε όλη η πηγή άνθρακα οδηγώντας στην παραγωγή ιδιαίτερα ικανοποιητικής συγκέντρωσης τελικού προϊόντος. Τέλος, αξιολογήθηκε η ικανότητα του στελέχους *Enterobacter* sp. FMCC-208 να αποχρωματίζει τη μελάσα σε αναερόβιες και αερόβιες συνθήκες, με το ποσοστό να ξεπερνά το 25% και στις δυο περιπτώσεις.

Λαμβάνοντας υπόψιν τα αποτελέσματα των παραπάνω ζυμώσεων, πραγματοποιήθηκαν ημι-συνεχείς τροφοδοτούμενες καλλιέργειες σε βιοαντιδραστήρα κάτω από διαφορετικές συνθήκες, με στόχο τη βελτιστοποίηση της βιοδιεργασίας. Οι δύο πρώτες ημι-συνεχείς τροφοδοτούμενες καλλιέργειες πραγματοποιήθηκαν στους 30 °C κάτω από αναερόβιες και αερόβιες συνθήκες, χρησιμοποιώντας ως πηγή άνθρακα μίγμα μελάσας και σακχαρόζης. Η συγκέντρωση του τελικού προϊόντος ήταν σημαντικά υψηλότερη στην περίπτωση των αερόβιων συνθηκών. Πιο συγκεκριμένα, η τελική συγκέντρωση της 2,3-βουτανοδιόλης ήταν 90.3 g l^{-1} και της ακετοΐνης 10 g l^{-1}. Οι τιμές αυτές μπορούν να χαρακτηριστούν ως αρκετά ικανοποιητικές σε σχέση με τη διεθνή βιβλιογραφία. Στη συνέχεια πραγματοποιήθηκε ημι-συνεχής τροφοδοτούμενη καλλιέργεια σε βιοαντιδραστήρα στους 37 °C χρησιμοποιώντας μελάσα και σακχαρόζη ως πηγή άνθρακα, έτσι ώστε τα αποτελέσματα να συγκριθούν με τα αντίστοιχα στους 30 °C. Αν και η τελική συγκέντρωση προϊόντος ήταν χαμηλότερη στους 37 °C, παρατηρήθηκε σημαντική αύξηση στην κατ' όγκο παραγωγικότητα του συστήματος ζύμωσης. Τέλος, πραγματοποιήθηκε ημι-συνεχής τροφοδοτούμενη καλλιέργεια σε βιοαντιδραστήρα στους 37 °C χρησιμοποιώντας τη μελάσα ως μοναδική πηγή άνθρακα. Η σημαντική μείωση της απόδοσης της ζύμωσης σχετίστηκε με την παρεμπόδιση λόγω αυξημένης παρουσίας προσμίξεων και τοξικών συστατικών οι οποίες υπάρχουν στο υπόστρωμα τύπου μελάσας. Τέλος, πραγματοποιήθηκαν δυο ημι-συνεχείς ζυμώσεις σε κωνικές φιάλες, χρησιμοποιώντας στην πρώτη περίπτωση αποστειρωμένο θρεπτικό μέσο και στη δεύτερη

περίπτωση μέσο που είχε υποστεί παστερίωση. Το στέλεχος *Enterobacter* sp. FMCC-208 παρουσίασε ικανοποιητική αύξηση και τελική παραγωγή προϊόντος ακόμα και στην περίπτωση παστεριωμένου θρεπτικού μέσου.

Η παρούσα μελέτη δεικνύει ότι τα δύο άγρια στελέχη που χρησιμοποιήθηκαν στα πειράματα, ήτοι τα στελέχη *K. oxytoca* FMCC-197 και *Enterobacter* sp. FMCC-208, μπορούν να αξιοποιηθούν ως μικροοργανισμοί για την παραγωγή 2,3-βουτανοδιόλης και ακετοΐνης, χρησιμοποιώντας μάλιστα μεγάλη ποικιλία υποστρωμάτων χαμηλού κόστους στην προτεινόμενη βιοδιεργασία.

Λέξεις κλειδιά: 2,3-βουτανοδιόλη, ακετοΐνη, μελάσα, μικροβιακή ζύμωση, *Klebsiella oxytoca*, *Enterobacter* sp.

LIST OF ABBREVIATIONS

BDO: 2,3-butanediol

Ace: Acetoin

TS: Total sugars

X_{max}: Maximum biomass concentration

DCW: Dry cell weight

$Y_{BDO,Ace}$: Bioconversion yield of substrate to BDO and Ace

$P_{BDO,Ace}$: Volumetric productivity of BDO and Ace production (g l^{-1} h^{-1})

Suc: Succinic acid

Lac: Lactic acid

Eth: Ethanol

DOT: Dissolved Oxygen Tension (%, v/v)

AIM OF THE STUDY

Each year, huge quantities of industrial or household wastes are produced, most of them being hazardous for the environment. On the other hand, the increasing energy demands require novel sustainable and "green" technologies for efficient conversion of renewable raw materials into bio-based chemicals and fuels. Such a chemical compound produced via microbial fermentation is that of 2,3-butanediol (BDO) and its derivative acetoin (Ace). Both the above-cited compounds, present an important number of applications in the manufacture of printing inks, perfumes, fumigants, moistening and softening agents, explosives, plasticizers, foods and pharmaceuticals.

During the first part of the research, nine food-derived, newly isolated and putatively non-pathogenic bacterial strains were cultivated in batch experiments using glucose and sucrose as carbon source, in order to estimate their ability to assimilate these types of carbon source and produce BDO. Two strains, namely *Klebsiella oxytoca* FMCC-197 and *Enterobacter* sp. FMCC-208 were selected for further investigations as they combined high bioconversion yields and productivity rates for both carbon sources.

The second part of the study focused on the evaluation of the ability of the strain *K. oxytoca* FMCC-197 to grow on different initial sucrose concentrations, different incubation temperature values and various carbon sources. These sets of experiments were conducted under aerobic conditions in shake flasks. *K. oxytoca* FMCC-197 was capable of growing even in high initial carbon source concentration (up to 90 g l^{-1}) without any substrate inhibition. Satisfactory results were also obtained in a wide range of temperature values (from 25 °C to 42 °C), whereas various carbon sources (glucose, fructose, mannose, arabinose, galactose and xylose) applied into the culture medium were converted into BDO and Ace. Different fed-batch fermentation strategies were carried out altering various parameters in order to optimize the process. For instance, 4 fed-batch experiments were conducted at bioreactors under anaerobic and aerobic conditions, altering the temperature value and the carbon source. In addition, 2 fed-batch experiments under aerobic conditions in shake flasks were also carried out using sterilized and pasteurized substrate, respectively.

During the third part of the study, different cultural parameters were investigated during batch cultures using the strain *Enterobacter* sp. FMCC-208, in order to optimize the BDO and Ace production process. Therefore, the impact of substrate (sucrose) concentrations, the temperature effect and the assimilation of different carbon sources were evaluated as regards biomass and BDO production during aerobic batch fermentations in shake flasks. The strain *Enterobacter* sp. FMCC-208 was capable of growing on a wide range of initial sucrose concentration without any substrate inhibition, even in values higher than 90 g l^{-1}. The strain was able to successfully grow in a wide range of incubation temperature values (from 25 °C to 42 °C), while several carbon sources

(glucose, fructose, mannose, arabinose, galactose and xylose) applied into the culture medium as individual substrates were assimilated leading to remarkable production of BDO and Ace. Thereafter, fed-batch bioreactor experiments were performed under different aeration modes and temperature values using molasses or molasses and sucrose blends as carbon sources, in order to maximize the final product accumulation into the medium. In addition, 2 fed-batch experiments under aerobic conditions in shake flasks were also carried out using sterilized and pasteurized substrate, respectively. The ability of the strain *Enterobacter* sp. FMCC-208 to decolorize molasses was also determined during anaerobic and aerobic experiments.

For both strains tested, BDO production was very satisfactory, comparable with the maximum values reported for wild-type strains deriving from official culture collections. In several cases, BDO and Ace production was even higher compared with the one achieved by genetically modified or mutant bacterial strains. From the results obtained, the newly isolated strains *K. oxytoca* FMCC-197 and *Enterobacter* sp. FMCC-208 are highly promising BDO and Ace producers. These strains can be successfully used in large scale bioprocesses for the bioconversion of wastes rich in carbohydrates into BDO and Ace.

1. Introduction

1.1. General approach of White Biotechnology

The abundance of energy supplies and organic chemical resources is the key for sustainability of human civilization. The world is currently facing severe energy crisis due to the incessant increase of energy demands and the gradual depletion of fossil fuels, while also, huge quantities of industrial or household wastes are produced each year, most of them hazardous for the environment. The problem becomes worst as many areas that are becoming industrialized do not yet have the resources or technology to dispose of waste with lesser effects on the environment and, therefore, untreated wastes are usually released in the seas or fields. The toxic pollutants which are usually contained in the wastes can cause serious problems to the local ecosystems. Therefore, new manufacturing concepts are being developed continuously for the production of fuels, organic chemicals, polymers, and materials from biomass and wastewaters, using complex processing technologies. These manufacturing concepts are analogous to today's integrated petroleum refinery and petrochemical industry commonly known as biorefinery.

Although microorganisms have been used for the production of various useful products since the ancient years, the first processes in large scale were performed the previous century for the production of acetic and citric acid, respectively. Moreover, Strecker (1854) observed for the first time the formation of propionic acid from sugar, while Pasteur (1861; 1879) demonstrated that the phenomena of fermentation, including the propionic and butyric acid fermentations, were due to the activities of living microbes. In 1905, Schardinger reported the production of acetone by *Bacillus macerans*. In addition, it was Fernbach and Strange (1911) who observed the production of both acetone and butanol by a *Bacillus* strain. In 1881, Freund, demonstrated microbial fermentations of the strain *Clostridium pasteurianum* producing 1,3-propanediol from glycerol (Freund, 1881). Since then, the field of bioprocess and systems metabolic engineering has made big steps for the production of numerous C2–C6 platform chemicals, through fermentation and biotransformation, which can be further used for the production of chemical intermediates, building block compounds and polymers.

Microbial Biotechnology studies the parameters, such as the medium composition, the process optimization, etc., for microbial growth and efficient production of desired metabolites. Moreover, it attempts to develop and study microbial strains on the basis of whole optimized bioprocess in order to maximize the production yield and productivity of the targeted chemical compounds, while minimizing overall operation costs that incur throughout the upstream and downstream processes. For this great challenge, intracellular metabolic fluxes are optimized towards the overproduction of the targeted chemical compounds by using various molecular and high-throughput techniques, including, but not limited to conventional gene knockout and overexpression (Jang et al., 2012a; 2012b), adaptive laboratory evolution (Li et al., 2016),

construction of novel metabolic pathways using promiscuous enzymes (Atsumi et al., 2008), sophisticated downregulation of gene expression levels (Yoo et al., 2013), multiple enzyme targets (Flowers et al., 2013), multiple genome engineering (Isaacs et al., 2011; Wang et al., 2009), synthetic regulatory circuits (Thieffry, 2007), omics analysis (Park et al., 2007) and in silico modeling and simulation (Yim et al., 2011). Table 1.1. summarizes the biofuels and chemicals produced via microbial fermentations.

Table 1.1. Representative fuels and chemicals recently produced using systems metabolic engineering.

Biofuels and chemicals		Production performances			Strains	References
		Titer ($g\ l^{-1}$)	Yield ($g\ g^{-1}$)	Productivity ($g\ l^{-1}\ h^{-1}$)		
Biofuels	Ethanol	48	0.37	0.79	*S. cerevisiae*	Ha et al. (2011)
	1-propanol	10.8	0.11	0.14	*E. coli*	Choi et al. (2012)
	1-butanol[a]	585.3	0.31	1.32	*C. acetobutylicum*	Jang et al. (2012)
Building block chemicals	Succinic acid[b]	36.1	0.47		*E. coli*	Yang et al. (2014)
	Fumaric acid[b]	28.2	0.39	0.448	*E. coli*	Song et al. (2013)
	Cadaverine[b]	9.61		0.32	*E. coli*	Qian et al. (2011)
	2,3-butanediol	73.8	0.41	1.19	*E. coli*	Yim et al. (2011)
Specialty chemicals	L-Lysine[b]	120	0.55	4	*C. glutamicum*	Becker et al. (2011)
	L-Valine[b]	61	0.30	2.1	*E. coli*	Park et al. (2011)
	L-Tyrosine[b]	13.8	0.12	0.38	*E. coli*	Santos et al. (2012)

[a] Fed-batch fermentation with *in situ* recovery.

[b] Fed-batch fermentation.

Low cost materials which are rich in carbohydrates can be successfully converted to organic acids and bio-based energy fuels. Lignocellulosic biomass is the most common substrate which is used in microbial fermentations (Asgher et al., 2013; Iqbal et al., 2013). For instance, the main components of woody biomass are lignin, cellulose and hemicellulose. Cellulose is a polymer of D-glucose, while hemicellulose is a polymer containing mostly D-xylose, L-arabinose and D-ribose (Rosenberg, 1980). Numerous studies so far have focused on the utilization of woody biomass as a by-product of industrial activities for the production of organic acids such as succinic acid and 2,3-butanediol (BDO). In addition, the two major by-products of oilseed-based biodiesel production processes are crude glycerol and oilseed cakes or meals (Koutinas et al., 2007; Papanikolaou & Aggelis, 2009; 2011; 2019; Lomascolo et al., 2012). Oilseed meals represent by-product streams remaining after oil extraction from oilseeds, such as rape-seed, soybean and sunflower. Glycerol is generated as a 10% (w/w) by-product that is generated following trans-esterification of triacylglycerols into fatty acid alkyl-esters in the presence of alcohol (Papanikolaou & Aggelis, 2009; 2011; Chatzifragkou & Papanikolaou, 2012; Lomascolo et al., 2012). Substrates enriched with these by-products can successfully be applied in microbial fermentations for the production of citric acid, 1,3-propanediol, 2,3-butanediol, polyols, PHAs, microbial oil, etc.

It is worth mentioning that microbial fermentations using food waste led to remarkable production of added-value products. In particular, fruit waste main fraction consists of carbohydrates (sucrose, fructose and glucose) while vegetables are rich in structural domains like lignin, cellulose and hemicelluloses with monosaccharides (glucose, xylose, galactose, mannose, arabinose) being their fundamental units. The ability of various natural bacterial strains to convert these substrates into organic compounds such as BDO (Liakou et al., 2018) has been well-studied with very promising results. Saha et al. (1999), has also achieved remarkable BDO production using an *Enterobacter cloacae* strain on different commercial carbon sources. Other industrial by-products which have been used in microbial fermentations are starch hydrolysates, sugarcane molasses and algea. For instance, Perego et al. (2000) has shown that the strain *Enterobacter aerogenes* NCIMB10102 could successfully convert wastes like corn starch hydrolysate, raw molasses and cheese whey into BDO.

Figure 1.1. presents the most common building blocks and platform chemicals which are obtained from the biorefineries. In conclusion, it should be stressed that the development of efficient microorganisms capable of producing target compounds at sufficiently high titers, yields, and industrial production levels remain significant challenges and major limiting steps. The optimization of downstream processes is interrelated with the development of host microorganisms and both will play important roles in reducing production costs and allowing bio-based processes to compete against the current petrochemical processes. Koutinas et al. (2014) have conducted a

techno-economic evaluation of the complete bioprocess of BDO production from renewable resources proving that efficient metabolite production can be achieved.

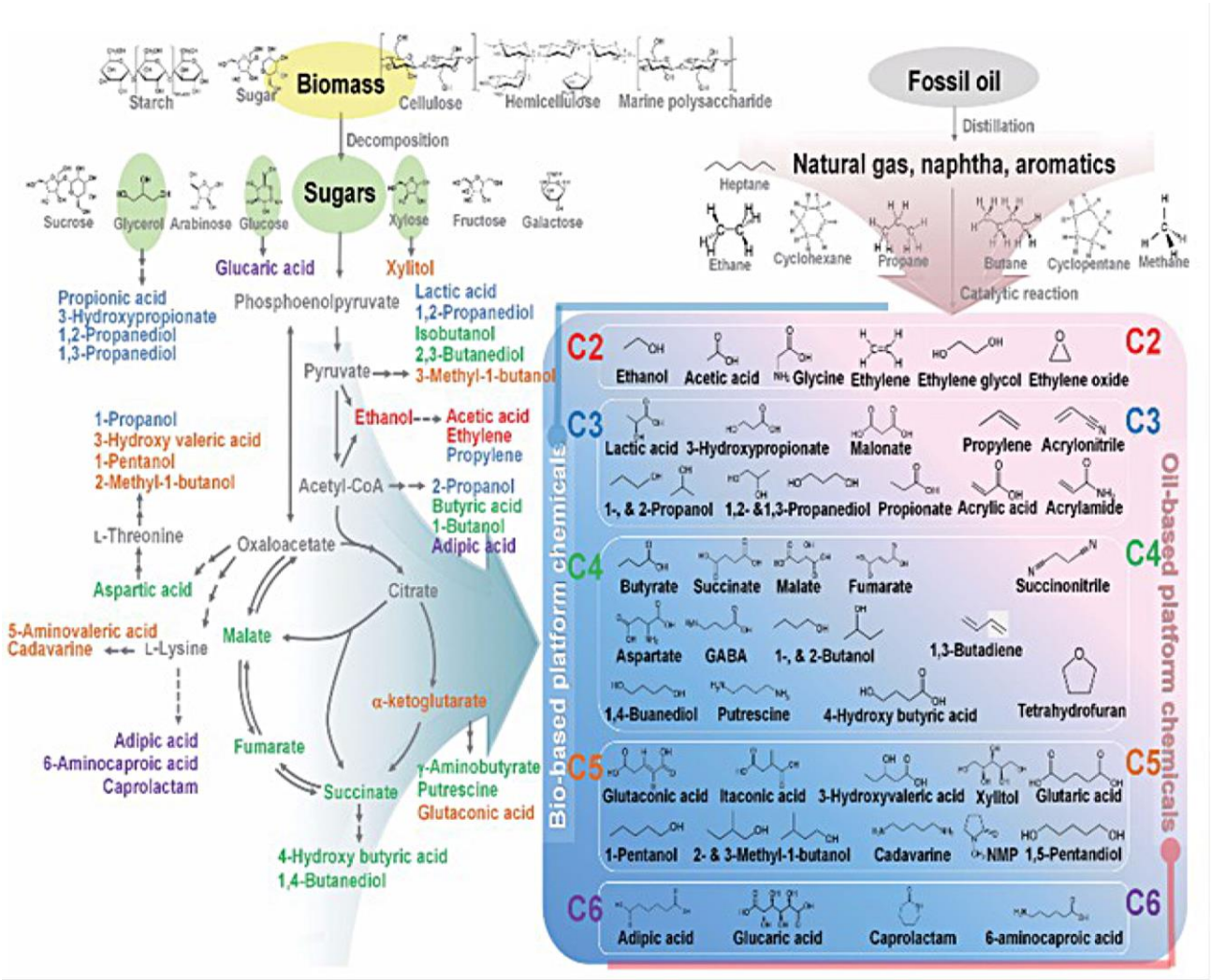


Figure 1.1. Platform chemical production trends are shifting away from petroleum-based methods to biology-based processes. Representative building blocks and platform chemicals that are currently obtained mostly from fossil oil are shown (right bottom box, not all platform chemicals currently produced are shown). Most of the platform chemicals currently produced, are generally derived from petroleum fractions. Chemicals obtained from the refineries are used as precursors for preparing plat form chemicals (red arrow). Many efforts are currently being directed toward developing new bio-based technologies (blue arrow, i.e., bio-refineries) capable of producing the same platform chemicals (blue shades replacing red shades) while addressing environmental concerns. Simplified biosynthetic networks (gray arrows) for producing platform chemicals (or corresponding intermediate chemicals) that can occur in a microorganism are illustrated (left portion of the figure). The end products and intermediates produced from various metabolic pathways vary by their carbon numbers. The different font colors represent platform chemicals with different carbon numbers: red, 2 carbons; blue, 3 carbons; green, 4 carbons; orange, 5 carbons; and purple, 6 carbons. The renewable carbon sources (or precursors) that can be used for producing such chemicals include sucrose, glycerol, arabinose, glucose, xylose, fructose, and galactose (green oval), which are obtained by decomposition of starch, sugarcane, plants, and algae (yellow oval).

1.2. Physicochemical characteristics of 2,3-butanediol

The colorless and odorless liquid 2,3-butanediol ($C_4H_{10}O_2$), which is also called 2,3-butylene glycol, dimethylethylene glycol or 2,3-dihydroxybutane, has a very high boiling point (180-184 °C) and low freezing point (-60 °C). Its molecular weight is 90.121 $g.mol^{-1}$. BDO can be found in three different isomers. Two of them, dextro- [L- (+)-] and levo- [D-(-)-] forms, are optically active. The third one is an optically inactive meso form (Celińska & Grajek, 2009). The differences among the three stereoisomers of BDO are presented in Figure 1.2. and Figure 1.3..

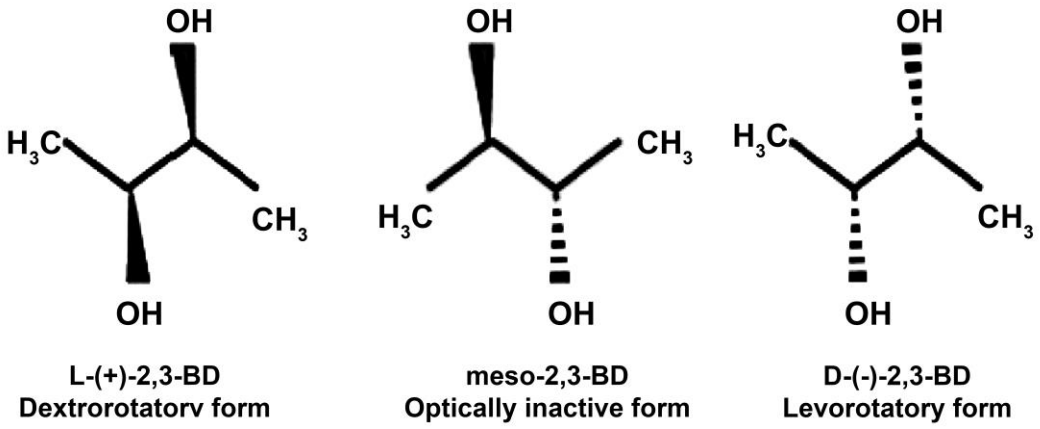


Figure 1.2. Stereoisomers of 2,3-butanediol (Celińska & Grajek, 2009).

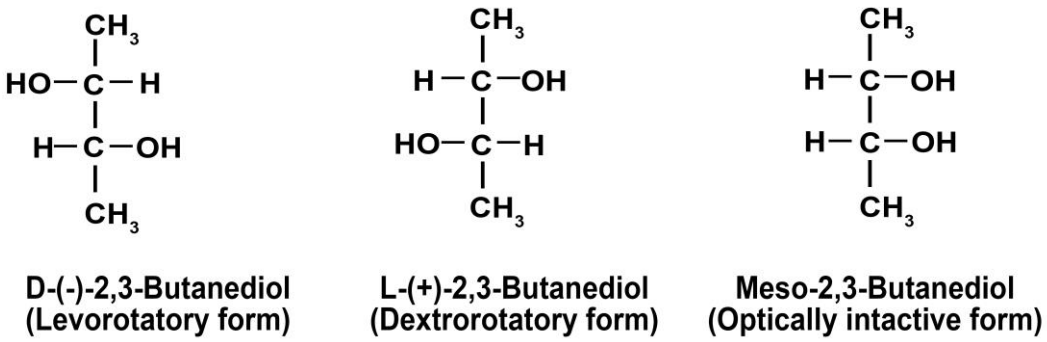


Figure 1.3. Stereoisomers of 2,3-butanediol (Celińska & Grajek, 2009).

1.3. Applications of 2,3-butanediol

BDO is a metabolic compound with various applications on several industrial fields. Although the fermentation through which BDO is produced was first described in the early part of the 20th century, great interest came during wartime, as it can be converted to 1,3-butadiene, which is used in synthetic rubber (Duan et al., 2015; Ji et al., 2010; Koutinas et al., 2014). Additionally, it has potential applications in the manufacture of printing inks, perfumes, fumigants, moistening and softening agents, explosives, plasticizers, foods, and pharmaceuticals (Garg & Jain, 1995; Syu, 2001). For instance, methyl-ethyl-ketone, the dehydration product of BDO can be used as an excellent organic solvent for resins and lacquers, while it can also find applications as liquid fuel having a higher heat of combustion than ethanol (Ji et al., 2010; Zeng & Sabra, 2011). BDO can be also dehydrogenated to form acetoin and diacetyl which are two high added-value compounds. Furthermore, BDO can be ketalized with acetone to produce a "tetramethyl" compound, which is a potential gasoline blending agent similar to the commonly used methyl tert-butyl ether (MTBE) (Ji et al., 2010; Koutinas et al., 2014). In another case, the dehydration of BDO led to the recovery of 3-buten-2-ol catalyzed by ZrO_2 (Duan et al., 2014). Figure 1.4. presents the various derivatives from BDO.

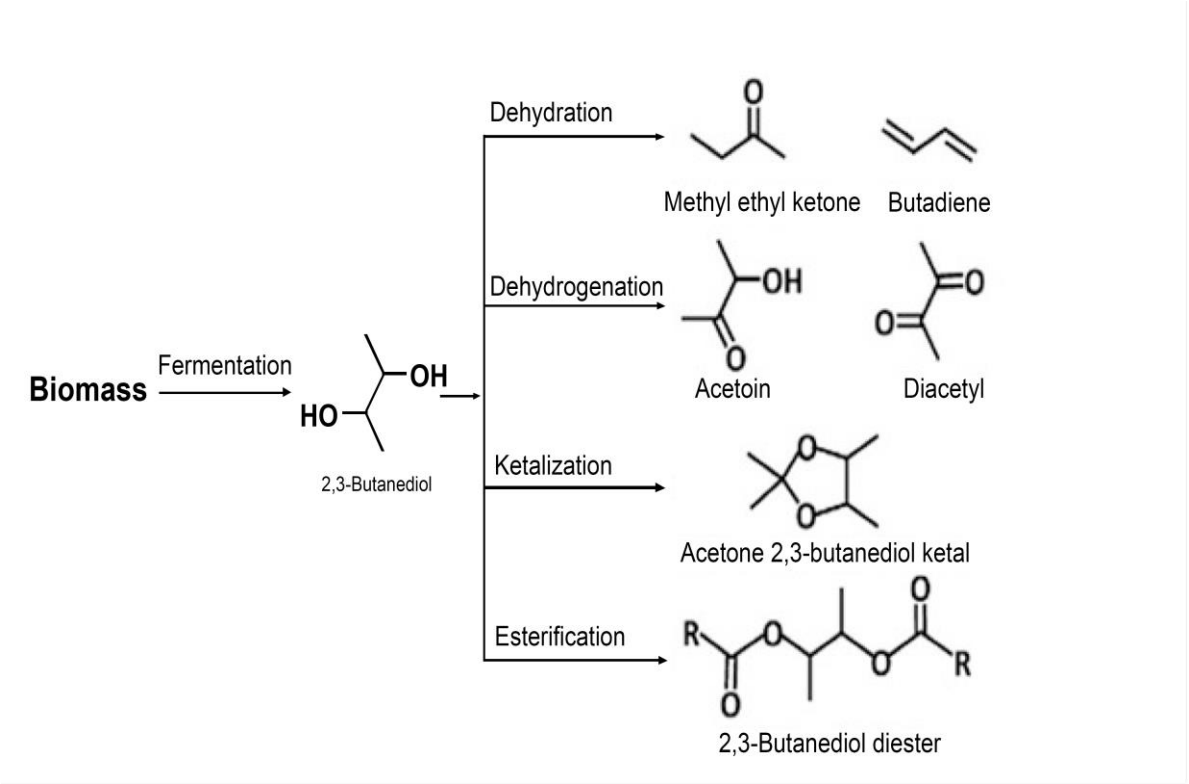


Figure 1.4. Derivatives of biologically produced 2,3-butanediol (Ji et al., 2011).

1.4. Biotechnological production of BDO through microbial fermentation

BDO can be produced via microbial fermentations, where mainly bacterial strains are implicated as microbial cell factories, when various commercial substrates, residues and low or negative cost materials are employed as carbon sources. The species which are predominately referred in the literature for their ability to accumulate into the medium BDO in significant quantities belong to the genera *Klebsiella*, *Enterobacter*, *Bacillus* and *Serratia* (Maddox, 1996). Additionally, various strains of the species *Lactobacillus* and *Lactococcus* have also the potential to produce BDO in remarkable concentrations (Celinska & Grajek, 2009). As it has already been mentioned, there are three isomers which can be produced via a bacterial fermentation, dextro- [L-(+)-] and levo- [D-(-)-] forms which are optically active and an optically inactive meso form. The isomer produced during bacterial fermentations depends from the microorganism which is used. Different microorganisms produce various isomers. In general, a mixture of two isomers is produced during a given bioprocess (Celinska & Grajek, 2009; Maddox, 1996). Most of the species such as *K. pneumoniae* (Ma et al., 2009), *K. oxytoca* (Cheng et al., 2010) and *E. aerogenes* (Zeng et al., 1991; Perego et al., 2000) produce the meso- and dextro- form. However, there are numerous studies in the literature which show that the strain *Paenibacillus polymyxa* has the ability to produce the pure levo- form that can be used as an antifreeze, due to the special properties of the isomer (Marwoto et al., 2004; Nakashimada et al., 1998; Soltys et al., 2001). The most common species able to produce BDO are presented in Table 1.2.. Recently however, numerous studies which focused on the BDO production from other species have been published. Thus, Qiu et al. (2016) have developed an engineered strain of *B. licheniformis* suited to produce high titers of the pure meso-BDO isomer. In a fed-batch fermentation, meso BDO titer reached 98.0 g l^{-1} with a purity of >99.0 % and a productivity of 0.94 g $l^{-1}h^{-1}$. In another case, a wild-type *B. subtilis* 168 was found to generate only D-(−)-BDO (purity >99 %) under low oxygen conditions (Fu et al., 2014). Furthermore, *Escherichia coli* was optimized to produce 73.8 g l^{-1} meso-BDO with a yield of 0.41 g g^{-1} glucose (Xu et al., 2014) and 54 g l^{-1} meso-BDO from glucose and xylose mixture with a productivity of 0.45 g l^{-1} h^{-1} using biomass-inducible chromosome-based expression system (Nakashima et al., 2014). The redirection of the carbon flow of the strain *Zymomonas mobilis* in order to produce not ethanol but BDO has been presented by Yang et al. (2016). The best gene combinations enabled *Z. mobilis* to reach a remarkable BDO production from glucose and xylose, as well as mixed C6/C5 sugar streams derived from the deacetylation and mechanical refining process.

McEwen et al. (2016) cultivated an obligate photoautotrophic cyanobacterium for enhanced production of BDO in continuous illumination, 12 h:12 h light-dark diurnal, and continuous dark conditions via supplementation with glucose or xylose. They achieved BDO production under

diurnal conditions comparable to production under continuous light conditions. The maximum BDO titer was 3.0 g l^{-1} in 10 days. Similar microorganisms have also been used in two other studies in order to demonstrate heterologous BDO production in heterologous hosts of the cyanobacteria *Synechococcus elongatus* (Nozzi et al., 2015; Oliver et al., 2014).

Table 1.2. Metrics of 2,3-butanediol production from different bacterial strains cultivated on various carbon sources and fermentation configurations.

Strain	Substrate	BDO ($g\ l^{-1}$)	Yield ($g\ g^{-1}$)	Fermentation mode	Reference
Bacillus polymyxa ATCC 12321	Glucose	24.7	0.38	Batch/ Shake flasks	de Mas et al. (1987)
	Xylose	3.1	0.14		
	Galactose	19.3	0.40		
	Mannose	20.6	0.37		
	Arabinose	21.9	0.34		
	Fructose	12.4	0.30		
Bacillus licheniformis DSM 8785	Glucose	127.4	0.37	Fed-batch /Bioreactor	Jurchescu et al. (2013)
Bacillus licheniformis DSM 8785	Glucose	100.6 (+5.7 Ace[#])	0.31	Fed-batch /Bioreactor	Jurchescu et al. (2013)
Bacillus licheniformis DSM 8785	Glucose	144.7	0.40	Fed-batch/ Shake flasks	Jurchescu et al. (2013)
Bacillus licheniformis NCIMB 8059	Apple pomace hydrolysates	113	0.49	Fed-batch/ Bioreactor	Białkowska et al. (2015a)
Bacillus licheniformis 10-1-A	Glucose	116	0.47	Fed-batch/ Bioreactor	Li et al. (2013)
Bacillus subtilis TUL 322	Sugarcane molasses	75	0.31	Fed-batch/ Bioreactor	Białkowska et al. (2015b)
Bacillus amyloliquefaciens	Cane sugar	127.3	0.50	Fed-batch/ Bioreactor	Maina et al. (2019)
Enterobacter cloacae NRRL B-23289	Glucose	18.6	0.37	Batch/ Shake flasks	Saha et al. (1999)
	Xylose	18.9	0.38		
	Galactose	18.9	0.38		
	Mannose	18.6	0.37		
	Arabinose	21.7	0.43		
Enterobacter cloacae CGMCC 605	Sugarcane molasses	90.8 (+8.7 Ace[#])*	0.39	Fed-batch/ Bioreactor	Dai et al. (2015)
Enterobacter cloacae CGMCC 605	Glucose	110.9 (+8.8 Ace[#])*	0.42	Fed-batch/ Bioreactor	Dai et al. (2015)
Enterobacter cloacae SDM	Corn stover	119.4	0.47	Fed-batch/ Bioreactor	Li et al. (2015)
Enterobacter aerogenes DSM 30053	Glucose	110	0.48	Fed-batch/ Bioreactor	Zeng et al. (1991)
Enterobacter aerogenes EMY-01 (KCTC ΔldhA)	Glucose	118.1	c. 0.46	Fed-batch/ Bioreactor	Jung et al. (2012)
Enterobacter aerogenes KCTC 2190-EMY68	Sugarcane molasses	98.7	0.37	Fed-batch/ Bioreactor	Jung et al. (2013)
Enterobacter aerogenes EMY-70SP	Sugarcane molasses	129.4	0.39	Fed-batch/ Bioreactor	Jung et al. (2015)
Enterobacter aerogenes EMY-70S	Sugarcane molasses	140.0	0.39	Fed-batch/ Bioreactor	Jung et al. (2015)
Enterobacter ludwigii FMCC-204	Fruit extract	18.2	0.36	Batch/Shake flasks	Liakou et al. (2018)
		50.1	0.40	Fed-batch /Bioreactor	
Klebsiella oxytoca ME-UD-3	Glucose	130	0.48	Fed-batch/ Bioreactor	Ji et al. (2010)
Klebsiella oxytoca M1	Glucose	19.0	0.32	Batch/ Shake flasks	Cho et al. (2013)
	Xylose	17.1	0.28		
	Galactose	15.1	0.25		

	Fructose	18.2	0.29		
Klebsiella oxytoca NBRF4	Glucose	34.2	0.35	Fed-batch/ Bioreactor	Han et al. (2013)
Klebsiella oxytoca M1	Glucose	118.5 (+42.1 Ace[#])	0.46	Fed-batch/ Bioreactor	Cho et al. (2015a)
Klebsiella oxytoca M3	Crude glycerol	131.5	0.44	Fed-batch/ Bioreactor	Cho et al. (2015b)
Klebsiella pneumoniae CICC 10011	Glucose	52.4	0.38	Batch/ Shake flasks	Qin et al. (2006)
K. pneumoniae SDM	Corn steep liquor	*c.* 151	0.48	Fed-batch/ Bioreactor	Ma et al. (2009)
Klebsiella pneumoniae CGMCC 1.9131	Sugarcane hydrolysate	17.35	0.43	Batch/ Shake flasks	Song et al. (2012)
K. pneumoniae G31	Glycerol	*c.* 70	0.39	Fed-batch/ Bioreactor	Petrov & Petrova (2010)

*: Non-aseptic experiment

#: Acetoin

Although bacterial strains have been used so far for the biotechnological production of BDO, other microorganisms can also ferment various substrates into BDO. The yeast *Saccharomyces cerevisiae* has been studied for its ability to produce BDO. The research has shown that in *S. cerevisiae*, acetaldehyde, pyruvate and α-acetolactate are the precursors of BDO. The biosynthetic pathway with diacetyl as intermediate is similar with that of bacteria. However, α-acetolactate decarboxylase is not found in the most strains. Instead the yeast can synthesize acetoin via the condensation of active acetaldehyde with acetaldehyde by pyruvate decarboxylase. In addition, the carboligase mechanism of pyruvate decarboxylase for the synthesis of acetoin from the reaction between pyruvate and acetaldehyde has been elucidated previously. Acetoin is then converted to BDO by butanediol dehydrogenase. Recently, an engineered *S. cerevisiae* strain was efficiently applied on xylose-based fermentation medium producing remarkable final BDO concentration (Kim et al., 2014). An engineered *S. cerevisiae* strain was also used for BDO production using glucose as the carbon source (Kim et al., 2013; Kim et al., 2015). In another case more than 100 g l^{-1} of BDO was synthesized from a mixture of glucose and galactose, two major carbohydrate components in red algae (Lian et al., 2014). Table 1.3. summarizes the microorganisms which have been studied so far for the BDO production.

Table 1.3. Microorganisms capable of producing 2,3-butanediol

Type of microorganisms	Name of microorganisms
Bacteria, cyanobacteria	*K. pneumoniae*, K. oxytoca*, Klebsiella terrigena, B. licheniformis*, B. amyloliquefaciens*, S. marcescens*, B. subtilis*, Bacillus stearothermophilus, Bacillus cereus, E. aerogenes, Peanibacillus polymyxa, Pseudomonas putida, Aeromonas hydrophilia, Aerobacter aerogenes, Brevibacillus brevis, Corynebacterium glutamicum, Lactobacillus brevis, Lactobacillus casei, Lactobacillus helveticus, Lactobacillus plantarum, Lactococcus lactis, Lactococcus lactis subsp. lactis bv. diacetylactis, Leuconostoc lactis, Lactococcus mesenteroides subsp. cremoris, Oenococcus oeni, Pediococcus pentosaceus, Raoultella planticola, Morganella morganii, Pantoea sp., Serratia plymuthica, Clostridium autoethanogenum, Clostridium ljungdahlii, Clostridium ragsdalei, Pseudomonas chlororaphis 06, Escherichia coli, Synechocystis sp., S. elongatus*
Yeasts	*Saccharomyces cerevisiae, Kloeckera apiculata, Zygosaccharomyces bailii, Saccharomycodes ludwigii, Hanseniaspora uvarum*
Marine microalgae	*Chlamydomonas perigranulata*

List of microorganisms was modified, based on Celinska & Grajek (2009), Nan et al. (2014), Hon-Nami (2006)

* The most efficient producers of 2,3-butanediol

A variety of monosaccharides, both hexoses and pentoses, can be converted to BDO (Syu, 2001). As shown in Figure 1.5., in bacterial metabolism, monosaccharides are initially converted to pyruvate before generation of major products. From glucose, pyruvate is formed via the Embden-Meyerhof pathway (glycolysis). In contrast, the production of pyruvate from pentoses must proceed via a combination of the pentose phosphate, the phospho-ketolase and the Embden-Meyerhof pathways (Jansen & Tsao, 1983; Papanikolaou & Aggelis, 2011; Koutinas et al., 2014; Sarris & Papanikolaou, 2016). In addition to BDO, a mixture of acetate, lactate, formate, succinate, acetoin, and ethanol are also produced through the mixed acid - BDO fermentation pathway (Magee & Kosaric, 1987; Maddox, 1996; Papanikolaou, 2009; Koutinas et al., 2014).

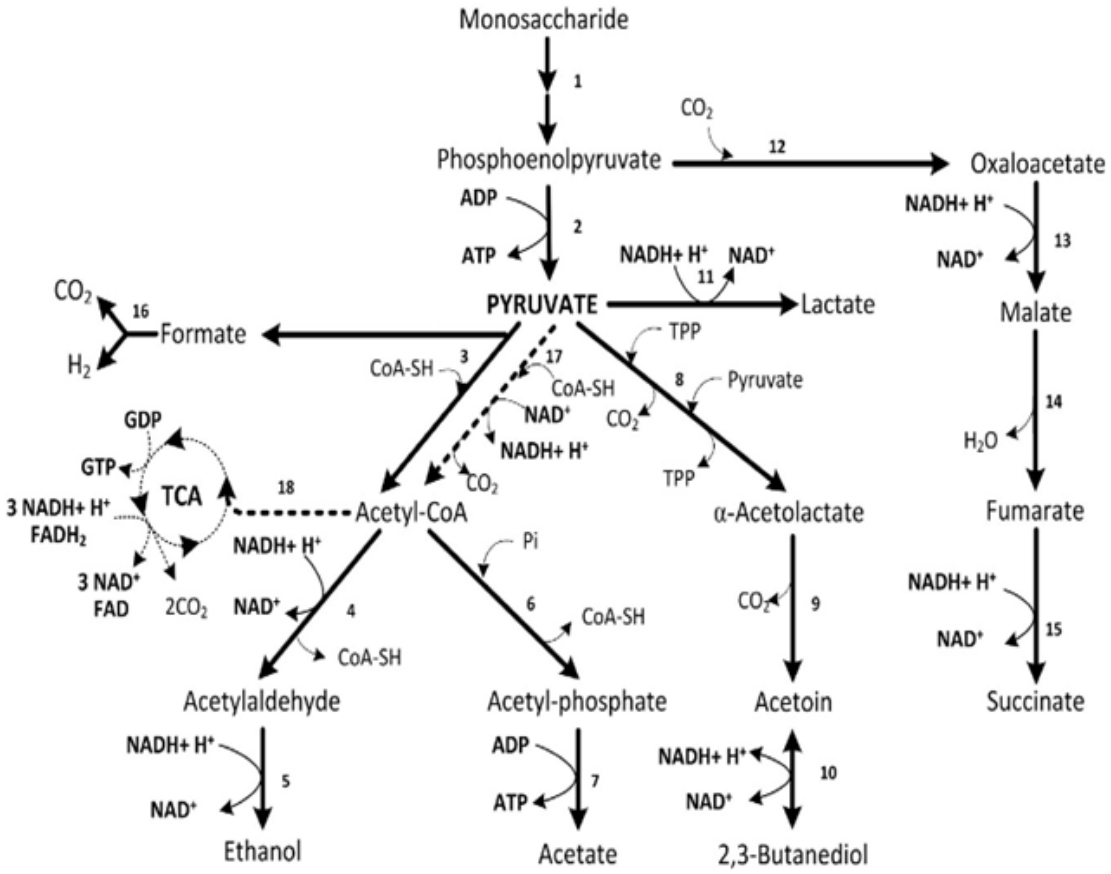


Figure 1.5. Mixed acid-2,3-butanediol pathway (modified, based on Maddox, 1996; Magee & Kosaric, 1987; dashed lines represent the pathways that are active only under the aerobic conditions). TCA, tricarboxylic acids cycle; 1, Embden–Meyerhof and pentose phosphate pathway enzymes; 2, pyruvate kinase; 3, pyruvate– formate lyase; 4, acetaldehyde dehydrogenase; 5, ethanol dehydrogenase; 6, phospho-transacetylase; 7, acetate kinase; 8,α-acetolactate synthase; 9, α-acetolactate decarboxylase; 10. acetoin reductase (2,3-butanediol dehydrogenase); 11, lactate dehydrogenase; 12, phosphoenolpyruvate decarboxylase; 13, malate dehydrogenase; 14, fumarase; 15, succ inate dehydrogenase; 16, formate-hydrogen lyase complex; 17, pyruvate dehydrogenase multi-enzyme complex; and 18, citroyl synthetase (Ji et al., 2011).

Figure 1.5. represents the mixed acid-2,3-butanediol pathway along with the enzymes which are involved in each conversion. There are three key enzymes involved in the BDO pathway, α-acetolactate synthase, α-acetolactate decarboxylase and 2,3-BDO dehydrogenase, also called acetoin reductase. The first, termed catabolic α-acetolactate synthase, has an optimum pH of 5.8 while the other enzyme, is called anabolic α-acetolactate synthase. As for α-acetolactate decarboxylase, it has an optimum pH of about 6.3 and catalyzes the decarboxylation of acetolactate to acetoin. Acetoin reductase catalyzes a reversible reduction of acetoin to BDO and an irreversible reduction of diacetyl to acetoin. At the end of the fermentation, the type of stereoisomer which is formed is related with the genes expressed. One of the initial models for stereoisomer formation was postulated by Taylor and Juni (1960) for *K. pneumoniae*. They proposed the existence of an acetoin racemase, L(+) BDO dehydrogenase and D(-) BDO dehydrogenase. The L(+) BDO dehydrogenase would convert L(+) acetoin to L(+) BDO and meso-BDO, whereas the D(-) BDO dehydrogenase would reduce D(-) acetoin to D(-) BDO and meso-BDO. A newer model for *K. pneumoniae* was similar to the earlier one, however in this model D(-) acetoin is converted to meso-BDO and L(+) acetoin is converted to L(+) BDO. This model is based on the purification and separation of the two acetoin reductases and the determination of their sterospecificity (Voloch et al., 1983). It should be stressed that research has revealed that the genes coding the three main enzymes for BDO pathway are clustered in one operon. The genes were sequenced and called “budABC”. The budABC operon appears to be regulated at the transcriptional level, as the highest amount of transcript was seen under conditions that favored BDO production (Blomqvist et al., 1993). The following figure (Fig. 1.6.) represents the BDO cycle in bacteria when glucose is the carbon source.

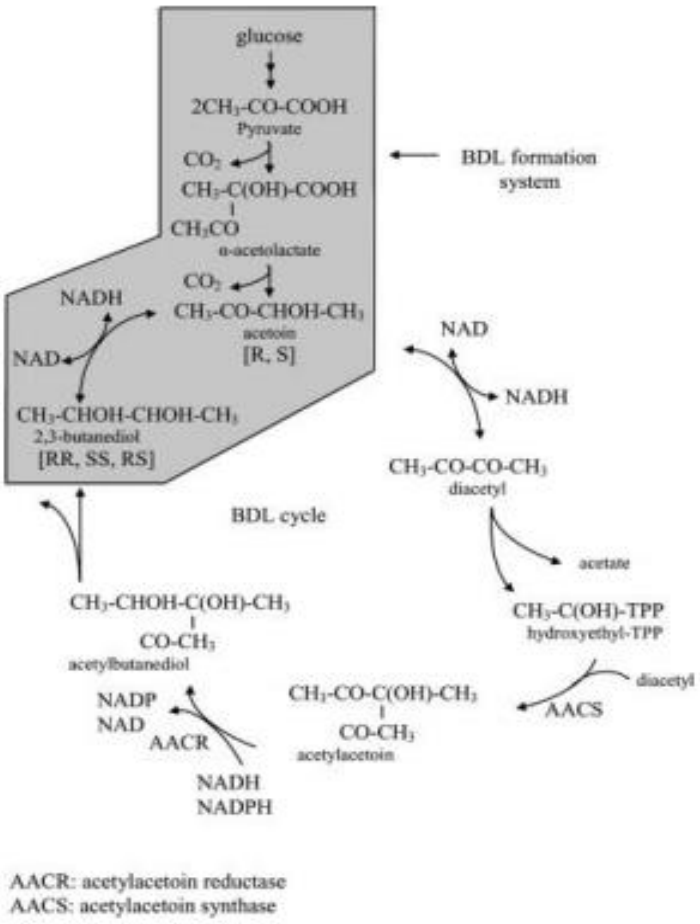


Figure 1.6. Metabolic pathway of BDO production from glucose and BDO cycle in bacteria as proposed by Juni and Heym (1956). The grey area represents the general BDO formation route in bacteria.

Another substrate that has recently been employed in the bioprocess of BDO production is that of glycerol which can also be converted into BDO leading to remarkable yields. Recently, *B. amyloliquefaciens* was engineered to produce 102.3 g l^{-1} from biodiesel-derived glycerol (Yang et al., 2015). Although BDO is the main product synthesized in several types of fermentations, in some cases it can also be produced along with other products that also present important biotechnological interest. The prospect of making both 1,3-propanediol and 2,3-butanediol in the same fermentation was proposed using *K. pneumoniae* and glycerol as substrate. (It must be taken into consideration that in most cases in which glycerol is assimilated under anaerobiosis by bacterial strains, 1,3-propanediol is one of the principal metabolites that is generated; see: Koutinas et al., 2014; Papanikolaou, 2009). In continuous culture, if pH was lowered stepwise BDO formation started at pH 6.6. This fermentation has the potential to be economically attractive as few by-products are formed (Biebl et al., 1998). The conversion of glycerol into the microbial cells under anaerobic or micro-aerobic conditions is shown in Figure 1.7..

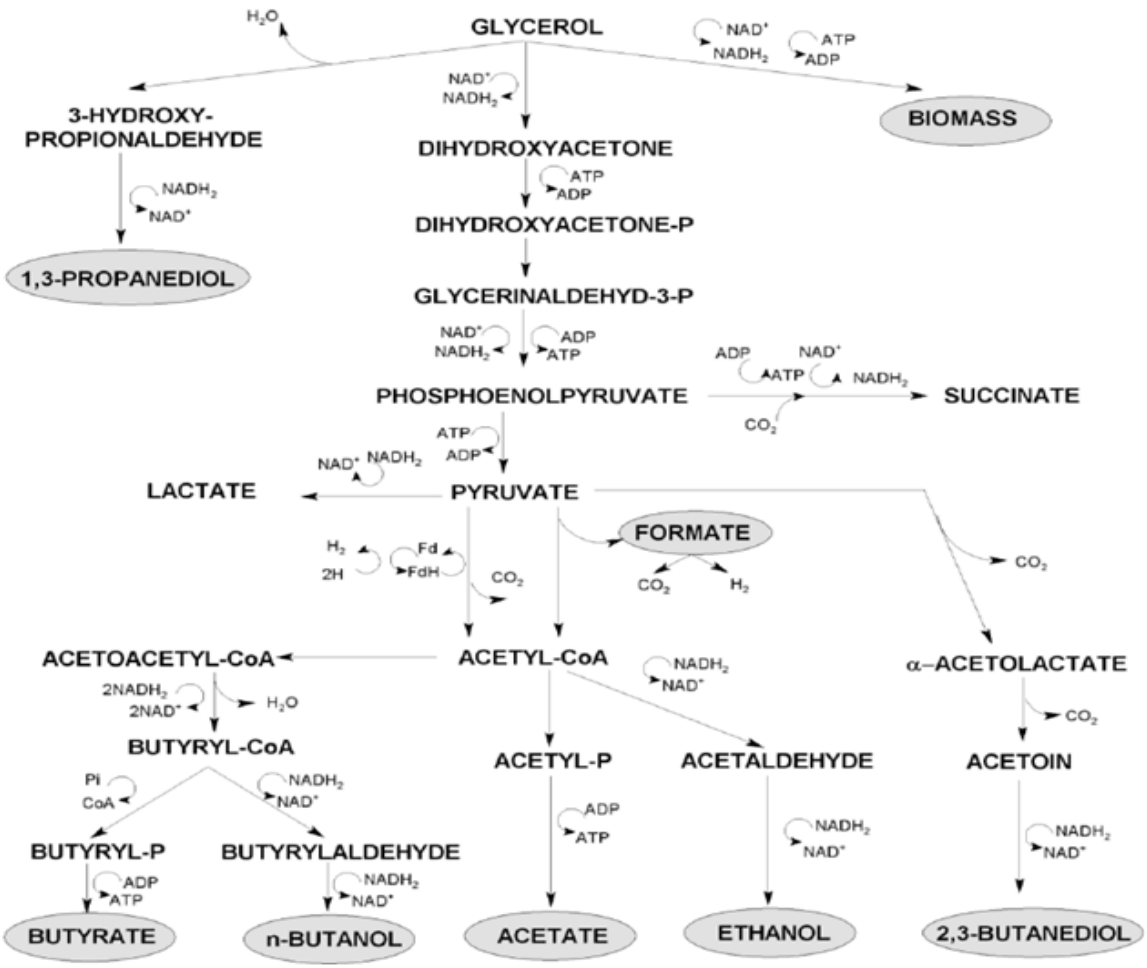


Figure 1.7. Biochemical pathway of glycerol fermentation (Leja et al., 2011).

Apart from 1,3-propanediol, BDO production can be accompanied by the biosynthesis of several other metabolites; for example, Collas et al. (2012) managed to simultaneously produce isopropanol, butanol, ethanol and BDO using engineered strains of the species *Clostridium acetobutylicum*. So far, the research has focused on the intracellular role of the metabolic pathway of BDO, proposing that there are three main reasons why bacteria assimilate various carbon sources and produce BDO along with other organic compounds. In particular, it seems that the production of BDO, which has a neutral pH value, protects the microorganism from intracellular acidification. In other words, the bacteria have the ability to alter the metabolism from acid to BDO production, in order to prevent from excessive acidification (van Houdt et al., 2007). Another function of the BDO pathway is related with the regulation of the NADH/NAD+ ratio in bacteria due to the reversible transformation between acetoin and BDO, coupled with the NADH/NAD+ conversion (Johansen et al., 1975; Magee & Kosaric, 1987). In addition, during the fermentations bacteria reutilize a part of the accumulated BDO when the carbon and energy sources have been depleted. Thus, the synthesis of BDO is regarded not only as a carbon- but also as an energy-storing strategy (Xiao & Xu, 2007).

1.5. Factors influencing BDO production through microbial fermentation

The factors influencing BDO production from bacterial strains can be either nutritional, like substrate and product concentrations as well as factors related with the implicated culture conditions i.e. temperature, pH, aeration and agitation. The appropriate inoculum preparation can also influence the final product concentration. Also, the special characteristics of the bioreactor and the operation mode play a predominant role on the final product synthesis and the bioconversion yield (for reviews see: Xiu & Zeng, 2008; Celinska & Grajek, 2009; Koutinas et al., 2014).

1.5.1. Aeration mode

The aeration which is applied during the fermentation is one of the most important parameters implicated in the BDO production bioprocess. Most of the studies so far have mentioned that BDO is produced under low O_2 supply or even under completely anaerobic conditions. As a result of the anaerobic conditions, an internal redox balance with respect to the pyridine nucleotide pool during glycolysis and biosynthesis is maintained (Converti et al., 2003). As it has already been referred, NADH from glycolysis is regenerated by BDH in a reversible reaction: acetoin↔BDO. Therefore, the NAD+/NADH balance is maintained by relative production of acetoin: BDO (Blomqvist et al., 1993).

Although it was found that under aerobic conditions α-ALS is rapidly and irreversibly inactivated, preventing BDO synthesis under high O_2 supply (Kosaric et al., 1992), some studies have shown that aeration increases BDO production (Barret et al., 1983; Converti et al., 2003; Ledingham & Neish, 1954; Long & Patrick, 1963; Nakashimada et al., 2000). This phenomenon was finely explained by Voloch et al. (1985). Most studies have focused on BDO production using *Klebsiella* sp. or *B. polymyxa*, both being facultative anaerobic microorganisms (Zeng & Sabra, 2011). Such microorganisms can obtain energy by two different pathways: respiration and fermentation. When the O_2 supply is limited, both pathways are active simultaneously. As a consequence, the final BDO concentration depends on the relative activities of each pathway. The yield of BDO can be maximized by minimizing the O_2 supply, because this limits the respiration. However, by lowering the availability of O_2, biomass production is also limited and therefore, indirectly BDO yield is reduced. This is the result of a direct relationship between the volumetric BDO productivity and the biomass concentration. Thus, increasing the O_2 supply rate leads to a higher cell density, and (in some way) to higher BDO yields.

In conclusion, decreasing the O_2 supply rate would increase the BDO yield, although decreasing the overall conversion rate due to a lower cell density. The ratio of O_2 demand and supply can control the proportions of metabolites produced (Voloch et al., 1985). In general, a high O_2 supply leads to high cell concentration and CO_2 formation at the expense of BDO biosynthesis. The reason for the shift in metabolism from production of biomass to production of BDO and other organic acids is the maintenance of NAD+/NADH balance. Under aerobic conditions, NADH from glycolysis is regenerated via respiration. Under anaerobic conditions, respiration cannot occur, so BDO and other fermentation pathways serve the same purpose. In the absence of O_2, usually ethanol is produced in approximately equimolar amounts with BDO (Voloch et al., 1985) but also formate, acetate, lactate, and acetoin are formed. Increasing the O_2 supply towards the value of O_2 demand increases the acetoin:BDO ratio (Voloch et al., 1985). If O_2 supply exceeds O_2 demand, the only products are biomass and CO_2. Therefore, in order to maximize BDO synthesis, a limited but non-zero supply of O_2 would be required (Voloch et al., 1985).

It is worth mentioned that the optimum aeration will also minimize the by-products formation, which may be inhibitory for the cells and the product recovery. Moes et al. (1985) proposed that acetoin production was higher with increase in O_2 supply. They found that acetoin was excreted, when dissolved O_2 (DO) level was above 100 ppb and BDO production was prevailing, when DO was below 100 ppb. The crucial DO level was 80– 90 ppb, when the product concentration ratio changed rapidly. Considering that the reaction acetoin↔BDO is reversible, switching the DO level in this range would cause conversion of one product to another in a

reversible manner. Figure 1.8. shows the effect of oxygen availability on the concentration of BDO and other organic acids during the cultivation of a *B. polymyxa* on glucose.

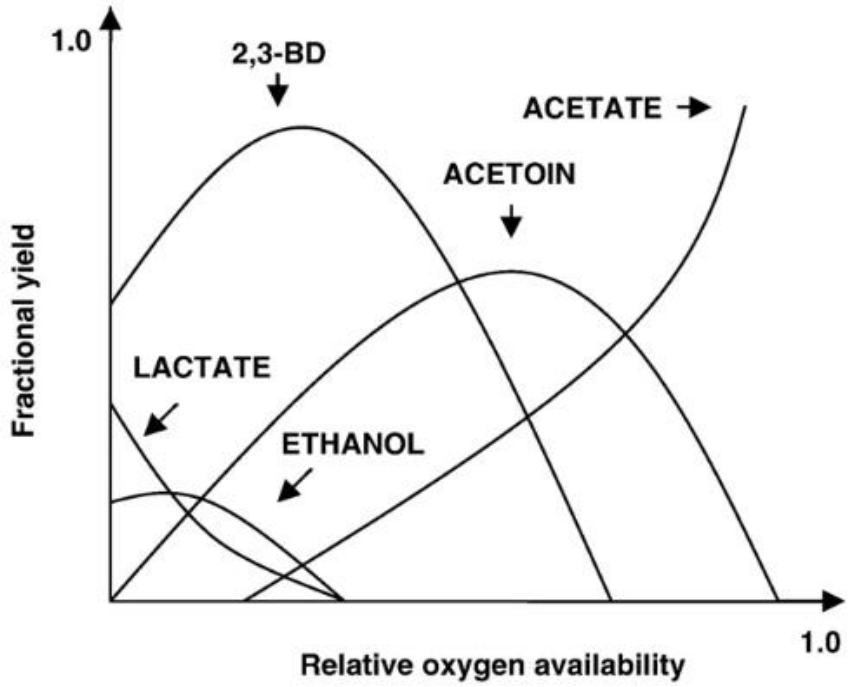


Figure 1.8. The effect of relative oxygen availability on fractional product yields in *B. polymyxa* (Figure is an approximation - modification, based on deMas et al., 1988).

The aeration applied is related to the agitation rate. The stirring action increases the efficiency of fermentation by continuously exposing new substrate to the culture and disseminating the metabolic end-products throughout the medium (Garg & Jain, 1995; Long & Patrick, 1963). Higher agitation seems to significantly increase the final BDO yield (Barret et al., 1983). It is worth mentioned that Nakashimada et al. (1998) found that optical purity of BDO produced by *B. polymyxa* was significantly decreased with an increase in O_2 availability during fermentation. When DO was high, the meso-form was increased. The optimal oxygen transfer rate (OTR) for this production was determined at 6.7 $mmol^{-1}h^{-1}$ but then the optical purity of the (R,R)-BDO form decreased to 93% compared with 98% under anaerobic conditions.

1.5.2. pH

Another important factor significantly influencing BDO production bioprocess is that of the medium pH. The optimum pH for diol production depends on the microorganism and the substrate

used. Most anaerobic fermentation processes are coupled with formation of organic acids, which acidify the cultural medium. In low pH values, when pH is not fixed, growth is finally inactivated by its own products (Biebl et al., 1998). Van Houdt et al. (2007) proposed that some microorganisms have evolved defensive strategies, like switching the metabolism to production of less toxic compounds, such as alcohols or glycols. Maddox (1996) referred that BDO pathway induction is caused by accumulation of acidic products into the medium rather than by altering the internal pH. The resulting transmembrane pH gradient causes accumulation of acetate, which would induce the enzymes involved in BDO synthesis. Hence, lowering the culture pH causes an increase in the pH gradient, and BDO production occurs before the external pH becomes too high and the culture is inactivated.

When *Klebsiella* sp. was cultivated at neutral pH, acetic acid and ethanol were synthesized, but below pH=6.0, BDO and ethanol were produced (Biebl et al., 1998). Moreover, in *K. pneumoniae* cultivated on glycerol employed as carbon source, at somehow lower pH values (i.e. pH *c.* 5.5), nearly 10% (25.3/221.8) of substrate (glycerol) was addressed to the synthesis of BDO and 44% (98.5/221.8) to the synthesis of 1,3-propanediol (Biebl et al., 1998; Menzel et al., 1997). According to the results of Biebl et al. (1998), the lower the pH into the medium (but however maintained to values that can support bacterial growth i.e. >5.0), the higher the BDO final concentration; however, as indicated, pH values lower than a critical value (i.e. 5.0) led to no BDO production and decreased bacterial growth. On the other hand, the highest amount of BDO was obtained when the pH was not controlled at all (changed continuously from the initial pH value of 7.0 to the final pH value of 5.5).

According to Voloch et al. (1985) the optimum pH value for BDO production by *K. oxytoca* is the pH range from 5.0 to 6.0. Furthermore, Stormer (1968) found that in *K. pneumoniae* a pH value above 6.0 causes a sharp decrease in the activity of one of the key enzymes (α-acetolactate synthase) in the BDO pathway. Perego et al. (2000) tested pH in the range from 4.0 to 8.0. The conversion yield was nearly constant within a range of pH 5.0↔6.5, and it sharply decreases either at lower or at higher pH values. The strongest effect was observed under acidic conditions, which stems from the fact that the lower pH value usually inhibits biomass growth as well as the bioprocess itself. However, during fermentation of *B. polymyxa* a different pH range was chosen for production of BDO (pH 6.3–6.8) (Nakashimada et al., 2000).

1.5.3. Temperature

In general, all bacterial fermentations are strictly temperature-dependent due to the strong dependence of the enzymatic activities and the cellular maintenance upon temperature (Garg & Jain, 1995). Most studies have shown that the range of 30-35 °C should be the optimum for the

bacterial fermentation, since this is the range of maximum biomass yield while there is no protein denaturation. Higher temperatures often lead to cell degradation. On the other hand, with suboptimal temperature, the regulation and rate of metabolism may fail. Biebl et al. (1998) have shown that in cultures of *K. pneumoniae*, lowering temperature from 35 °C to 30 °C resulted in a substantial reduction in ethanol synthesis in favor of BDO formation. Therefore, according to that investigation, a temperature value of not more than 30 °C is recommended. In contrast, during batch fermentation of *E. aerogenes*, a temperature of 39 °C was considered as optimal (Converti et al., 2003). Perego et al. (2000) performed various experiments in a wide range of temperature values using another strain of *E. aerogenes*; the study covered a temperature range from 23 to 46 °C. The rate of glucose conversion into BDO and acetoin as well as the final product concentration, were both positively correlated with the increase of the incubation temperature. Due to the acceleration of enzymatic processes, the fermentation time progressively decreased with increasing temperature. The BDO volumetric productivity gradually increased with the temperature up to 39 °C (0.36 mmol l^{-1} h^{-1}) and decreased over this threshold. Thus, the authors concluded that, a temperature of 39 °C is optimal for this process. Barret et al. (1983) examined the effects of temperature in the range of 30 °C to 37 °C on BDO production using two different strains, *K. pneumoniae* and *E. aerogenes*. A temperature of 33 °C appeared to be optimal for *K. pneumoniae*, while BDO formation was enhanced at 37 °C for *E. aerogenes*. In addition, in cultivations using *B. polymyxa* the optimum temperature was fixed at 30 °C (Hespell, 1996; Nakashimada et al., 2000). In conclusion, the research has shown that different strains are characterized by different optimal values suggesting that the temperature should be established individually for each strain and substrate used.

1.5.4. Substrate

The culture medium composition along with the initial concentration of the carbon source, can significantly affect microbial growth, substrate consumption and metabolites production. As for the substrate composition, many toxic substances can be found in raw materials such as wood or starch hydrolysates. Frazer and McCaskey (1991) focused on the effect of toxic compounds from acid-hydrolyzed wood on BDO production by *K. pneumoniae*. They found that, although it did not affect microbial growth, sulphate concentration of up to 0.2% (wt vl^{-1}) reduced the BDO yield by 30%. Furfural, another toxic compound, has shown similar impact upon BDO production. Phenolic compounds (syringealdehyde and vanillin) were also inhibitory for BDO production (at the concentration of 0.05% (wt vl^{-1}) and growth (0.1%, wt vl^{-1}). Moreover, according to Nakashimada et al. (2000), the substrate supplementation with acetate, propionate, pyruvate, and succinate enhanced BDO production by *B. polymyxa*, while addition of butyrate, valerate, formate, lactate, and malate gave no observable effect. Yu and Saddler (1982) studied the effect of acetate

supplementation on BDO and acetoin production. The authors found that growth and BDO yield from wood hemicellulose was favored in cultures supplemented with low levels of acetic acid.

As for the substrate concentration, a number of studies so far proposed that there is high correlation between cell growth and initial carbon source quantity. For instance, when synthetic media were supplemented with pure sugars like analytical glucose, and no inhibitory compounds were presented into the medium, initial sugar concentrations of up to 200 g l^{-1} could be fermented leading to remarkable BDO production. In another study, using *B. polymyxa*, the growth inhibition occurred at substrate concentration higher than 150 g l^{-1} (de Mas et al., 1988). On the other hand, in several other types of studies, lower initial concentrations of sugars (or glycerol) should be used in order to support significant BDO production. For instance, a moderate initial sugar concentration (exceeding 50 g l^{-1}) was reported to inhibit BDO formation and sugar utilization (Yu & Saddler, 1983). Accordingly, at not excessively high initial sugar concentrations (i.e. 4 to 6% wt vl^{-1}), the fermentation of sucrose in sugar beet molasses proceeded rapidly to completion in 24 h (McCall & Georgi, 1954). On the other hand, at higher sucrose concentrations (up to 17%) fermentations were incomplete and less efficient.

In conclusion, similar to the other operational factors which play a predominant role on BDO production, it seems that the influence of the substrate type and concentration on BDO production is species-dependent.

1.6. Biotechnological production of acetoin through microbial fermentation and its applications

Acetoin (Ace) is a metabolic compound produced via bacterial fermentations through the mixed-acid BDO fermentation pathway which was previously described. In particular, Ace is an intermediate product of the BDO fermentation pathway. As it has previously been mentioned, BDO transformation into acetoin is the result of the reversible reaction catalyzed by the enzyme acetoin reductase along with the simultaneous reduction of NAD^+ to NADH. The produced NADH can then be used in order to generate ATP through the electron transport system. Acetoin is also mentioned as 3-hydroxy-2-butanone or acetyl methyl carbinol.

The parallel production of acetoin along with BDO can be a beneficial bioprocess as long as acetoin is also a high added-value product with various applications in various edible (i.e. dairy) products, in cosmetology applications and in several pharmaceutical preparations (Zeng & Sabra, 2011). Acetoin fermentation has shown many exciting results through strain screening, while enhancement fermentation strategies related with the control of pH and aeration have been achieved to increase acetoin production (Xiao & Lu, 2014). From the results obtained so far it is shown that this bioprocess can have industrial applications in various fields. For instance, acetoin is widely

used in foods, plant growth promoters, and biological pest controls. It can be used as an aroma carrier in flavors and essences. In addition, it can also be used as a precursor for a variety of chemical compounds, such as diacetyl and alkyl pyrazines (Xiao & Lu, 2014). Its versatile usage and its potential for bulk industrial production make acetoin one of the 30 platform chemicals that are given priority in development and utilization by the United States Department of Energy (Werpy et al., 2004).

In most studies acetoin appears as a minor by-product of BDO fermentation process. For instance, the final acetoin accumulation into the medium noted was between 5 and 15 g l^{-1} in *S. marcescens* (Zhang et al., 2010) and *K. oxytoca* (Ji et al., 2008). However, in one study, a mutant of *B. pumilus* produced 63 g l^{-1} of acetoin from 200 g l^{-1} glucose (Xu et al., 2009) while a mutant of *B. subtilis* produced 43.8 g l^{-1} and 46.9 g l^{-1} from 100 g l^{-1} in shake flask and bioreactor experiment respectively (Xu et al., 2011). In another case, Liu et al. (2011) managed to produce *c.* 41 g l^{-1} acetoin from 100 g l^{-1} glucose using a newly isolated *B. licheniformis* strain. Wang et al. (2011) used metabolic engineered *B. subtilis* strains to enhance not only acetoin production but also acetoin productivity. The results were very satisfying as the highest volumetric productivity achieved was 0.273 g l^{-1} h^{-1}.

Zhang et al. (2013) have achieved one of the highest acetoin production values so far using the newly isolated strain *B. amyloliquefaciens* FMME 044. In order to enhance acetoin production by manipulating the carbon flux distribution, a two-stage agitation speed control strategy was proposed: during the first 24 h, the agitation speed was set to 350 rpm to achieve a high BDO concentration and then the speed was increased to 500 rpm to reverse transform BDO to acetoin. At the end of the fermentation 51.2 g l^{-1} of acetoin were produced reaching a yield of 0.43 g g^{-1}. Another highly interesting study focused on the synthesis of (3R)-acetoin and BDO isomers by metabolically engineered *Lactococcus lactis*. The fermentations led to efficient production of (3R)-acetoin, meso-BDO and R-BDO from processed whey waste, with titers of 27, 51, and 32 g l^{-1} respectively (Kandasamy et al., 2016). An engineered *S. marcescens* strain was used by Bai et al. (2015) in order to produce (3R)-acetoin and R-BDO and the results were also very satisfying.

It should be stressed that acetoin can also be produced by wine yeasts. For instance, yeasts belonging to the genus *Saccharomyces* produce α-acetolactate in considerable amounts during fermentation and this highly labile compound may easily be converted to diacetyl or to acetoin, particularly in the presence of oxygen (Haukeli et al., 1972). Xiao et al. (2007) used molasses and soybean meal hydrolysate in cultivations of *B. subtilis* CICC 10025. They achieved to produce 37.9 g l^{-1} of acetoin in flask fermentation and 35.4 g l^{-1} of acetoin in bioreactor fermentation. In another case, enhanced acetoin production by *Serratia marcescens* H32 was achieved with expression of a

water-forming NADH oxidase. By fed-batch culture of the engineered strain, the final acetoin titer up to 75.2 g l^{-1} with the productivity of 1.88 g l^{-1} h^{-1}) was obtained (Sun et al., 2011).

Considering the promising results obtained so far for acetoin production, it is essential to further focus on the optimization of the process in order to achieve higher titers and yields.

1.7. Downstream process for 2,3-butanediol and acetoin purification

One of the major aims for an economically efficient BDO production is the development of an appropriate downstream processing approach. In order to commercialize the microbial BDO production it is essential to design separation techniques for the recovery of BDO as well as acetoin from the culture medium. The separation of these products from fermentation broth makes more than 50% of the total costs in their microbial production.

At the end of a microbial fermentation there are numerous impurities which must be removed. For instance, nutrients, pH buffering agents and by-products from the microbial metabolism must be separated from the fermentation broth. Suspended solids, such as cells, can be easily removed from fermentation broth through simple filtration or centrifugation processes, while the removal of dissolved impurities, especially organic acids and inorganic salts, requires more complicated processes. Many methods, such as ion exchange, electrodialysis and membrane filtration have been proposed to remove or extract the dissolved organic acids and inorganic salts from fermentation broth. Xiu and Zeng (2008) presented different methods of diols extraction and proposed improvements for more efficient separation technologies.

In most studies, the separation techniques which were applied following the microbial fermentation were distillation (Afschar et al., 1993), steam stripping (Garg & Jain, 1995), reverse osmosis (Xiu & Zeng, 2008), and pervaporation (Qureshi et al., 2001). Some of the above-mentioned techniques successfully reached pilot-scale. Other methods which have been applied on a laboratory scale were solvent extraction (Eiteman & Gainer, 1989), recovery based on chemical conversion of BDO (Xiu & Zeng, 2008), salting out (Afschar et al., 1993) and countercurrent steam stripping (Garg & Jain, 1995). In the last decade the aqueous two-phase extraction (Ghosh & Swaminathan, 2003, 2004; Jiang et al., 2009; Li et al., 2010; Sun et al., 2009), in situ recovery (Anvari et al., 2009), integrated solvent extraction and pervaporation (Shao & Kumar, 2009a, b) have also been studied with promising results. The following figures (Fig. 1.9.; 1.10.) present the diagram of a typical recovery process and the results obtained during a study using alcohol precipitation and vacuum distillation (Jeon et al., 2014).

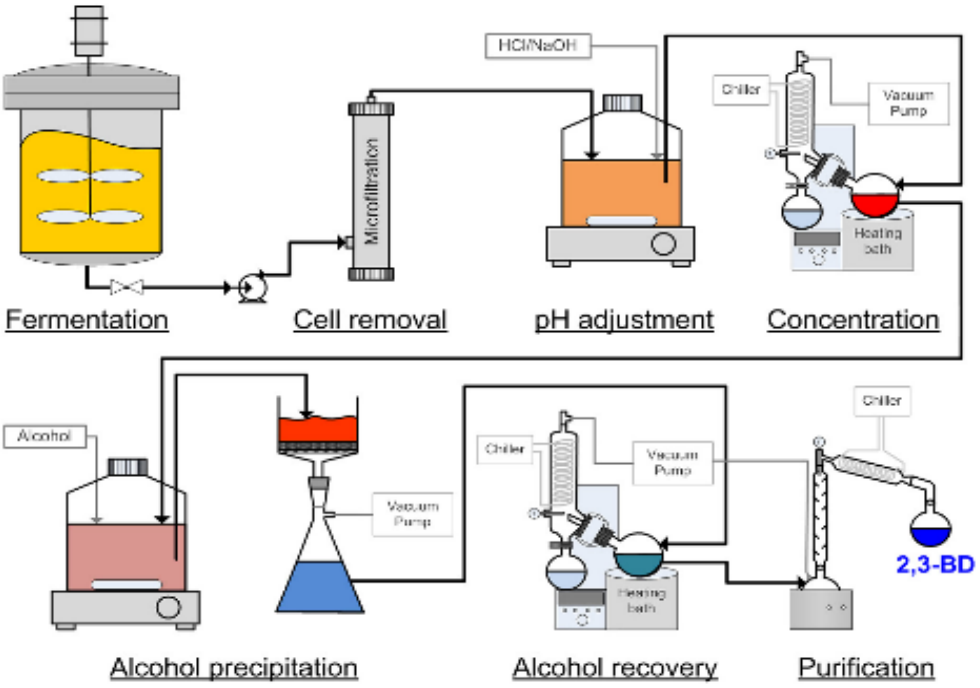


Figure 1.9. Schematic diagram of BDO fermentation and recovery system (Jeon et al., 2014)

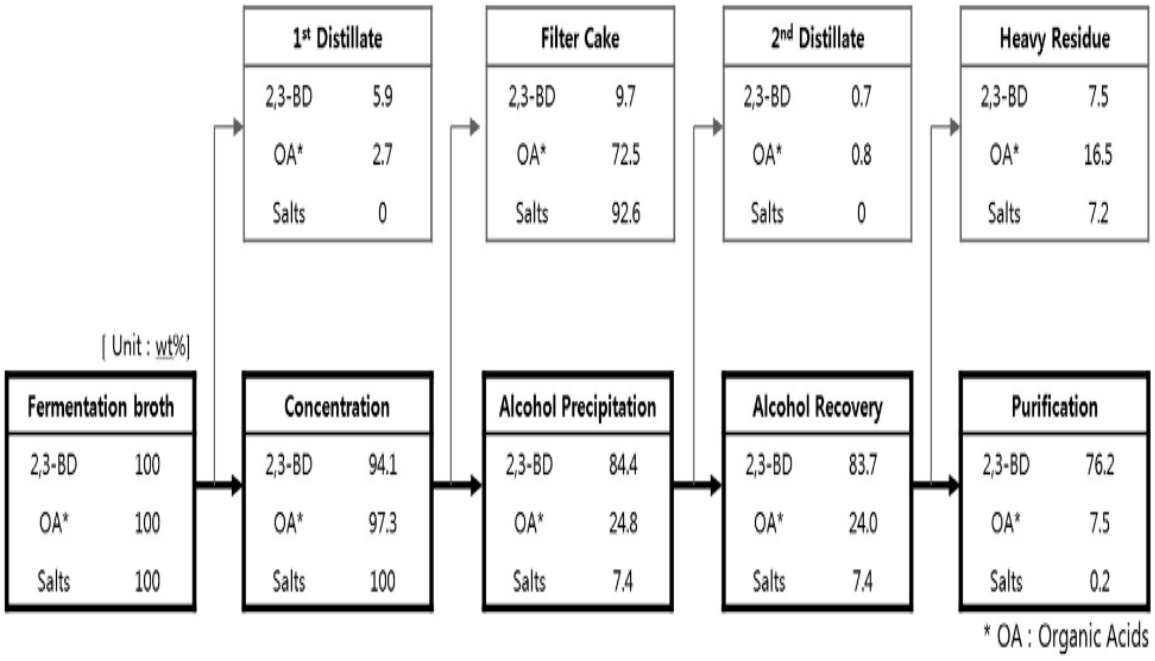


Figure 1.10. Effect of isopropanol amount on the removal efficiency of organic acids and inorganic salts by precipitation. BDO concentration (closed triangles) and mass ratio of total organic acids (closed circles) and inorganic salts (open circles) to BDO. (Jeon et al., 2014)

1.8. Molasses as an industrial by-product

The production of cane sugar comes from the cultivation of sugarcane (*Saccharum officinarum*) in tropical and sub-tropical countries like Brazil, India and Thailand. In 2017 and 2018, approximately 191.81×10^6 t of sugar produced with sugarcane accounts for about 80% of the total production. Brazil is the predominant sugar producer, producing 34% of the world's sugarcane.

Molasses are collected from the liquid by-product of the sugar cane or sugar beet processing into sugar, containing sugars (44-60% w/w) and various minerals (Curtin, 1983; Chen & Chou, 1993). The high molecular weight polymer melanoidin is the dark brown pigment found in the molasses wastewaters (Kumar & Chandra, 2006; Plavšić et al., 2006). The following figure represents the process flow diagram for typical cane sugar production.

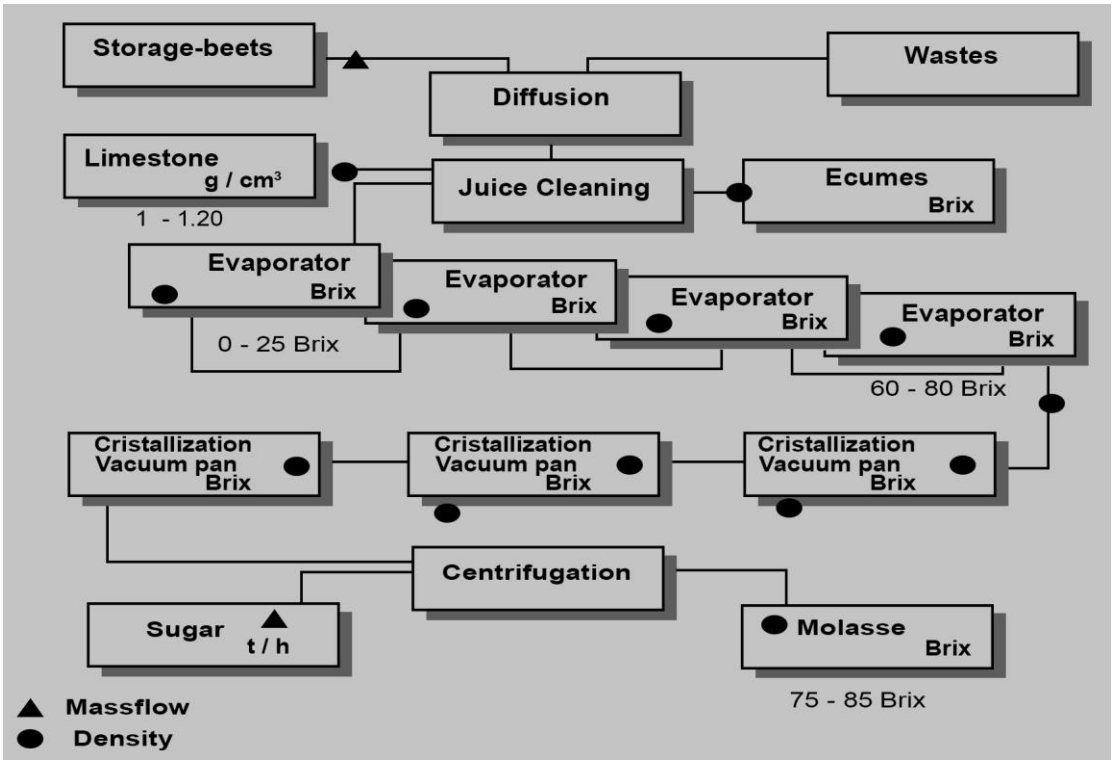


Figure 1.11. Schematic diagram of sugar cane molasses refining process

As shown, the cane is mechanically grinding and then mixed with water in order to enhance the extraction of the juice. Following, the juice is clarified to remove large particles and then goes to the evaporators for the concentration of the juice. The syrup passes through vacuum pans for crystallization. The final step is the centrifuging process in which sugar is separated from molasses (Sahu, 2018).

1.8.1. Composition and polluting power of molasses

Molasses varies by amount of sugar, method of extraction, and age of plant. A typical range of all the components which can be found in molasses is presented in Table 1.4..

Table 1.4. Typical composition of sugar cane molasses

Component	Usual range
Brix (%)	79.5
Density (g/cm^3)	1.38 – 1.52
Water (%)	17-25
Total solids (%)	75 - 88
Total sugars (%)	44 - 60
Sucrose (%)	30 - 40
Glucose (%)	4 - 9
Fructose (%)	5 - 12
Other reducing substances (%)	1 - 5
Other carbohydrates (%)	2 - 5
Ash (%)	7 - 15
Nitrogenous compounds (%)	2 - 6
Non-nitrogenous acids (%)	2 - 8
Wax, sterols and phospholipids (%)	0.1 - 1
Calcium (%)	0.2 - 0.8
Phosphorus (%)	0.03 - 0.08
Potassium (%)	2.4 - 4.7
Sodium (%)	0.2 - 1.0
Chlorine (%)	0.9 - 1.4
Sulphur (%)	0.5

(Chen & Chou, 1993; Teclu et al., 2009)

Apart from the sugarcane molasses, other forms of molasses are also produced like sugar beet molasses and unsulphured molasses. Molasses are characterized by heavy organic load and include high concentrations of nutrients and minerals like nitrogen phosphorus and potassium which may lead to eutrophication phenomena. FitzGibbon et al. (1998) have mentioned that molasses dark color hinders photosynthesis by blocking sunlight, which can cause serious damage to aquatic life. Moreover, molasses can also harm soil and water (Evershed et al., 1997). Another reason why molasses can be harmful for the environment is its low pH and its strong odor (Satyawali & Balakrishnan, 2008).

1.8.2. Biotechnological uses of molasses

Due to the high content in sugars, molasses have been used as growth medium for the production of various high-added value products through microbial fermentations. On the other hand, molasses are a cheap substrate which can be easily found in high and continuously increasing quantities. There is a huge number of studies in the literature which have focused so far on the microbial fermentations using molasses as carbon source from various microbial strains and species. Apart from BDO production, other microbial products with high biotechnological interest that have been obtained through molasses fermentations, are ethanol, citric acid, fructo-oligosaccharides (FOS), pullulan, succinic acid, gluconic acid, single cell oil, bacteriocins etc. (Roukas, 1996; El-Enshasy et al., 2008; Liu et al., 2008; Sharma et al., 2008; Chatzifragkou et al., 2010). For instance, remarkable ethanol production using molasses as fermentation substrate has been described so far in numerous publications (Roukas, 1996; Nahvi et al., 2002; Baptista et al., 2006; Kopsahelis et al., 2007; 2012; Cáceres-Farfán et al., 2008). The production of citric acid has been noted when *Aspergillus niger* was cultivated on molasses employed as microbial substrate. In particular, Hamissa and Radwan (1977) and Qazi et al. (1990) produced a maximum concentration of citric acid of 60.8 and 67.0 g l^{-1}, respectively. Other studies have also resulted to the production of citric acid in remarkable conversion yields and final product concentrations (Pera & Callieri, 1997; Ikram-ul et al., 2004; Garg & Sharma, 1991). As for the production of succinic acid, *Actinobacillus succinogenes* is the most common representative. Liu et al., 2008 achieved the production of maximum succinic acid concentration ~51 g l^{-1}.

Single cell oil (SCO) as mentioned in the previous paragraph, is another added-value product that can be obtained from cultivation of oleaginous microorganisms using molasses. Therefore, Chatzifragkou et al. (2010) cultivated the Zygomycete fungi *Cunninghamella echinulata* and *Mortierella isabellina* on sugar-based media including molasses and satisfactory lipid accumulation was achieved. It is worth mentioned that the medically and nutritionally important γ-linolenic acid (GLA) were synthesized. In another case, a *Yarrowia lipolytica* strain was cultivated on molasses leading to remarkable single cell oil accumulation (Gajdoš et al., 2015).

Although molasses can be consumed by different microbial strains for the biotechnological production of added-value products, recent research has shown that it is an appropriate supplement for animal feeding. Two studies, Bórquez et al. (2009) and Xandé et al. (2010), proposed that molasses can enhance the quality of animal food. Table 1.5. summarizes the various products which are derived through microbial cultivation on molasses.

Table 1.5. Biotechnological production of metabolites using molasses as carbon source

Products	Microorganism	References
Ethanol	*Saccharomyces cerevisiae*	Ghorbani et al. (2011)
	Zymomonas mobilis	Cazetta et al. (2007)
	Saccharomyces cerevisiae	Roukas (1996)
	Saccharomyces cerevisiae	Echegaray et al. (2000)
	Saccharomyces cerevisiae	Rattanapan et al. (2011)
Single cell oil (SCO)	*Cunninghamella echinulata*	Chatzifragkou et al. (2010)
	Yarrowia lipolytica	Gajdoš et al. (2015)
	Mortierella isabellina	Chatzifragkou et al. (2010)
	Trichosporon fermentans	Zhu et al. (2008)
Citric acid (CA)	*Aspergillus niger*	Milson and Meers (1985)
	Aspergillus niger	Qazi et al. (1990)
	Aspergillus niger	Adham (2002)
Gluconic acid	*Aspergillus niger*	Sharma et al. (2008)
Fructooligosaccharides (FOS)	*Aureobasidium pullulans*	Shin et al. (2004)
Succinic acid	*Actinobacillus succinogenes*	Liu et al. (2008)
Erythromycin	*Saccharopolyspora erythraea*	El-Enshasy et al. (2008)
Bacteriocins	*Leuconostoc mesenteroides*	Metsoviti et al. (2011)

1.8.3. Molasses and molasses waste waters

The waters remaining after the microbial conversions and the downstream processing and the subsequent recovery of the biotechnological products resulted after the fermentation of molasses are called molasses wastewaters (MWWs). These wastes are characterized by high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) values, strong odor and dark brown color. This color is originated from melanoidins which are dark brown to black pigments and are produced by condensations carried out by non-enzymatically catalyzed reactions between sugars and amino-acids, so-called "Maillard reactions" during the thermal process of sugar cane or sugar beet for the production of crystal sugar (Painter, 1998).

The degradation of melanoidins can be achieved using various processes. For instance, pyrolysis, which is the most common physicochemical process, has been described by Yaylayan and Kaminsky (1998). Moreover, Kim et al. (1985) described the degradation of melanoidins (derived from glucose and glycine) by applying ozonolysis at -1 °C. The decolorization reached a value of 84% (after 10 minutes) and 97% (after 90 minutes). There was also a reduction of the average molecular weight of melanoidins from 7000 to 3000 (after 40 minutes). On the other hand, biological processes are preferred as they are more environmentally friendly and financially more feasible, compared to the physicochemical processes. Bacteria (Kumar et al., 2006), yeasts (Tondee et al., 2008a) and fungal strains (Miranda et al., 1996) have been successfully applied for the decolorization of molasses and MWWs melanoidins. They have also been successfully applied on the biotechnological conversion of several types of phenol-containing wastewaters like olive-mill wastewaters (Aggelis et al., 2003; Tsioulpas et al., 2002). On the other hand, substrate decolorization was noted when the fungi *C. echinulata* and *M. isabellina* were cultivated on molasses, reaching up to ~75% for *C. echinulata* (400 h of culture) and ~20% for *M. isabellina* (200 h after inoculation), simultaneously with the biotechnological conversion of molasses into mycelial mass rich in fungal oils containing nutritionally important poly-unsaturated fatty acids (Chatzifragkou et al., 2010). Ohmomo et al. (1988) used *Aspergillus oryzae* which absorbed in its mycelia low molecular weight melanoidins. Another *Aspergillus* species, *A. niger* led to 83% decolorization of MWWs (Miranda et al., 1996). Furthermore, Metsoviti et al. (2011) have proposed that species belonging to lactic acid bacteria like strains of the microorganism *Leuconostoc mesenteroides* have the ability to produce bacteriocin during growth of molasse-based media, while simultaneously with the biotechnological conversion, a non-negligible decolorization of the medium (up to ~27%) was noted.

A large number of publications have mentioned the potential of bacterial strains to reduce COD and decolorize several molasse-based substrates. For instance, strains which belong to the genera *Bacillus*, *Pseudomonas*, *Enterobacter*, *Stenotrophomonas*, *Aeromonas*, *Acinetobacter* and

Klebsiella have achieved remarkable results. For instance, Kumar and Chandra (2006) used mixed cultures of *B. thuringiensis* MTCC 4714, *B. brevis* MTCC 4716 and *Bacillus* sp. MTCC 6506 on substrates containing synthetic melanoidins and reached a maximum decolorization of 50%. In another case, a *Lactobacillus plantarum* strain has shown high potential for use in decolorization of MWWs reaching a maximum yield of 68.12% within 7 days (Tondee & Sirianuntapiboon, 2008b). The cultivation of *P. putida* led to 24% decolorization whereas when immobilized cells were used, an increase of the decolorization yield was achieved (Ghosh et al., 2009). Last but not least, three Cyanobacterial-type microorganisms, namely *Oscillatoria boryana*, *Lyngbya* sp. and *Synechocystis* sp. were able to decolorize the molasses substrate reaching in very satisfying yields. In particular, Kalavathi et al. (2001) achieved degradation of the melanoidins up to 96% using *O. boryana*, while the other two species were studied by Patel et al. (2001), leading to a decolorization yield of 81% and 26% respectively.

1.9. Genetic modifications for production enhancement

Although there are huge numbers of publications in the literature focusing on the cultivation of wild strains for BDO production, genetic modification in order to enhance the final production has been achieved so far. The aim of introducing a mutation is to overexpress the enzymes involved in BDO pathway. The most common species which have been modified belong to the species *E. coli*, *K. pneumoniae*, *B. polymyxa* and *L. lactis.* For instance, Ui et al. (2004) focused their research on the genetic modification of the strain *E. coli* JM109 in order to construct a completely new BDO pathway, with BDO being the main product. One construct (pFLAG-CTC-based) with two genes combined together in tandem, meso-BDH of *K. pneumoniae* and L-BDH of *B. saccharolyticum*, was introduced into the engineered *E. coli* strain. Furthermore, *K. oxytoca* mutants were isolated from the wild type strain, ME-303, after mutagenesis with UV coupled with diethyl sulfate and the following modified proton suicide method (Ji et al., 2008). A modification in the mixed acid fermentation pathway was achieved and the authors assumed that a disturbance in lactic and acetic acid synthesis should improve the availability of the substrate (acetoin) for BDO production. The mutant, ME-UD-3, produced 7.8% more BDO with the corresponding by-products, lactic and acetic acid, decreased by 88% and 92%, respectively. Another study of Jing et al. (2016) presented the results of a *B. subtilis* strain which was engineered to produce chiral pure meso-BDO. Under microaerobic oxygen conditions, the best strain BSF9 produced 103.7 g l^{-1} meso-BDO with a yield of 0.487 g g^{-1}glucose in the 5-L batch fermenter. Wang et al. (2012) developed mutants of a newly isolated *B. licheniformis* strain and achieved the production of D-(-)-BDO in an optical purity of more than 98%.

In another case, Mallonee and Speckman (1988) developed a mutant strain ($AlsR^{-}$) of *B. polymyxa* which displays a constitutive production of α-acetolactate synthase, an enzyme normally produced only in the late log or stationary phase. The mutant was obtained by treating the wild type with nitrosoguanidine followed by the penicillin counter selection procedure. The $AlsR^{-}$ strain started production of acetoin and BDO earlier and the production was still apparent after 40 h of growth and it used less substrate. The wild type grew faster, but the production of BDO was stopped before 40 h. During prolonged fermentation (up to 96 h), mutants produced 4-fold more of acetoin and BDO than the wild type. Zhang et al. (2016) developed mutants of four genes in *Serratia* sp. T241.

Apart from bacterial strains, other microorganisms have also been genetically modified in order to produce BDO in high titers. *S. cerevisiae* was engineered to produce 72.9 g l^{-1} from glucose with a yield of 0.41 g g^{-1} glucose (Kim et al., 2015) and more than 100 g l^{-1} d-(−)-BDO from a mixture of glucose and galactose in up to 300 h (Lian et al., 2014).

1.10. The family of Enterobacteriaceae

The strains that belong to the family of Enterobacteriaceae, which are also referred as enterobacteria, have been well-studied for their properties for decades. There are huge numbers of studies in the literature which focus on the special characteristics of the members of Enterobacteriaceae family and their potential to be used in the industry. Members of Enterobacteriaceae are rod-shaped (bacilli) and are typically 1–5 μm in length. They are gram-negative stains and they are facultative anaerobes, fermenting sugars to produce lactic acid and various other end products. Moreover, most of the species have flagella used to move about, but a few genera are non-motile. They are not spore-forming. Catalase reactions vary amongst bacterial members of the Enterobacteriaceae family. Various of the species are pathogenic disease-causing bacteria.

The enterobacteria can be isolated from different places including human body or animals, where they are part of the gut flora as well as from water or soil. It should be stressed that the strain *E. coli* and the genus *Salmonella* sp. are the most important model organisms as they have been studied for their applications in the fields of Genetics and Biochemistry. Other members of the family which have predominately been used so far in the literature belong to the genera *Enterobacter*, *Klebsiella*, *Serratia* and *Citrobacter*. Figure 1.12. represents the relatedness among the members of the family Enterobacteriaceae.

In this study, after an initial screening process, two newly isolated strains which belong to the family of Enterobacteriaceae have been used. The first one was a *K. oxytoca* strain while the second one a strain of *Enterobacter* sp.. Figure 1.13. presents the scientific classification of both strains.

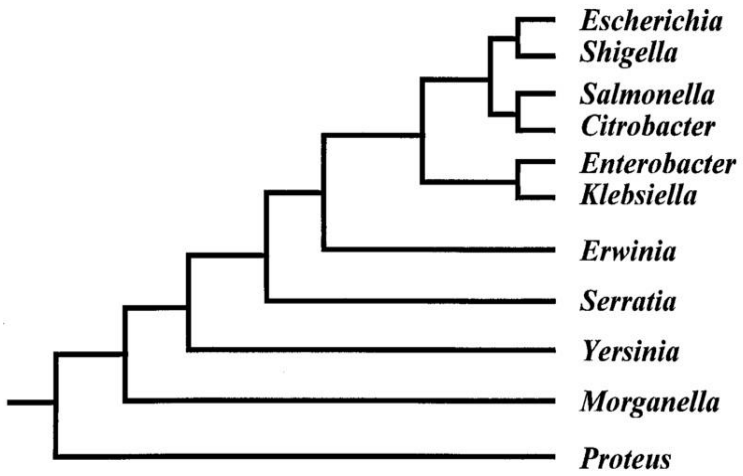


Figure 1.12. Phylogenetic tree of the family Enterobacteriaceae (Mcnamara & Wolfe, 1997).

Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Gammaproteobacteria
Order:	Enterobacteriales
Family:	Enterobacteriaceae
Genus:	*Klebsiella*
Species:	***Klebsiella oxytoca***

Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Gammaproteobacteria
Order:	Enterobacteriales
Family:	Enterobacteriaceae
Genus:	*Enterobacter*
Species:	***Enterobacter* sp.**

Figure 1.13. Scientific classification of the species *Klebsiella oxytoca* and *Enterobacter* sp.

1.11. Economic approach of BDO production

As the microbiological production of BDO becomes more likely to be applied on an industrial scale, techno-economic analysis for efficient manipulation of the microorganisms which are promising producers is needed. For this reason, in order to design an economically feasible process, one should take into account factors such as the process yield, the volumetric productivity, the final BDO production, and, certainly the related costs, including the cost of manufacturing (e.g., utilities, raw materials, nutrient supplements, labor) as well as the fixed capital investment (e.g., equipment procurement and installation, downstream separation facility).

One interesting techno-economic evaluation of the complete bioprocess of BDO production from renewable resources has been conducted by Koutinas et al. (2016), including the separation step, therefore, BDO has been recovered and purified. In this study, the data of fermentations using three feedstocks (glycerol, sucrose and sugarcane molasses) which are produced in large and continuously increasing quantities worldwide were evaluated. Cost analysis was based on a bioprocess involving *K. pneumoniae, S. marcescens* and *E. aerogenes* strains utilizing the three above-mentioned substrates respectively. In the case of glycerol employed as the carbon source, the data which were used for the economical analysis were based on fermentation with a final BDO production of 55 g l^{-1} reaching a bioconversion yield (Y_{BDO}) of 0.35 g g^{-1} and a volumetric productivity rate (P_{BDO}) of 0.57 g l^{-1} h^{-1}. The final bioconversion yield and BDO concentration revealed that the method may be attractive only with a significant increase in the efficiency of the fermentation and a remarkable decrease in the market price of glycerol; crude glycerin originated from biodiesel production processes could reduce costs, by removing the impurities from the fermentation broth in order to minimize any growth inhibition.

In addition, when sucrose and molasses were used as carbon sources, 140 g l^{-1} (Y_{BDO}= 0.41 g g^{-1}; P_{BDO}=3.33 g l^{-1} h^{-1}) and 99 g l^{-1} (Y_{BDO}= 0.37 g g^{-1}; P_{BDO}=2.74 g l^{-1} h^{-1}) of BDO was produced respectively. The study concluded that the existing microorganisms are particularly efficient. However, raw materials costs, including costs of nutrient supplements, are significant in both cases and they should be replaced by less costly sources of nutrients in order to reduce the cost of the whole process.

It should be stressed that thanks to a better understanding of metabolic engineering and synthetic biology, genetic modification of strains makes it feasible to improve the natural BDO producers and design full catabolic pathways in efficient and ecologically favorable systems. Taking into consideration the existing knowledge, cost-competitive bioprocesses for BDO and acetoin production can be designed.

2. Materials and Methods

2.1. Microorganisms

During the initial screening process, nine newly isolated and putatively non-pathogenic strains which belong to the family of Enterobacteriaceae were used in order to assess their ability to consume glucose and sucrose and produce 2,3-butanediol (BDO). In particular, the strains which were cultivated were *Enterobacter ludwigii* FMCC-204, *Enterobacter aerogenes* FMCC-9, *Enterobacter aerogenes* FMCC-10, *Citrobacter freundii* FMCC-207, *Klebsiella oxytoca* FMCC-197, *Citrobacter freundii* FMCC-8, *Citrobacter farmeri* FMCC-5, *Citrobacter farmeri* FMCC-7 and *Enterobacter* sp. FMCC-208.

All the strains have been isolated from foodstuffs, grapes and meat (Doulgeraki et al., 2011; Nisiotou et al., 2011) by using the selective medium Violet Red Bile Glucose Agar (VRBGA) employed exclusively for the isolation of enterobacterial saprophytic (Drosinos et al., 2005; Argyri et al., 2011) and have been identified and characterized in the Department of Food Science and Technology and have been deposited in the culture collection of this Department (Drosinos et al., 2007; Paramithiotis et al., 2010; Doulgeraki et al., 2011). Although for several species/genera of the family of Enterobacteriaceae attention is generally paid due to their pathogenic properties, some members of the family constitute only an important spoilage group when conditions favor their growth over that of pseudomonads and other pathogens. This group includes most strains of *Serratia liquefaciens, Hafnia alvei, Klebsiella oxytoca* and *Enterobacter* sp. (Garcia Lopez et al., 1998). Pathogenic microorganisms isolated from food stuffs belonging to the above-mentioned family (Enterobacteriaceae) include mostly the species/genera *S. marescens*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella* sp. and *Proteus mirabilis* and certainly microorganisms belonging to other families (i.e. *Pseudomonas aeruginosa*, *Clostridium perfrigens*, etc) (Narashisma Rao et al., 1998).

Long-term storage took place at -80 °C in Tryptic Soy Broth, supplemented with 20% glycerol (Sigma Chemical, St. Louis, MO, USA). Before each experimental use, the strain was cultured in Tryptic Soy Broth and incubated at the optimum temperature for 24 h. Petri dishes were inoculated using this culture and incubated at T=30 °C for 24h in order to be used for the preparation of the pre-cultures.

2.2. Substrate and cultural conditions

2.2.1 The culture media

The culture media (MRS) which was used both in the pre-cultures and in the main fermentations contained in g l^{-1}: peptone 5; meat extract 5; yeast extract 2.5; K_2HPO_4 2; CH_3COONa 5; $MgSO_4$ 0.41. The carbon source used in the pre-cultures, was 10 g l^{-1} of analytical

grade glucose. As for the main fermentations carried out, the carbon source which was used as well as the initial substrate concentration varied, depended from the experiments performed. In any case, the carbon sources which were used throughout this study were glucose, fructose, sucrose, mannose, galactose, xylose, arabinose and cane molasses, that are the principal residue-stream deriving from sugar-processing facilities. Sucrose (purity ≈98%) was purchased from Hellenic Industry of Sugar S.A., Thessaloniki; Greece (cost of sucrose is 0.1 $/kg). Sugarcane molasses was provided by the sugarcane industry Cruz Alta (Guarani, Sao Paulo, Brazil), containing in w w^{-1}: sucrose 43.5%; glucose 7.3%; fructose 6.3%; protein (expressed as total Kjeldahl nitrogen ×6.25) 3.2%; moisture 29.7%; solids 70.4%. As for the element composition of sugarcane molasses, it was analyzed and determined in the laboratory and is presented in Table 2.1.. All other carbon sources which were used during the fermentations were analytical-grade (purity ≈99.5%).

Table 2.1. Element composition of sugarcane molasses employed as carbon source throughout the current study

Elements	ppm
Ca	6.67
S	5.23
Mg	4.70
K	1.75
P	0.21
Fe	0.12
Mn	0.03
Zn	0.003
Co	<0.0005

2.2.2. Pre-culture preparation

For the preparation of each pre-culture, the MRS media were supplemented with 10 g l^{-1} of analytical-grade glucose. The pre-cultures in all cases were conducted in 250-ml shake flasks containing 80 ml medium volume inoculated with a single colony preserved in fresh petri dishes at 4°C. The shake flasks were incubated in an orbital shaker (Lab-Line, Illinois, USA) for 18-20 h at T=30 °C with an agitation of 180 rpm (Lab-Line, Illinois, USA). The initial pH value of culture medium after autoclaving was 7.0±0.2 while the final pH value of the pre-culture was around 6.0 in all cases.

2.2.3. Screening process using anaerobic batch fermentations in Duran bottles

Preliminary assessment of glucose and sucrose consumption and BDO production from various strains was conducted in 1-l Duran bottles under anaerobic conditions. The MRS broth was

supplemented with ≈30 g l^{-1} of glucose or commercial sucrose. The final fermentation volume was 800 ml. Cultures were inoculated with 10% (v/v) of the pre-culture inoculum. The initial pH value of culture medium after autoclaving was 7.0±0.2 and it remained uncontrolled until the end of the fermentation, reaching a value of 6.0±0.2. In order to achieve anaerobic conditions during the fermentations, the medium in Duran bottles was sparged with N_2 for 30 minutes before autoclaving. The Duran bottles were incubated in an orbital shaker (Lab-Line, Illinois, USA) at an agitation speed of 180 rpm and T=30 °C. Samples were taken every 2 hours under aseptic conditions in the laminar flow. Figure 2.1. was taken in the laboratory and represents a 1-l Duran bottle filled with the fermentation broth after sterilization without the pre-culture inoculum.

Figure 2.1. Anaerobic batch fermentation in 1-l Duran bottle (The photo was taken in the laboratory).

2.2.4. Anaerobic batch fermentations in Duran bottles in different initial total sugars concentrations using sucrose and molasses as carbon source

From the results obtained from the screening process in the Duran bottles, two strains which have combined the highest yield and productivity, namely *K. oxytoca* FMCC-197 and *Enterobacter* sp. FMCC-208, were employed for further investigations. Preliminary tests in 1-l Duran bottles were performed on a synthetic (MRS) medium using commercial sucrose or cane molasses. In the case of *Enterobacter* sp. FMCC-208 the initial concentration of total sugars was ≈15, ≈30 and ≈60 g l^{-1} both when sucrose and molasses were used as carbon source. When *K. oxytoca* FMCC-

197 was cultivated in sucrose and molasses, 30, 60, 90, 120 and 150 g l^{-1} of total sugars were used as initial carbon source concentration. The culture conditions were the same as previously described. Samples were taken every 2 or 3 hours, depending from the experiment, under aseptic conditions in the laminar flow.

2.2.5. Aerobic batch fermentations in shake flasks

In order to further assess the ability of the strains *K. oxytoca* FMCC-197 and *Enterobacter* sp. FMCC-208 to produce BDO, various batch fermentations in shake flasks were conducted. Different parameters (i.e. employment of various commercial carbon sources, incubation temperature, initial carbon source concentration, evaluation of potential substrate inhibition) were studied in order to identify the optimum values for the strain growth along with the maximum achievable product synthesis. As far as the different carbon sources employed were concerned, glucose, fructose, mannose, arabinose, galactose and xylose, were added into the MRS medium in an initial concentration adjusted to around 30 g l^{-1} (all these sugars were analytical-grade; purity ≈99.5%) using both strains. Shake flask experiments were conducted in 500-ml shake flasks, the working volume of the fermentation was 100±2 ml and flasks were inoculated with 10% (v/v) of a pre-culture. The fermentations took place at T=30 °C. The agitation rate was 180 rpm and the initial pH value was 7.0±0.2 after autoclaving and it remained uncontrolled until the end of the fermentation. Samples were taken every 2 to 3 hours, depending from the experiment, under aseptic conditions in a laminar flow.

In order to evaluate the optimum temperature for growth and BDO production, trials with 6 different incubation temperatures (i.e. T=25, 30, 34, 37, 40, 42 °C) were carried out in shake-flask cultures using commercial sucrose as carbon source in different initial concentrations. As previously, the fermentations were conducted in 500-ml non-baffled flasks and samples were taken every 2 hours under sterile conditions in a laminar flow.

Likewise, bacterial growth of *Klebsiella oxytoca* FMCC-197 and *Enterobacter* sp. FMCC-208 was studied using a wide range of initial sucrose concentration in order to identify the maximum value in which there is no substrate inhibition. In particular, 10 different initial sucrose concentrations (viz. 5, 10, 15, 20, 40, 60, 80, 110, 130 and 150 g l^{-1}) were used in shake-flask experiments at T=30 °C and T=37 °C using the strains *Klebsiella oxytoca* FMCC-197 and *Enterobacter* sp. FMCC-208, respectively. Samples were taken every 30 minutes and the μ_{max} value of each of the fermentations carried out was calculated. The specific growth rate of each sample was calculated by fitting the equation $\ln\left(X/X_0\right) = f(t)$ on the experimental data within the early

exponential growth phase (X is the dry cell weight – DCW concentration in g l^{-1}). For each case, μ_{max} was the slope of the trendline occurred.

2.2.6. Aerobic batch fermentations in shake flasks under non-aseptic conditions

In the case of *Enterobacter* sp. FMCC-208, a non-aseptic fermentation (viz. in previously pasteurized media) was conducted in 500-ml non-baffled flasks, aiming to investigate the potential of growth to surpass the growth of other microorganisms in the culture medium. Sucrose was used as carbon source in the MRS medium in an initial concentration ≈40 g l^{-1} and the working volume was 100 ml. The culture medium was thermally treated to T=80 °C for 15 min and the inoculum volume was 15% (v/v) of the final working volume. The non-aseptic fermentations were conducted in an orbital shaker as previously. In all cases the pH remained uncontrolled throughout the whole fermentation and samples were taken every 2 hours.

2.2.7. Aerobic batch fermentations in bioreactor

The study has also focused on the evaluation of the optimum pH value for growth of the strains and the production of BDO. For this type of evaluation, four batch experiments in 2-l bioreactor (Infors HT, Type Labfors, Switzerland) using an initial sucrose concentration of 40 g l^{-1} and pH values of 5.0, 6.0, 6.5 and 7.0. As for the strain *K. oxytoca* FMCC-197 the incubation temperature was T=30 °C and for the strain *Enterobacter* sp. FMCC-208 it was T=37 °C. The pH value was measured with a selective pH-meter (Jenway 3020, UK) and was controlled by automatic addition of 5M NaOH. The working volume for each bioreactor experiment was 800 ml; a 10% (v/v) inoculum was employed while the agitation rate was adjusted 180±5 rpm. In order to achieve aerobic conditions, the medium was sparged with air with a rate of 1 vvm throughout the whole experiment.

2.2.8. Fed-batch bioreactor experiments

In order to enhance the final BDO production, both strains were cultivated in fed-batch fermentations applying different culture conditions. Two preliminary fed-batch experiments at 30 °C under anaerobic conditions were conducted. In order to achieve anaerobic conditions, the medium was sparged with N_2 for 20 minutes before sterilization. Thereafter, growth proceeded under anaerobic conditions through self-generated anaerobiosis (Metsoviti et al., 2012a; 2012b). The following fed-batch experiments were carried out under aerobic conditions at 30 °C and 37 °C. Molasses was used as initial carbon source and when the carbon source concentration was low, pulses of a concentrated sucrose solution (600 g l^{-1}) containing also 5% (w/v) yeast extract were

added into the bioreactor vessel. Moreover, molasses was used as the sole carbon source in another fed-batch experiment. In the case of aerobic conditions 1 vvm of aeration was provided into the culture medium while the DOT was constantly ≥20% v/v, achieved with a cascade agitation rate from 180 rpm to 400 rpm. All fed-batch experiments were performed in 2-l bioreactor (Infors HT, Type Labfors, Switzerland) in which the working volume was adjusted at 0.8 l. The initial pH was adjusted to value 7±0.2 before autoclaving and was controlled by automatic addition of 5M NaOH when the value was under 6.0. A selective pH meter (Jenway 3020, UK) was used during the process. Samples were taken every two or three hours. Figure 2.2. represents a fed-batch bioreactor experiment carried out in the laboratory using molasses as the carbon source.

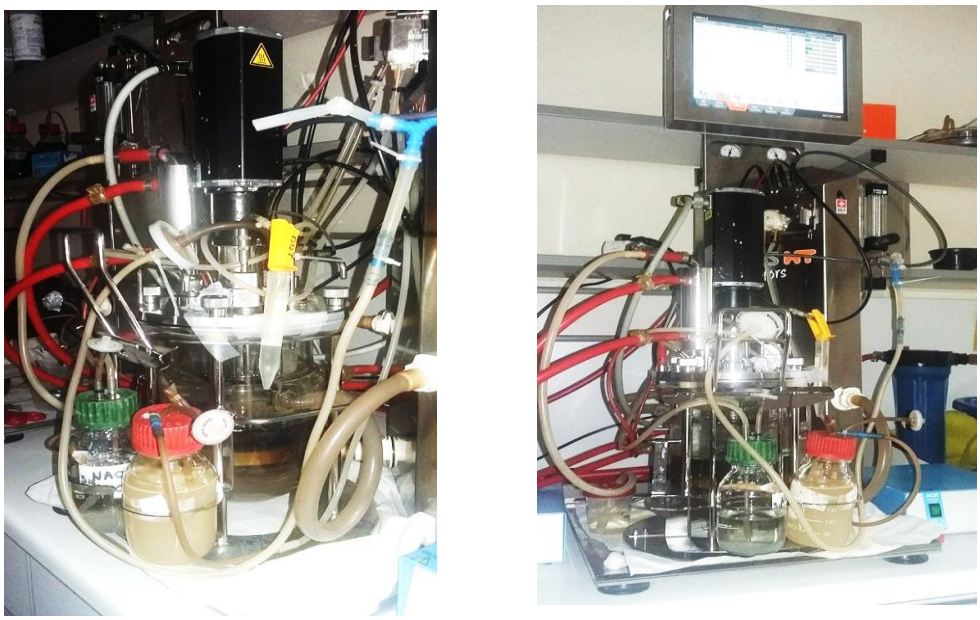


Figure 2.2. Fed-batch bioreactor fermentations using molasses and sucrose as the carbon sources (The photo was taken in the laboratory).

2.2.9. Fed-batch shake-flask experiments under aseptic and non-aseptic conditions

In order to further assess the potential of the process a fed-batch experiment for each of the two strains was conducted in a 2-l shake flask presenting 500 ml working volume, in previously pasteurized media (T=80 °C for 15 min). Rapidly after the thermal treatment conducted and when temperature became ambient, the flask was inoculated with the pre-culture, with an inoculation volume of 15% v/v, of the final working volume of the flask. The experiment was conducted at T= 30 °C and T=37 °C for both *K. oxytoca* FMCC-197 and *Enterobacter* sp. FMCC-208 respectively, in an orbital shaker (Lab-Line, Illinois, USA) at an agitation speed of 180 rpm. The pH was adjusted to value 7.0 before autoclaving and was controlled by automatic addition of 5M NaOH

when the value was lower than 6.0. In parallel, fed-batch fermentation in a 2-l shake flask under aseptic conditions was conducted in order to compare the growth and final product accumulation with the one under non-aseptic conditions.

2.3. Analytical methods

2.3.1. Determination of biomass production

Cell concentration (X, g l^{-1}) was determined through dry cell weight (DCW) measurement (wet and washed biomass put at 90±5 °C until constant weight). Cells were collected by centrifugation (9000 × g/15 min, 9 °C) in a Hettich Universal 320-R (Germany) centrifuge and washed twice with distilled water.

2.3.2. Determination of substrate consumption and metabolite production

Concentrations of glucose, fructose, mannose, arabinose, galactose, xylose, 2,3-butanediol (BDO), acetoin (Ace), ethanol (Eth), succinic acid (Suc) and lactic acid (Lac) (in g l^{-1}) were determined with High Performance Liquid Chromatography (HPLC) analysis (Waters 600E) with an Aminex HPX-87H (300 mm × 7.8 mm, BioRad, USA) column coupled to a differential refractometer (RI Waters 410) and a UV detector (Waters 486). Operating conditions were as follows: sample volume 20 μL; mobile phase 0.010 M H_2SO_4; flow rate 0.6 mL min^{-1}; column temperature T=45 °C. The determination of sucrose and total sugars in the molasses fermentations was carried out via hydrolysis of sucrose to glucose and fructose prior to HPLC analysis. This was achieved by mixing 100 μl of 10% (v/v) H_2SO_4 solution with 500 μl supernatant followed by heating the mixture at 100 °C for 30 min. The concentration of sucrose or carbohydrates found in the molasses is expressed as total sugars, including the concentration of both glucose and fructose while the dilution of the supernatant mixed with the acid was taken into consideration. All data presented are the average of two independent experiments performed under the same culture conditions.

2.3.3. Determination of molasses decolorization

Decolorization of the medium which contained molasses was determined by measuring the decrease of absorbance at 475 nm (Hitachi U-2000, Japan) as described in previous studies (Dahiya et al. 2001; Metsoviti et al., 2011). The absorbance (475 nm) of the fermentation medium (diluted 10 times) before inoculation was determined as set-point. The difference between the absorbance of the set-point and each experimental point was expressed in %.

2.3.4. Dissolved oxygen tension (DOT) and specific oxygen consumption rate determination

For the determination of DOT (%, v/v) of the shake-flask cultures carried out using glucose, fructose and sucrose as carbon source, a Lonibond Sensodirect OXI 200 (Dortmund, Germany) oxygen-meter was used, with the experimental protocol being explained explicitly in Papanikolaou et al. (2004). Precisely, before harvesting, the shaker in which the shake flask trials were carried out was stopped and the oxygen measuring probe was placed into the flask. Attention was paid in order for the probe not to touch to the bottom of the flask. Then, the shaker was again switched on and the measurement was taken after DOT equilibration (usually within the next 10 min after the shaker was again switched on). The measurement of DOT was represented by both oxygen saturation (% v/v) and dissolved oxygen concentration into the liquid medium (in mg l^{-1}). Figures 2.3 and 2.4. represent the linear equation / correlation between the dissolved oxygen saturation (%, v/v) and the dissolved oxygen concentration (in mg l^{-1}) for the strains *Enterobacter* sp. FMCC-208 and *Klebsiella oxytoca* FMCC-197, respectively.

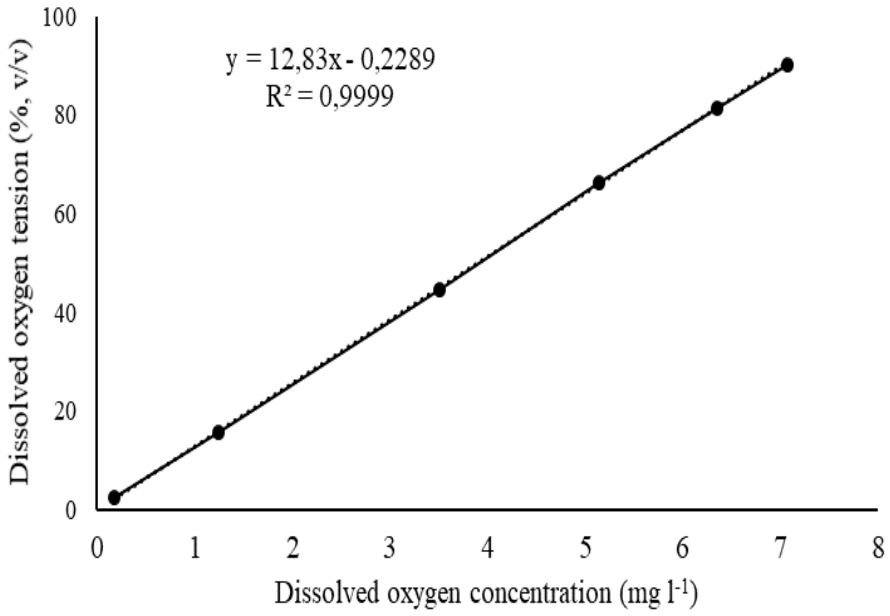


Figure 2.3. Dissolved oxygen tension (DOT, % v/v) (●) vs dissolved oxygen concentration (mg l^{-1}) during growth of *Enterobacter* sp. FMCC-208 on sucrose in shake-flask experiments. Culture conditions: growth in 500-ml flasks filled with 100 ml, 180 rpm agitation rate, initial pH=7.0, various initial concentrations of sucrose employed. Each point is the mean value of two independent measurements.

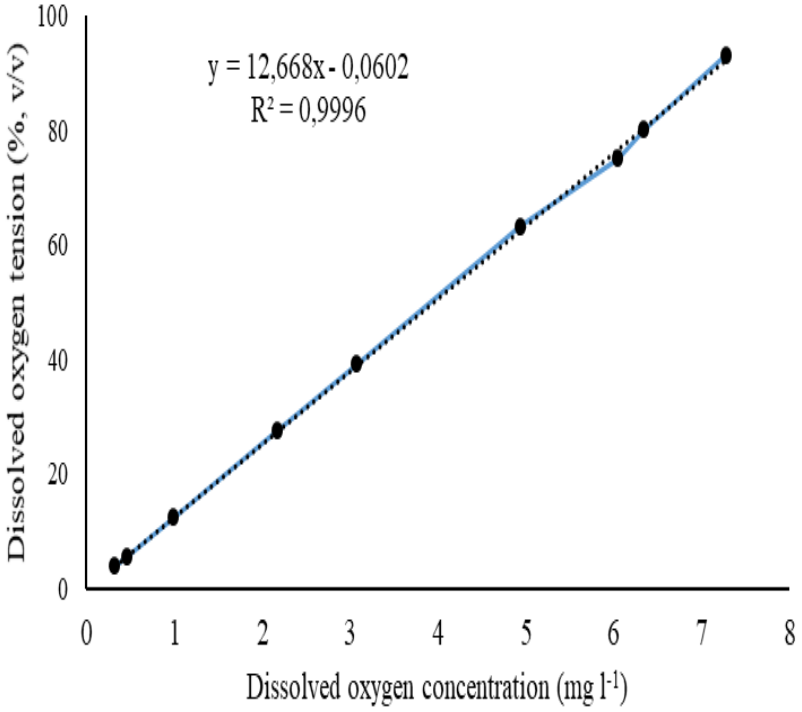


Figure 2.4. Dissolved oxygen tension (DOT, % v/v) (●) vs dissolved oxygen concentration (mg l^{-1}) during growth of *Klebsiella oxytoca* FMCC-197 on sucrose in shake-flask experiments. Culture conditions: growth in 500-ml flasks filled with 100 ml, 180 rpm agitation rate, initial pH=7.0, various initial concentrations of sucrose employed. Each point is the mean value of two independent measurements.

For the measurement of the specific oxygen consumption rate (q_{O_2} – g (g h)$^{-1}$), after DO equilibration the shaker was again switched off and oxygen concentration (mg l^{-1}) was measured every 5 sec. The slope of the curve $[O_2]=f(t)$ showed the oxygen consumption rate (r_{O_2}, g (l h)$^{-1}$), and the value of q_{O_2} was found after division of the oxygen consumption rate by the respective DCW (X in g l^{-1}) value. The following figures represent an example of the reduction of the concentration of oxygen noted every 5 sec for 30 sec, using the two species employed in the current investigation. The samples were taken after 2 hours of fermentation using glucose as carbon source in an initial concentration of 30 g l^{-1} in both cases.

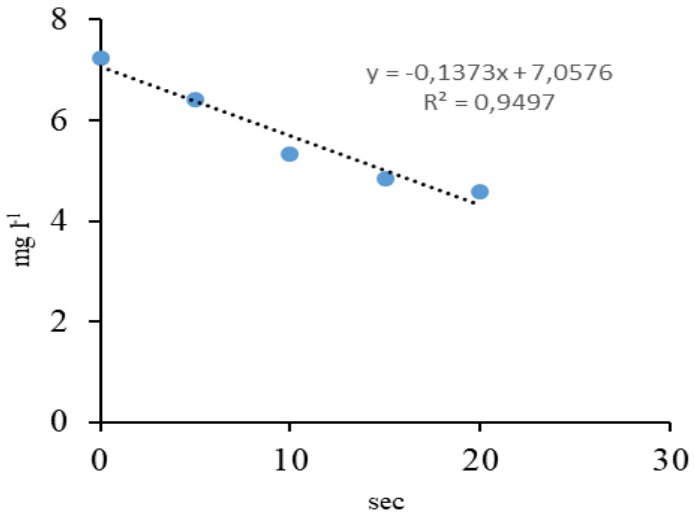


Figure 2.5. Reduction of dissolved oxygen noted every 5 sec for 30 sec during cultivation of *Enterobacter* sp. FMCC-208 on glucose in shake-flask experiments. Culture conditions: growth in 500-ml flasks filled with 100 ml, 180 rpm agitation rate, T=30 °C initial pH=7.0, ≈30 g l^{-1} initial glucose concentration. Each point is the mean value of two independent measurements.

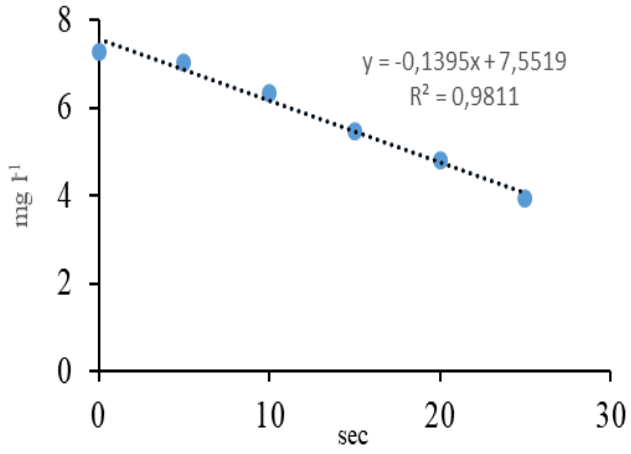


Figure 2.6. Reduction of dissolved oxygen noted every 5 sec for 30 sec during cultivation of *Klebsiella oxytoca* FMCC-197 on glucose in shake-flask experiments. Culture conditions: growth in 500-ml flasks filled with 100 ml, 180 rpm agitation rate, T=30 °C initial pH=7.0, ≈30 g l^{-1} initial glucose concentration. Each point is the mean value of two independent measurements.

3. Results

3.1. Initial screening using newly isolated strains for the evaluation of their ability to grow on analytical-grade glucose and commercial sucrose and produce 2,3-butanediol

3.1.1. Introduction

At the first part of this study, a screening study was carried out in anaerobic cultures using nine newly isolated, natural bacterial strains. In all cases, two different carbon sources, *viz*. analytical-grade glucose and commercial sucrose, were used in order to evaluate the ability of the strains to consume the substrate and produce 2,3-butanediol (BDO) and potentially other interesting value-added microbial metabolites. From the results obtained through these initial cultivations, two strains were selected for further experiments which are presented in the second and third part of this study.

3.1.2. Results

The initial trials of this study focused on the cultivation of nine newly isolated and potentially non-pathogenic strains which belong to the family of *Enterobacteriaceae* on commercial glucose and sucrose. These strains are derived from various food stuffs (i.e. raw meat and grapes) and are genetically non-modified. The aim of this set of experiments was to evaluate the ability of these strains to assimilate glucose and sucrose for growth and BDO production. In particular, the strains which were cultivated were *Enterobacter ludwigii* FMCC-204, *Enterobacter aerogenes* FMCC-9, *Enterobacter aerogenes* FMCC-10, *Citrobacter freundii* FMCC-207, *Klebsiella oxytoca* FMCC-197, *Citrobacter freundii* FMCC-8, *Citrobacter farmeri* FMCC-5, *Citrobacter farmeri* FMCC-7 and *Enterobacter* sp. FMCC-208 and they have never been used before for the production of BDO from commercial sucrose. The fermentations took place under anaerobic conditions in 1-l Duran bottles for 24 hours and the carbon source consumption along with biomass and BDO production were evaluated by taking samples every 2 hours. Table 3.1. summarizes the results obtained from the screening process. As shown, six strains could efficiently consume both glucose and sucrose, producing BDO in highly promising yields and productivity rates. For instance, the strain *E. ludwigii* FMCC-204 assimilated 29.2 g l^{-1} of sucrose and the final BDO titer after 20 hours was 12.5 g l^{-1}. The final bioconversion yield and volumetric productivity obtained were 0.43 g g^{-1} and 0.63 g l^{-1} h^{-1} respectively. The strains *E. aerogenes* FMCC-9 and FMCC-10 gave also very promising results as they successfully consumed the whole substrate quantity within 20 hours presenting bioconversion yield values of 0.43 g g^{-1} and 0.40 g g^{-1} respectively. The strains *C. freundii* FMCC-207 and *Enterobacter* sp. FMCC-208 reached the highest bioconversion yield, 0.44 g g^{-1}, however the productivity rate was remarkably higher in the case of *Enterobacter* sp. FMCC-

208. The highest volumetric productivity value was noted for *K. oxytoca* FMCC-197 reaching a value of 0.72 g l^{-1} h^{-1}.

It should be stressed that in all the previous anaerobic experiments, no substrate remained unconsumed at the end of the fermentation. Although BDO was the main fermentation product, slight production (~1 g l^{-1}) of organic acids was also detected.

On the other hand, three strains, namely *C. freundii* FMCC-8, *C. farmeri* FMCC-5 and *C. farmeri* FMCC-7 could only consume glucose, however, around 5 g l^{-1} of the carbon source remained unconsumed at the end of the fermentation. In addition, no BDO production was detected and acetic acid was the only metabolite produced at a final concentration lower than 10 g l^{-1}. On the other hand, the three bacterial strains were not revealed capable to assimilate sucrose as the substrate concentration after 24 hours of fermentation was almost the same as the initial one. Slight biomass production was noted and no organic acids were detected in the fermentation broth.

Taking into consideration the results obtained from the initial trials in anaerobic cultivations using glucose or sucrose as the sole carbon source, two strains, *Enterobacter* sp. FMCC-208 and *K. oxytoca* FMCC-197, were selected for further investigation as they combined satisfactory bioconversion yield and relatively enhanced volumetric productivity values in both carbon sources employed as substrates.

Table 3.1. Growth parameters, substrate consumption, final BDO concentration, conversion yield and productivity in batch fermentations under anaerobic conditions in 1-l Duran bottles. Culture conditions: T=30 °C; agitation rate =180 rpm; initial pH=7.0. Each point is the mean value of two independent measurements.

Strain	Carbon source	Substrate consumed ($g\ l^{-1}$)	X_{max} ($g\ l^{-1}$)	BDO ($g\ l^{-1}$)	Y_{BDO} ($g\ g^{-1}$)	P_{BDO} ($g\ l^{-1}\ h^{-1}$)	Fermentation time (h)
Enterobacter ludwigii FMCC-204	Sucrose	29.2±0.4	2.5±0.2	12.5±0.3	0.43±0.01	0.63±0.01	20
	Glucose	27.9±0.3	3.2±0.1	13.3±0.2	0.48±0.02	0.72±0.02	18
Enterobacter aerogenes FMCC-9	Sucrose	30.0±1.0	3.1±0.1	13.0±0.5	0.43±0.01	0.65±0.03	20
	Glucose	29.3±0.2	3.3±0.1	12.7±0.2	0.43±0.01	0.71±0.01	18
Enterobacter aerogenes FMCC-10	Sucrose	29.8±1.1	3.3±0.5	12.0±1.0	0.40±0.02	0.60±0.05	20
	Glucose	31.1±1.2	2.9±0.2	14.2±1.2	0.46±0.03	0.79±0.02	18
Citrobacter freundii FMCC-207	Sucrose	28.7±2.2	2.5±0.0	12.5±0.3	0.44±0.04	0.50±0.01	25
	Glucose	29.8±1.2	3.5±0.2	12.3±0.3	0.41±0.02	0.68±0.02	18
Klebsiella oxytoca FMCC-197	Sucrose	31.5±0.8	2.1±0.3	13.0±0.8	0.41±0.02	0.72±0.03	18
	Glucose	29.1±1.6	3.9±1.0	14.0±0.5	0.48±0.02	0.88±0.04	16
Citrobacter freundii FMCC-8	Sucrose	-	-	-	-	-	24
	Glucose*	25.3±0.5	3.5±0.2	-	-	-	24
Citrobacter farmeri FMCC-5	Sucrose	-	-	-	-	-	24
	Glucose*	24.2±0.2	2.5±0.2	-	-	-	24
Citrobacter farmeri FMCC-7	Sucrose	-	-	-	-	-	24
	Glucose*	26.3±0.5	3.0±0.2	-	-	-	24
Enterobacter sp. FMCC-208	Sucrose	25.3±1.0	3.2±0.2	11.2±0.6	0.44±0.01	0.62±0.01	18
	Glucose*	29.9±1.5	4.2±0.2	14.2±0.5	0.47±0.02	0.89±0.02	16

*At the end of the fermentation acetic acid was produced at a final concentration c.10 $g\ l^{-1}$

3.2. Optimization of 2,3-butanediol (BDO) production in *Klebsiella oxytoca* FMCC-197 cultures using analytical-grade or low-cost carbohydrate-based substrates

3.2.1. Introduction

The first strain which was selected for further investigation was *Klebsiella oxytoca* FMCC-197. From the preliminary results obtained through the screening process, the strain *K. oxytoca* FMCC-197 was proven to be a highly promising BDO producer. For instance, it was able to totally convert glucose and sucrose into BDO, reaching remarkable values of bioconversion yield and volumetric productivity. During the initial trial in Duran bottle using glucose as carbon source, the whole substrate, 29.1 g l^{-1}, was totally consumed after 16 hours of fermentation and 14.0 g l^{-1} of BDO were produced. The final yield was 0.48 g g^{-1} while the productivity was 0.88 g l^{-1} h^{-1}. Furthermore, when sucrose was used as the carbon source in the culture medium in Duran bottles, 31.5 g l^{-1} of total sugars were consumed, which corresponded to the complete substrate quantity employed into the medium, and 13.0 g l^{-1} of BDO were produced. The bioconversion yield was 0.41 g g^{-1} and the volumetric productivity achieved was 0.72 g l^{-1} h^{-1}.

The following step was to investigate different parameters in order to achieve high BDO and acetoin (Ace) production with satisfactory yields and productivity rates. For instance, batch experiments were conducted in the next steps of this chapter using different initial sucrose concentrations, various carbon sources employed as individual substrates and several incubation temperature values in order to optimize the bioprocess.

Taking into consideration the results obtained from the mentioned fermentations, four fed-batch bioreactor experiments were conducted changing different parameters in order to achieve high product synthesis. Fed-batch shake flask experiments were also performed in aseptic and non-aseptic conditions, using sucrose as the sole carbon source into the culture medium.

3.2.2. Initial trials under anaerobic and aerobic conditions demonstrating sugar assimilation and BDO production

Considering that the strain *K. oxytoca* FMCC-197 is a highly promising BDO producer in cultivations using glucose or sucrose as carbon source, the following experiments were focused on the BDO and Ace production using different initial substrate concentrations, in which mostly sucrose was employed as carbon source. Moreover, the impact of the aeration (i.e. growth in aerobic shake-flask or anaerobic Duran-bottle cultures) was investigated, given that aeration constitutes a parameter of major importance for the efficiency of BDO production process (Maddox, 2008). Therefore, during this set of experiments, sucrose was used as the sole carbon source in batch experiments, under anaerobic and aerobic conditions. As for the anaerobic

fermentations, when 30 g l^{-1} and 60 g l^{-1} of sucrose were added into the culture medium, the whole quantity of carbon source was consumed leading to the production of BDO in yields of 0.41 g g^{-1} and 0.44 g g^{-1}, respectively. When 90 g l^{-1} of initial carbon source was used, around 10 g l^{-1} remained unconsumed and the conversion yield was reduced. At higher initial concentrations imposed (i.e. >120 g l^{-1}), a considerable part of the carbon source remained unconsumed and final yield and productivity rate was also reduced.

On the other hand, higher productivity values were noted during aerobic cultivations, although the bioconversion yields were similar to those obtained under anaerobic conditions. It should be stressed that the whole substrate was consumed when 30, 60 and 90 g l^{-1} of initial sucrose concentration were used into the medium; however in higher initial concentrations employed (*viz*. >90 g l^{-1}) a remarkable part of the substrate remained unconsumed. For instance, the highest bioconversion yield, 0.43 g g^{-1}, was noted at initial sucrose concentrations of 60 g l^{-1} and 90 g l^{-1}, while the productivity values achieved were higher than 1.30 g l^{-1} h^{-1}. When higher substrate concentrations were added into the culture medium (i.e. 120 g l^{-1} and 150 g l^{-1}), a considerable amount of carbon source remained unconsumed, lowering the final bioconversion yield of the fermentation. Table 3.2. summarizes the results obtained during the anaerobic and aerobic cultures using different initial carbon source concentrations. It should be stressed that although anaerobic fermentations resulted remarkable BDO and Ace production, the forthcoming batch experiments using the strain *K. oxytoca* FMCC-197 were performed under aerobic conditions, since as it has been demonstrated (Table 3.2.) higher productivity and substrate consumption values were achieved in these culture conditions. Aerobic conversion of sugars and related substrates (i.e. polysaccharides, glycerol) into BDO is considered as a somehow surprising as result since BDO production process, in general, is considered as a bioprocess that is mostly carried out under mostly anaerobic conditions (Zeng & Sabra, 2011).

Table 3.2. Cultures of *Klebsiella oxytoca* FMCC-197 on Duran bottles under anaerobic conditions and in shake flasks under aerobic conditions using commercial sucrose as carbon source. Representation of maximum biomass production (X_{max}), substrate consumption, final 2,3-butanediol concentration (BDO), conversion yield on sugars consumed ($Y_{BDO,Ace}$) and productivity in batch fermentations. Culture conditions: T=30 °C; agitation rate =180 rpm; initial pH=7.0. Each point is the mean value of two independent measurements.

Cultivation mode	Initial substrate concentration (g l^{-1})	Total sugars consumed (g l^{-1})	X_{max} (g l^{-1})	BDO (g l^{-1})	Ace (g l^{-1})	$Y_{BDO,Ace}$ (g g^{-1})	$P_{BDO,Ace}$ (g l^{-1} h^{-1})	Fermentation time* (h)
Duran-bottle (anaerobic) cultures	31.5±0.8	31.5±0.8	2.1±0.3	11.0±0.8	2.0±0.2	0.41±0.02	0.72±0.03	18
	62.2±0.8	62.2±0.8	3.8±0.3	23.5±1.5	4.0±0.5	0.44±0.02	1.15±0.06	24
	92.0±2.0	83.0±3.0	4.3±0.3	26.7±1.2	5.1±0.6	0.38±0.01	1.14±0.04	28
	124.2±2.5	95.3±4.5	4.9±1.0	30.0±2.0	5.5±1.5	0.37±0.03	1.04±0.04	34
	149.8±2.5	101.3±0.7	5.4±0.6	28.1±1.5	7.5±1.1	0.35±0.01	0.74±0.03	48
Shake-flask (aerobic) cultures	37.1±0.1	37.1±0.1	6.4±0.2	13.0±0.1	1.0±0.1	0.38±0.01	1.40±0.01	10
	67.9±1.3	67.9±1.3	6.8±0.0	24.2±0.3	4.9±0.3	0.43±0.00	1.32±0.01	22
	91.8±2.5	91.8±2.5	7.5±0.5	32.8±2.0	6.8±0.6	0.43±0.01	1.37±0.07	29
	122.1±3.5	100.5±2.5	6.3±1.0	29.8±2.5	5.9±1.5	0.36±0.01	1.02±0.05	35
	164.5±3.5	101.5±3.5	6.0±0.6	31.0±0.0	7.0±1.0	0.37±0.01	0.76±0.00	50

*Fermentations were extended after the indicated time and no more sugar has been consumed

3.2.3. Evaluation of the ability of the strain to assimilate different sugars

Taking into consideration that most of the industrial and housekeep wastes contain various types of carbohydrates, and in order to present and demonstrate a sustainability aspect of the present study, aerobic shake flask cultures were conducted using different carbon sources, in order to evaluate the ability of the strain *K. oxytoca* FMCC-197 to produce BDO and Ace. For instance, eight carbon sources (i.e. glucose, fructose, mannose, xylose, arabinose, galactose, sucrose and molasses) were added into the culture media at an initial concentration of 30 g l^{-1}. Therefore, interesting results as regards substrate assimilation and BDO and Ace production during growth on the above-mentioned compounds would demonstrate the potential of the strain *K. oxytoca* FMCC-197 to valorize waste streams containing these sugars (i.e. lignocellulosic hydrolysates, spent sulfite liquor, food waste hydrolysates and sugar-rich wastewaters, etc; see Koutinas et al. 2014; Papanikolaou and Aggelis 2019).

As shown in Table 3.3., in most cases the final product synthesis was satisfying, accompanied by high yields and volumetric productivities. In particular, the highest bioconversion yield, namely 0.47 g g^{-1}, was noted when arabinose was used as carbon source although the concomitant productivity rate was low (0.58 g l^{-1} h^{-1}). The strain successfully converted glucose, fructose, mannose, galactose and molasses into BDO and Ace in yields higher than 0.40 g g^{-1}. On

the other hand, reduction in final product accumulation into the medium with low bioconversion yield (0.30 g g^{-1}) and productivity rate (0.22 g l^{-1} h^{-1}) was observed when xylose was used in the substrate. Additionally, the highest productivity rates were achieved when sucrose and molasses were added into the culture media.

Table 3.3. Growth of *Klebsiella oxytoca* FMCC-197 in shake flasks using various sugars employed as sole carbon source under aerobic conditions. Representation of maximum biomass production, substrate consumption, final 2,3-butanediol, acetoin and other organic products concentration, conversion yield and productivity in batch fermentations under aerobic conditions using different carbon sources. Culture conditions: growth in 500-ml flasks filled with 100 ml, T=30°C; agitation rate =180 rpm; initial pH=7.0. Each point is the mean value of two independent measurements.

Carbon source	Total sugars consumed ($g\ l^{-1}$)	X_{max} ($g\ l^{-1}$)	BDO ($g\ l^{-1}$)	Ace ($g\ l^{-1}$)	Ethanol ($g\ l^{-1}$)	Succinic ($g\ l^{-1}$)	Lactic ($g\ l^{-1}$)	$Y_{BDO,Ace}$ ($g\ g^{-1}$)	$P_{BDO,Ace}$ ($g\ l^{-1}\ h^{-1}$)	Fermentation time* (h)
Glucose	27.7±1.1	6.6±0.4	10.9±0.2	1.5±0.1	2.3±0.3	2.4±0.1	0.4±0.1	0.45±0.01	1.03±0.02	12
Fructose	28.3±0.9	5.8±0.1	10.7±1.2	1.2±0.1	2.3±0.1	2.5±0.1	0.5±0.1	0.42±0.01	0.99±0.10	12
Mannose	23.8±1.1	5.8±0.2	9.8±0.2	0.9±0.1	2.2±0.2	2.2±0.1	0.5±0.1	0.45±0.03	0.82±0.02	13
Xylose	16.0±0.7	6.0±0.1	4.2±0.2	0.2±0.0	0.0±0.0	0.8±0.1	0.0±0.0	0.30±0.01	0.22±0.01	22
Arabinose	17.4±1.5	7.0±0.4	7.7±0.2	0.5±0.1	0.8±0.1	1.1±0.3	0.0±0.0	0.47±0.02	0.58±0.01	14
Galactose	28.5±0.7	6.8±0.0	10.7±0.3	1.1±0.1	2.1±0.4	1.5±0.4	0.0±0.0	0.41±0.01	0.98±0.02	12
Sucrose	37.1±0.1	6.4±0.2	13.0±0.1	1.0±0.1	1.3±0.1	1.6±0.1	0.2±0.0	0.38±0.01	1.40±0.01	10
Molasses	35.2±1.5	5.9±0.1	12.2±0.2	1.9±0.2	1.1±0.1	1.9±0.3	0.2±0.0	0.40±0.01	1.41±0.01	10

*Fermentations were extended after the indicated time and no more sugar had been consumed after the given fermentation point

3.2.4. Temperature effect on growth and BDO production during aerobic fermentations using sucrose as carbon source

During this part of the study, aerobic shake-flask experiments were performed using various temperature values in order to identify the most suitable temperature for the studied fermentation. Six different incubation temperature values (i.e. 25, 30, 34, 37, 40 and 42 °C) were applied in order to examine the effect of temperature upon the final biomass and product synthesis (Table 3.4.). As shown, high bioconversion yields were achieved at values 25, 30 and 34 °C, while higher temperatures led to reduction of the final conversion yield. The highest volumetric productivity value (0.40 g l^{-1} h^{-1}) was achieved at 30 °C, when 13.0 g l^{-1} of BDO and 1.0 g l^{-1} of Ace were produced after cultivation of 10 h.

Temperature values higher than 37 °C (i.e. 40 or 42 °C) seem to favor biomass production instead of product synthesis. This could be explained as high temperature values reduce the enzymatic activity altering the mixed-acid BDO pathway to biomass production (Maddox, 2008; Perego et al., 2000).

Table 3.4. Temperature effect in total sugars consumption and 2,3-butanediol production, yield and productivity in shake-flask fermentations carried out with c. 40 g l^{-1} of initial sucrose concentration employed as substrate by *Klebsiella oxytoca* FMCC-197. Culture conditions: growth in 500-ml flasks filled with 100 ml, agitation rate =180 rpm; initial pH=7.0. Each point is the mean value of two independent measurements.

T	Total sugars consumed	X_{max}	BDO	Ace	Ethanol	Succinic	Lactic	$Y_{BDO,Ace}$	$P_{BDO,Ace}$	Fermentation time*
◦C	(g l^{-1})	(g l^{-1})	(g l^{-1})	(g l^{-1})	(g l^{-1})	(g l^{-1})	(g l^{-1})	(g g^{-1})	(g l^{-1} h^{-1})	(h)
25	37.5±0.7	4.9±0.7	14.8±1.3	2.0±0.2	1.4±0.3	1.4±0.1	0.2±0.0	0.45±0.03	0.93±0.07	18
30	37.1±0.1	6.4±0.2	13.0±0.1	1.0±0.1	1.3±0.1	1.6±0.1	0.2±0.1	0.38±0.01	1.40±0.01	10
34	33.9±2.7	4.8±0.4	12.4±0.9	2.4±0.4	1.1±0.1	1.5±0.3	0.1±0.0	0.42±0.01	1.20±0.08	12
37	39.8±1.6	4.8±0.4	11.1±0.6	1.5±0.2	2.5±0.0	2.1±0.1	0.7±0.1	0.32±0.02	1.15±0.01	11
40	40.0±0.4	5.1±0.2	11.7±0.6	2.1±0.2	1.5±0.0	1.9±0.1	0.3±0.1	0.35±0.02	0.86±0.07	16
42	26.4±1.4	7.2±0.8	7.2±0.9	0.8±0.1	0.5±0.1	0.0±0.0	0.0±0.0	0.30±0.02	0.31±0.03	26

*Fermentations were extended after the indicated time and no more sugar has been consumed

3.2.5. Impact of sucrose concentration upon the microbial growth of *Klebsiella oxytoca* FMCC-197

The following experiments focused on the further optimization of the bacterial growth as regards the initial sugar concentration employed. Therefore, trials with 10 different initial sucrose concentrations (viz. 5, 10, 15, 20, 40, 60, 80, 110, 130 and 150 g l^{-1}) were carried out in shake-flask experiments at T=30 °C, in order to further identify the impact of sucrose concentration upon the physiological and kinetic behavior of the strain, in conjunction with previous obtained results (see also Table 3.2.). In the current set of experiments, it was mostly interesting to evaluate the μ_{max} during growth on the several initial sucrose concentration media, and for this reason the curve of $\ln\left(\frac{X}{X_0}\right) = f(t)$ was fitted on the available experimental data within the early exponential growth phases of the trials. On the other hand, in this part of the work we were not interested only in the BDO production and the Y_{BDO} value achieved, but also on the lag phase duration, and also on the yield of dry cell weight produced per unit of sugar consumed ($Y_{X/S}$), that are kinetic parameters demonstrating the effect of substrate inhibition exerted towards the studied strain (Matsoviti et al., 2013a; 2013b; Tchakouteu et al., 2015). The obtained results are shown in Table 3.5.. High values of specific growth rate (μ_{max}>0.65 h^{-1}) were obtained when the initial substrate concentration was lower than 40 g l^{-1}. In higher initial sucrose concentrations employed, μ_{max} values decreased suggesting substrate inhibition exerted due to the relatively increased initial sucrose concentration added into the medium. It is worth mentioning that irrespective of the (sufficiently high in several trials) concentration of sucrose found into the medium, satisfactory BDO quantities (up to 41.0 g l^{-1} with simultaneous yield $Y_{BDO,Ace}$≈0.41-0.44 g g^{-1}) were recorded. On the other hand, yield $Y_{X/S}$ constantly decreased towards the whole range of sucrose concentration employed, while the lag phase duration slightly increased with the increment of sucrose quantity into the medium. It is interesting to indicate, in conjunction with the results previously reported (Table 3.2.) that despite the high initial concentrations of sucrose found into the medium (i.e. ≈150 g l^{-1}), noticeable sugar assimilation and remarkable BDO production occurred, demonstrating the potential of the employed strain towards this type of bioconversion. On the other hand, given that *K. oxytoca* FMCC-197 was unable to assimilate sucrose quantities >100 g l^{-1} in batch experiments (see Tables 3.2. and 3.5.), fed-batch strategies would be revealed necessary in order to maximize BDO (and Ace) production.

Table 3.5. Growth of *Klebsiella oxytoca* FMCC-197 in shake flasks using various initial sucrose concentrations employed. Representation of the maximum specific growth rate of the strain in shake flask fermentations carried out at T=30 °C. Culture conditions: growth in 500-ml flasks filled with 100 ml, agitation rate =180 rpm; initial pH=7.0. Each point is the mean value of two independent measurements.

Initial sucrose concentration ($g\ l^{-1}$)	μ_{max} (h^{-1})	Sugar consumed ($g\ l^{-1}$)	X_{max} ($g\ l^{-1}$)	$Y_{X/S}$ ($g\ g^{-1}$)	BDO ($g\ l^{-1}$)	Lag phase duration (h)	Fermentation time (h)
≈5	0.80	4.5	2.0	0.44	2.1	0.5	3.5
≈10	0.82	9.7	3.8	0.39	4.5	0.5	4.5
≈15	0.84	16.2	4.2	0.26	6.8	1.0	6.0
≈20	0.85	19.3	4.5	0.23	8.0	1.5	6.5
≈40	0.65	42.0	6.4	0.15	18.1	1.5	8.0
≈60	0.32	62.3	6.5	0.10	28.2	1.5	24.0
≈80	0.27	78.4	7.0	0.09	37.0	2.0	28.0
≈110	0.14	87.2*	6.2	0.07	38.8	2.5	32.0*
≈130	0.12	90.2**	5.8	0.06	36.2	3.5	36.0**
≈150	0.10	98.5**	5.5	0.06	41.0	5.0	40.0**

*Initial sugars at 110.4 $g\ l^{-1}$, fermentation was extended after 32 h and no more sugar has been consumed

**Initial sugars at 133.2 $g\ l^{-1}$, fermentation was extended after 36 h and no more sugar has been consumed

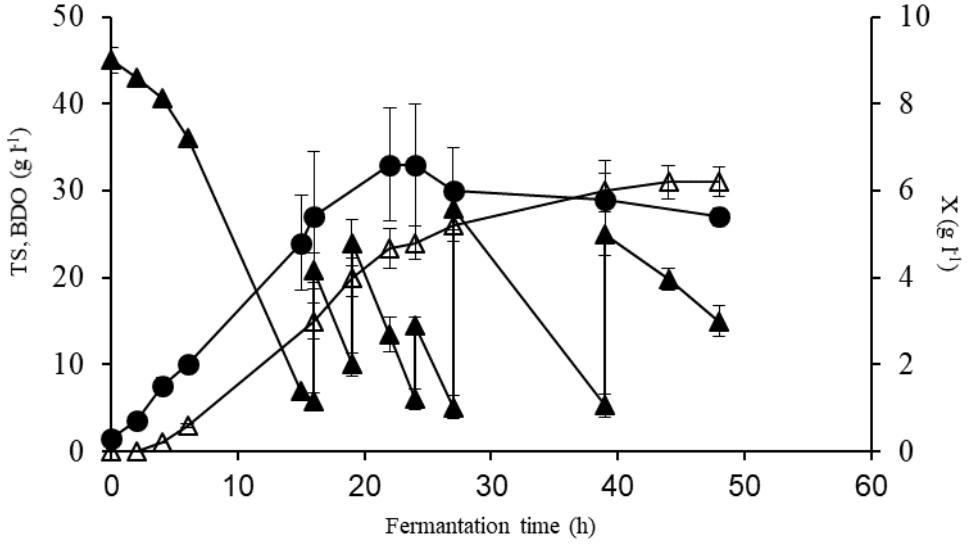
*** Initial sugars at 148.2 $g\ l^{-1}$, fermentation was extended after 40 h and no more sugar has been consumed

3.2.6. Fed-batch experiments for enhanced 2,3-butanediol production

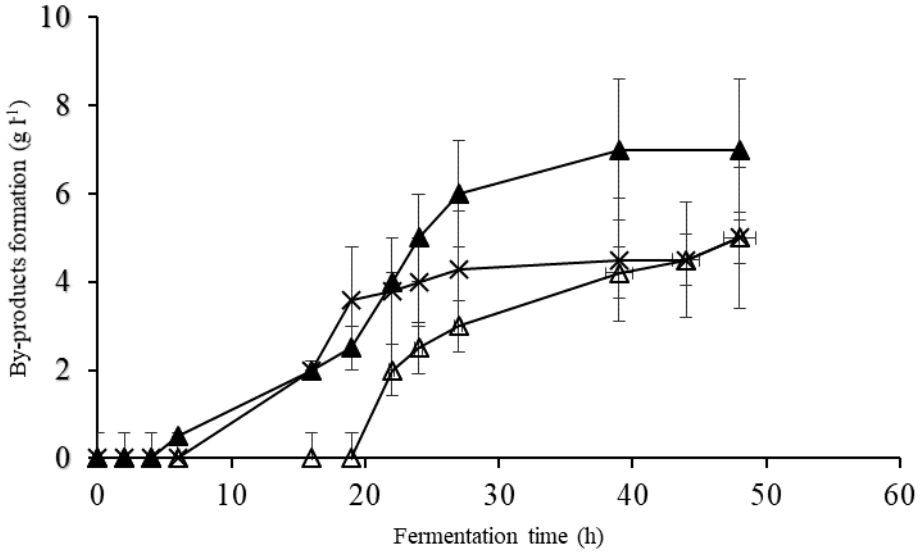
As previously indicated, in batch cultures performed by *K. oxytoca* FMCC-197, the quantity of sugar consumed (and, thus, the quantity of BDO produced) could not be excessively high. Therefore, the following part of the study using the above-mentioned strain was carried out in order to maximize the final BDO and Ace production in fed-batch bioreactor experiments, in which different parameters were changed.

In the first fed-batch bioreactor experiment, the microbial culture was conducted under anaerobic conditions (i.e. sparging with N_2 30 min before inoculation, and self-generated anaerobiosis after the inoculation) using molasses as the initial carbon source and pulses of concentrated sucrose were performed when needed. The experiment was conducted at 30 °C. As shown in Table 3.6., a quantity of 110.1 $g\ l^{-1}$ of total sugars was consumed in 48 h, leading to the production of 31.0 $g\ l^{-1}$ of BDO and 7.0 $g\ l^{-1}$ of Ace. The total bioconversion yield was 0.35 $g\ g^{-1}$ and the volumetric productivity achieved was 0.89 $g\ l^{-1}\ h^{-1}$. Other organic acids were also produced

in low concentrations. In Figure 3.1., the kinetics of substrate consumption along with bacterial growth and products accumulation into the growth medium are presented.



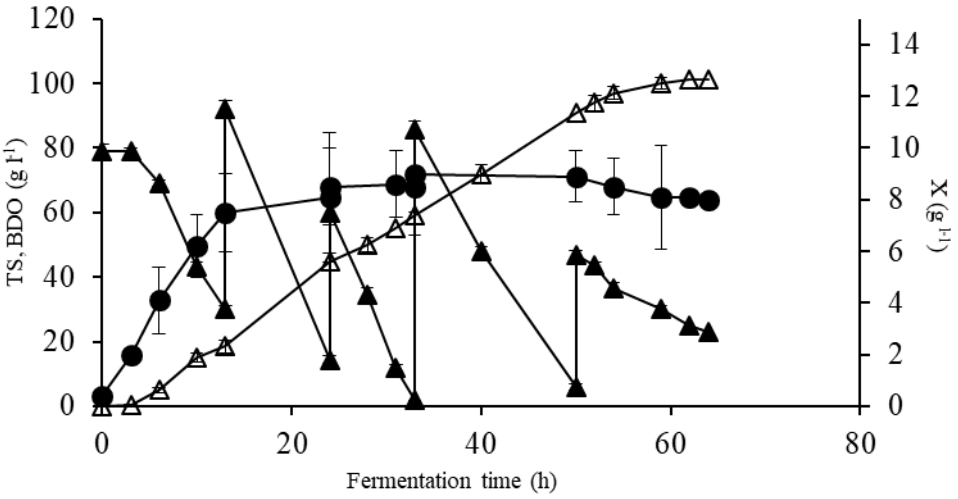
(a)



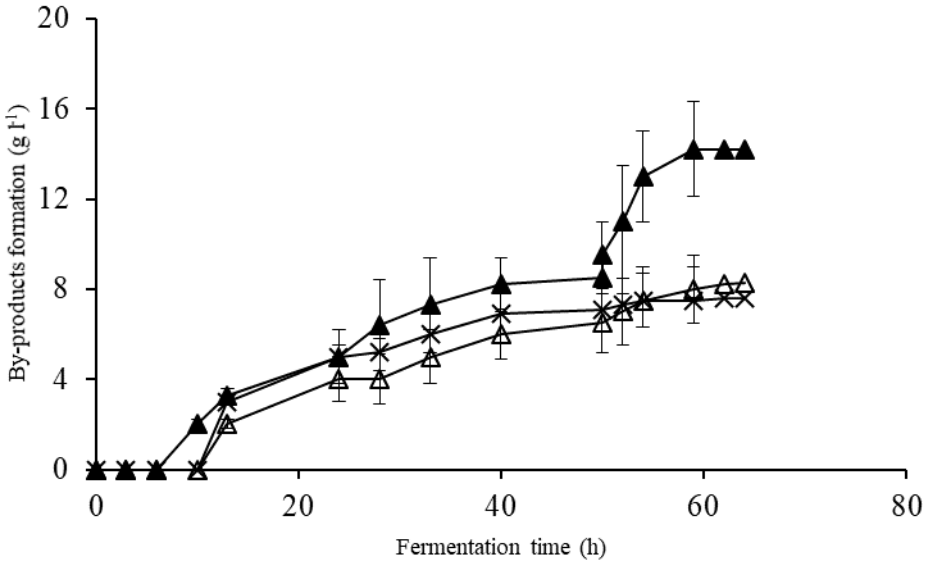
(b)

Figure 3.1. Kinetics of evolution of total sugars (TS, g l^{-1}) (▲), 2,3-butanediol (BDO, g l^{-1}) (△), biomass (X, g l^{-1}) (●) (a) and acetoin (Ace, g l^{-1}) (▲), lactic acid (Lac, g l^{-1}) (△) and ethanol (Eth, g l^{-1}) (×) (b) during growth of *Klebsiella oxytoca* FMCC-197 on molasses and sucrose in fed-batch bioreactor experiments. Culture conditions: anaerobic trial, 180 rpm agitation rate, pH fluctuating from 7.0 to 6.0, T=30 °C, growth on 2-l bioreactor. Each point is the mean value of two independent measurements.

The second fed-batch bioreactor experiment was conducted using molasses and sucrose as carbon sources at 30 °C. However, aerobic conditions (1 vvm of aeration and a cascade agitation rate from 180 rpm to 400 rpm; DOT constantly≥20% v/v) were applied into the culture medium, in order to compare the final product accumulation into the medium with the one under anaerobic conditions. After 64 h, 288.8 g l^{-1} of total sugars were converted into 101.1 g l^{-1} of BDO and 14.2 g l^{-1} of Ace, reaching a bioconversion yield for the last fermentation point ≈0.40 g g^{-1}. The production of a total quantity of 115.3 g l^{-1} of BDO+Ace is one of the highest values reported in the international literature so far, where non-genetically modified or mutant strains are implicated in the process (Celińska & Grajek, 2009; Jurchescu et al., 2013; Koutinas et al., 2014; Jung et al., 2015). Remarkable increase in the productivity rate was noted under aerobic conditions employed, with a value of 1.80 g l^{-1} h^{-1}. The production of other organic acids was higher than in the case of anaerobic conditions. The following figure (Fig. 3.2.) presents the kinetics of total sugars consumption along with biomass and product accumulation into the medium.



(a)



(b)

Figure 3.2. Kinetics of evolution of total sugars (TS, g l^{-1}) (▲), 2,3-butanediol (BDO, g l^{-1}) (△), biomass (X, g l^{-1}) (●) (a) and acetoin (Ace, g l^{-1}) (▲), lactic acid (Lac, g l^{-1}) (△) and ethanol (Eth, g l^{-1}) (×) (b) during growth of *Klebsiella oxytoca* FMCC-197 on molasses and sucrose in fed-batch bioreactor experiments. Culture conditions: 1 vvm aeration, agitation rate from 180 to 400 rpm, pH fluctuating from 7.0 to 6.0, T=30 °C, growth on 2-l bioreactor. Each point is the mean value of two independent measurements.

The global yield of BDO+Ace produced per unit of sugar consumed for the whole aerobic fed-batch bioreactor trial (employment of blends molasses and sucrose as substrates, T=30 °C) is 0.35 g g^{-1} (Fig. 3.2.c).

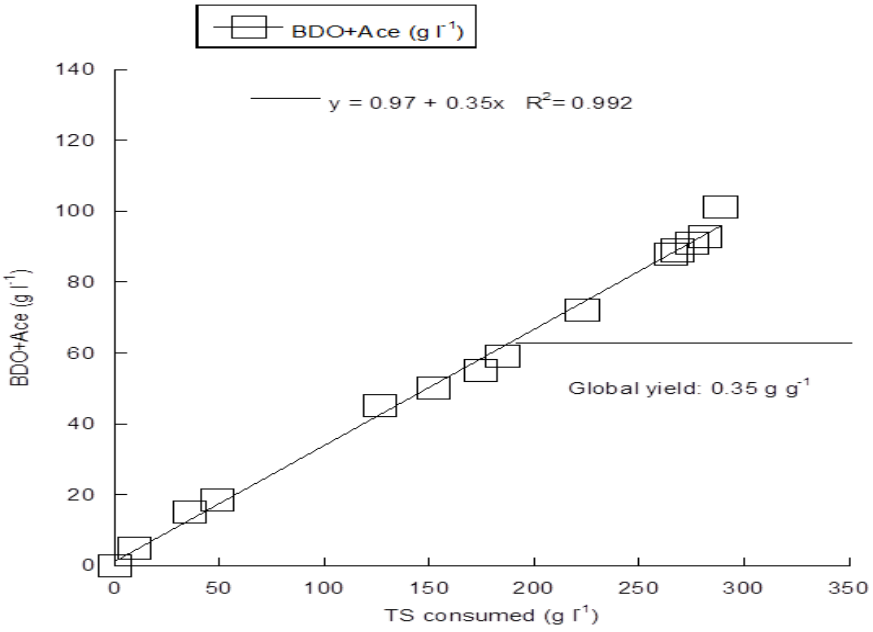
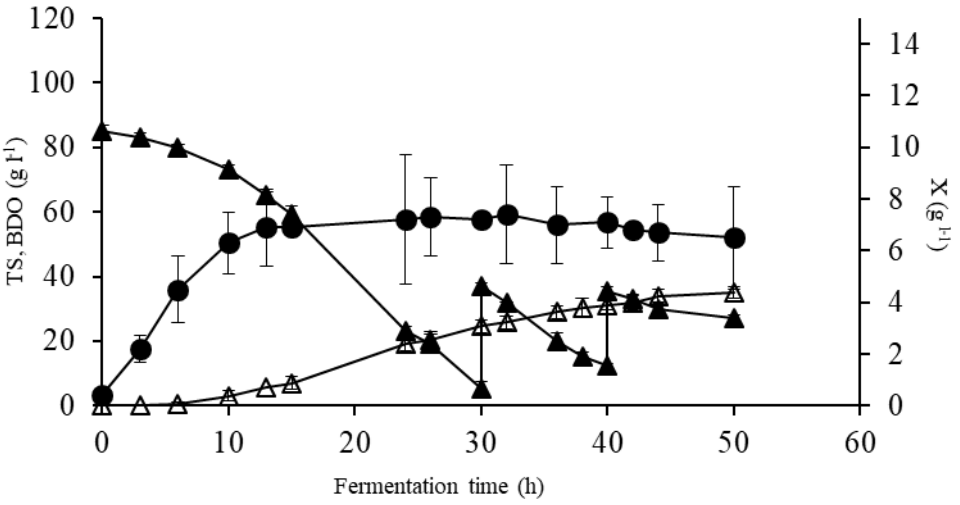
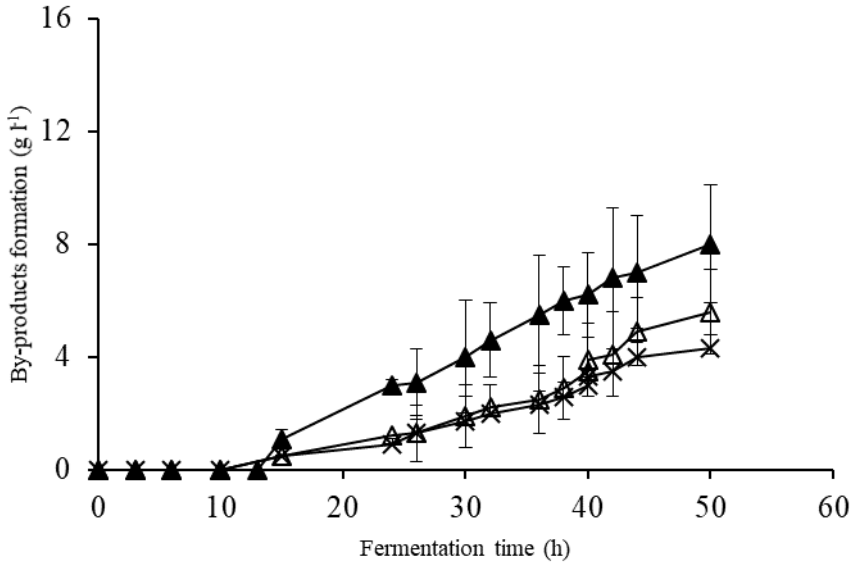


Figure 3.2. (c) Representation of 2,3-butanediol and acetoin production (BDO+Ace; g l^{-1}) per total sugars consumed (TS; g l^{-1}) during fed-batch bioreactor culture of *Klebsiella oxytoca* FMCC-197 on molasses and sucrose. Culture conditions: 1 vvm aeration, agitation rate from 180 to 400 rpm, pH fluctuating from 7.0 to 6.0, *T*=30 °C, growth on 2-l bioreactor. Each point is the mean value of two independent measurements.

In a next approach, a fed-batch bioreactor experiment was conducted using molasses as the sole carbon source under aerobic conditions at 30 °C. A solution of concentrated molasses had been previously prepared and, pulses of this concentrated solution were added into the bioreactor when need. As presented in Table 3.6., 112.8 g l^{-1} of total sugars were consumed in 50 h leading to the production of 35.1 g l^{-1} of BDO and 8.0 g l^{-1} of Ace. Although the final bioconversion yield was the same with the fermentation using molasses and sucrose as carbon source (0.38 g g^{-1}), the volumetric productivity rate was considerably lower (0.86 g l^{-1} h^{-1}). As for the by-products, lactic acid production was slightly favored compared to that of ethanol and succinic. Figure 3.3. shows the kinetics of total sugars consumption along with bacterial growth and metabolites production in the fed-batch trial in which molasses were used as the sole carbon source of the process.



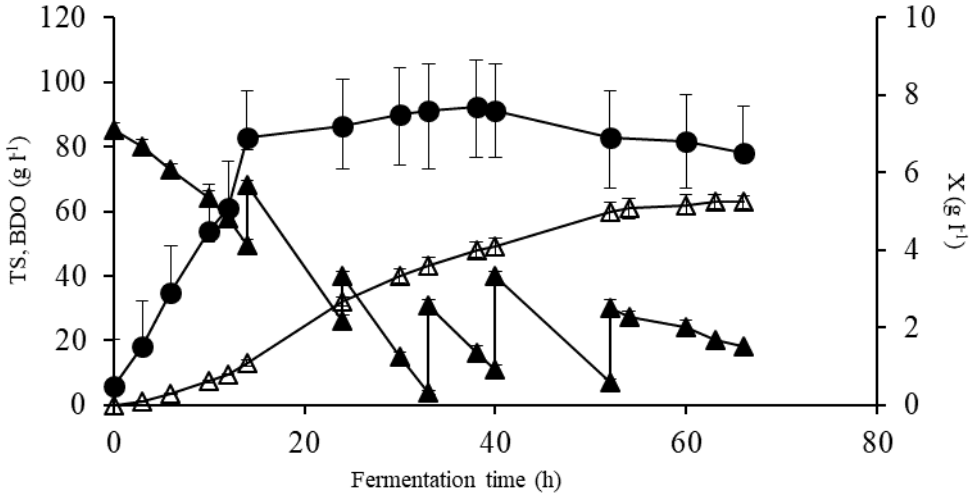
(a)



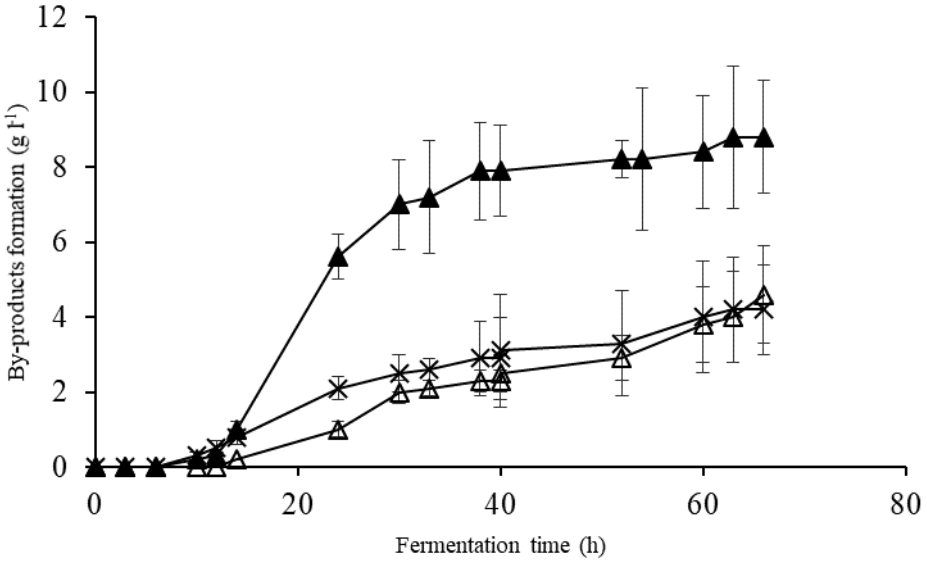
(b)

Figure 3.3. Kinetics of evolution of total sugars (TS, g l^{-1}) (▲), 2,3-butanediol (BDO, g l^{-1}) (△), biomass (X, g l^{-1}) (●) (a) and acetoin (Ace, g l^{-1}) (▲), lactic acid (Lac, g l^{-1}) (△) and ethanol (Eth, g l^{-1}) (×) (b) during growth of *Klebsiella oxytoca* FMCC-197 on molasses in fed-batch bioreactor experiments. Culture conditions: 1 vvm aeration, agitation rate from 180 to 400 rpm, pH fluctuating from 7.0 to 6.0, T=30 °C, growth on 2-l bioreactor. Each point is the mean value of two independent measurements.

The last fed-batch bioreactor experiment was conducted under aerobic conditions using molasses and sucrose as the carbon sources. The temperature applied into the culture medium was adjusted to 37 °C, in order to compare the final BDO and Ace production to the one at 30 °C. After 66 h of fermentation, 178.2 g l^{-1} of total sugars had been assimilated and were converted into 63.0 g l^{-1} of BDO and 8.8 g l^{-1} of Ace. The final bioconversion yield was slightly higher (0.40 g l^{-1}) compared with the trial carried out at 30 °C, with a concomitant non-negligible volumetric productivity achieved of 1.09 g l^{-1} h^{-1}. Figure 3.4. presents the kinetics of carbon source consumption along with biomass and metabolites production.



(a)



(b)

Figure 3.4. Kinetics of evolution of total sugars (TS, g l^{-1}) (▲), 2,3-butanediol (BDO, g l^{-1}) (△), biomass (X, g l^{-1}) (●) (a) and acetoin (Ace, g l^{-1}) (▲), lactic acid (Lac, g l^{-1}) (△) and ethanol (Eth, g l^{-1}) (×) (b) during growth of *Klebsiella oxytoca* FMCC-197 on molasses and sucrose in fed-batch bioreactor experiments. Culture conditions: 1 vvm aeration, agitation rate from 180 to 400 rpm, pH fluctuating from 7.0 to 6.0, T=37 °C, growth on 2-l bioreactor. Each point is the mean value of two independent measurements.

Another set of fed-batch experiments under aerobic conditions at 30 °C was conducted using the strain *K. oxytoca* FMCC-197 in shake-flask trials (flask volume of 2000 ml). Commercial sucrose was added into the culture medium as the sole carbon source. For instance, the medium of the first fed-batch shake flask experiment was previously sterilized while the medium of the second one was previously pasteurized (pasteurization of the medium at 80 °C for 15 min). It is evident that the ability of the strain to perform the BDO bioprocess production under non-aseptic conditions in previously pasteurized media, presents high economic significance, as regards the potential of the scale-up of the process in larger-scale operations (Koutinas et al. 2016). Table 3.6. presents the substrate consumption and biomass and metabolites production in these culture configurations. As shown, when the culture medium was previously sterilized, 145.4 g l^{-1} of total sugars were consumed within 58 h while simultaneously 51.0 g l^{-1} of BDO and 9.0 g l^{-1} of Ace had been produced. A remarkable yield of 0.41 g g^{-1} was achieved, while the productivity rate was 1.03 g l^{-1} h^{-1}. In the case of previously pasteurized medium, 164.8 g l^{-1} of substrate was converted into 48.0 g l^{-1} of BDO and 10 g l^{-1} of Ace. The bioconversion yield and productivity rate of the process were reduced as 0.35 g g^{-1} and 0.94 g l^{-1} h^{-1} were noted. Nevertheless, the accomplishment of the fermentation with satisfactory results under not previously sterilized media, demonstrates the potential of the strain *K. oxytoca* FMCC-197 concerning the conversion of sucrose (and, thus,

sucrose-containing wastewaters and residues) into 2,3-butanediol and acetoin in larger-scale operations that could be envisaged in the future.

Table 3.6. Comparative values of maximum biomass production, substrate consumption, final 2,3-butanediol concentration, conversion yield and productivity in fed-batch trials of *Klebsiella oxytoca* FMCC-197 using molasses and sucrose as carbon source, under anaerobic and aerobic conditions. Culture conditions for the bioreactor experiments: T=30 °C or 37 °C; aeration rate =1 vvm (aerobic conditions); agitation rate: from 180 rpm to 400 rpm (aerobic conditions); agitation rate =180 rpm (anaerobic conditions); pH fluctuating from 7.0 to 6.0. Culture conditions for the shake-flask experiments: growth in 2-l flasks filled with 500 ml, T=30 °C; agitation rate: 180 rpm; pH fluctuating from 7.0 to 6.0.

Fermentation mode	Aeration mode	T	Total sugars consumed	X_{max}	BDO	Ace	Ethanol	Succinic	Lactic	$Y_{BDO,Ace}$	$P_{BDO,Ace}$	Fermentation Time*
		◦C	(g l^{-1})	(g l^{-1})	(g l^{-1})	(g l^{-1})	(g l^{-1})	(g l^{-1})	(g l^{-1})	(g g^{-1})	(g l^{-1} h^{-1})	(h)
Bioreactor	Anaerobic[a]	30	110.1±2.0	6.6±1.4	31.0±1.7	7.0±1.6	5.0±1.6	3.5±0.5	5.0±1.2	0.35±0.01	0.79±0.01	48
	Aerobic[a]	30	288.8±2.0	9.0±1.5	101.1±1.8	14.2±2.1	7.5±1.5	5.5±1.5	8.0±1.5	0.40±0.02	1.80±0.02	64
	Aerobic[b]	30	112.8±1.8	7.4±1.9	35.1±1.8	8.0±2.1	4.0±0.5	4.5±1.0	7.0±1.5	0.38±0.01	0.86±0.01	50
	Aerobic[a]	37	178.2±3.0	7.9±2.1	63.0±2.0	8.8±1.0	4.2±1.0	4.5±1.1	4.6±1.6	0.40±0.01	1.09±0.02	66
Shake flask	Aerobic[c]	30	145.4±4.0	9.5±1.5	51.0±2.0	9.0±1.5	4.5±1.0	4.0±0.5	4.0±0.2	0.41±0.01	1.03±0.03	58
	Aerobic[d]	30	164.8±5.1	13.2±2.5	48.0±1.5	10.0±1.0	3.8±0.2	5.1±1.1	5.5±1.5	0.35±0.02	0.94±0.01	62

*Fermentations were extended after the indicated time and no more sugar has been consumed

a: Molasses and sucrose as carbon source

b: Molasses as the sole carbon source

c: Sucrose as the sole carbon source; previously sterilized medium

d: Sucrose as the sole carbon source; previously pasteurized medium

3.2.7. Color removal occurring during molasses fermentation

Besides BDO production, trials carried out on molasses employed as the sole carbon source were accompanied by non-negligible decolorization of the residue; therefore, anaerobic cultures performed in Duran bottles, were accompanied by a decolorization of the medium of 40%. On the other hand, aerobic growth occurring in shake-flask experiments, resulted in somehow higher decolorization of the medium (50%) (Fig. 3.5.). In both aerobic and anaerobic experiments performed, decolorization seemed to be a completely growth-associated process. Figure 3.6. represents biomass production during the experiments.

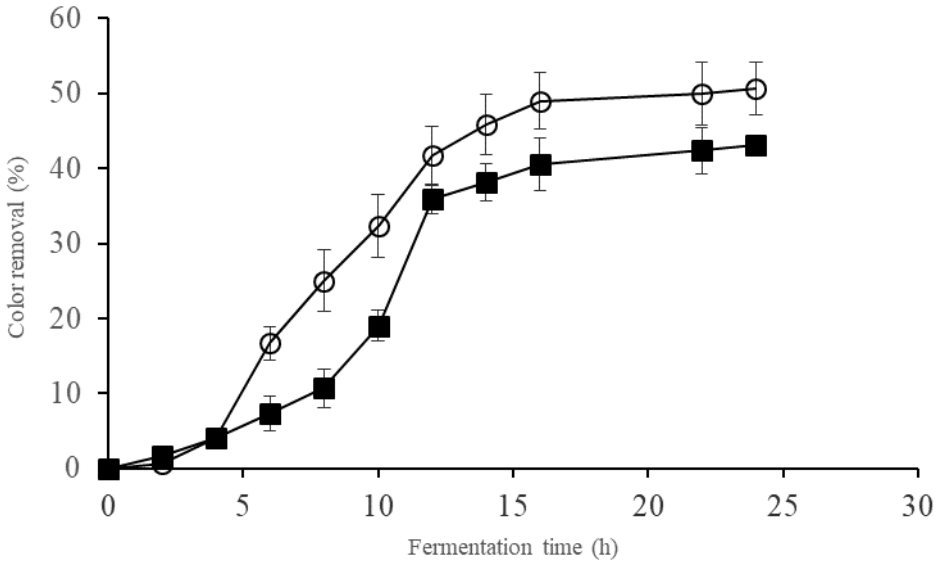


Figure 3.5. Color removal during growth of *Klebsiella oxytoca* FMCC-197 on molasses under anaerobic (■) and aerobic (○) conditions. Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0, initial total sugars concentration ≈30 g l^{-1}. Anaerobic experiments were conducted in Duran bottles and aerobic experiments on 500-ml shake flasks. Each point is the mean value of two independent measurements.

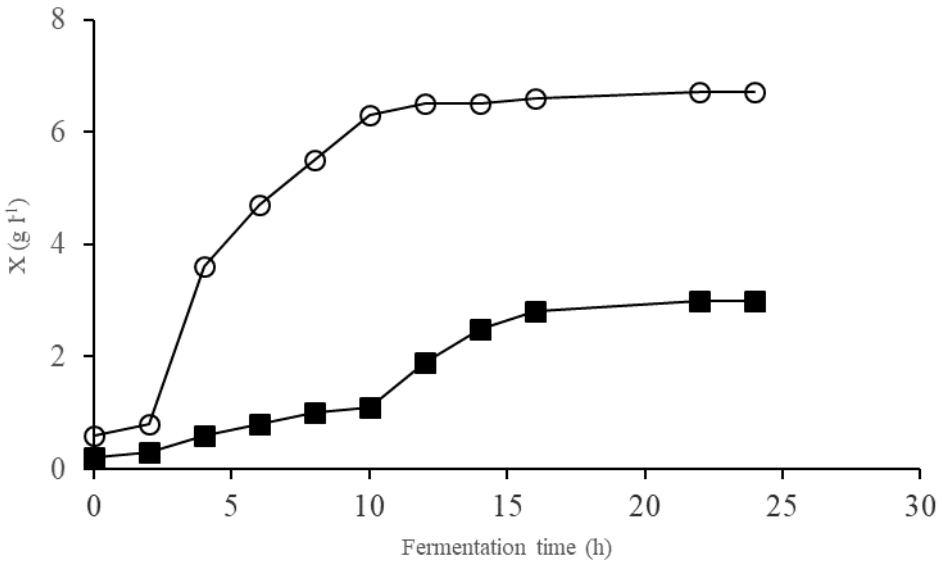
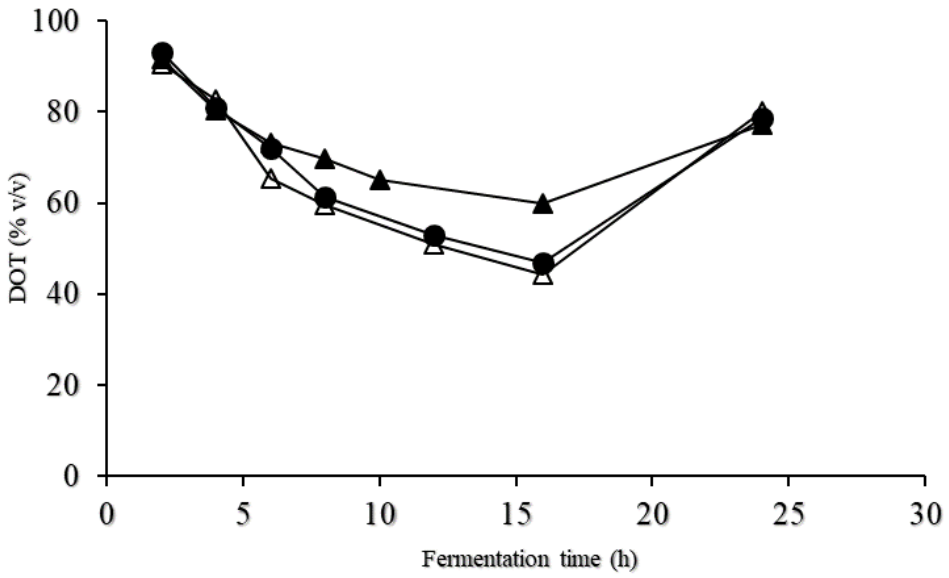


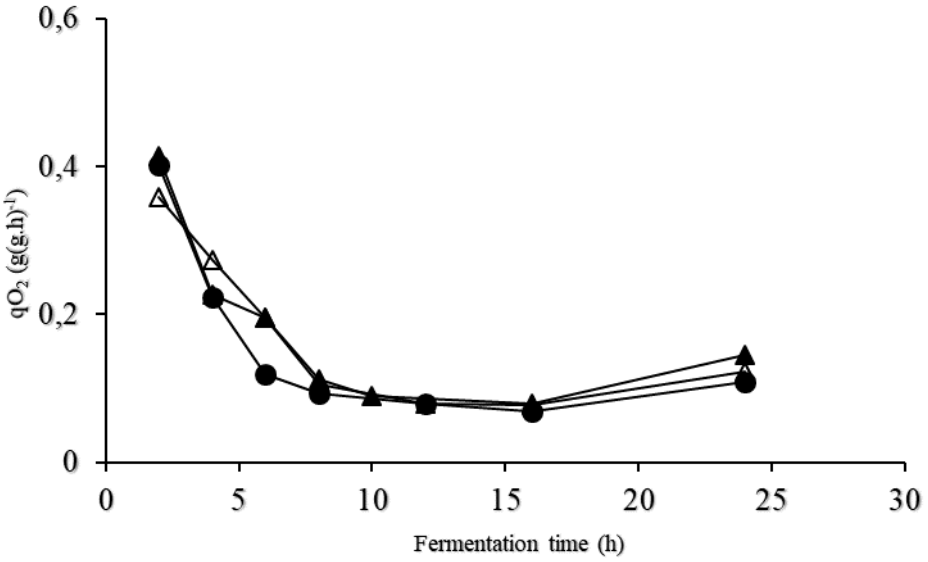
Figure 3.6. Growth of *Klebsiella oxytoca* FMCC-197 on molasses under anaerobic (■) and aerobic (○) conditions. Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0, initial total sugars concentration ≈30 g l^{-1}. Anaerobic experiments were conducted in Duran bottles and aerobic experiments on 500-ml shake flasks. Each point is the mean value of two independent measurements.

3.2.8. Effect of dissolved oxygen concentration upon the BDO production bioprocess carried out by *Klebsiella oxytoca*

In the next part of the manuscript, it was desirable to study the effect of the oxygen concentration and the oxygen uptake rate upon the performed bioprocess, when shake-flask experiments were carried out. Therefore, during the initial set of shake-flask trials, three different carbon sources (*viz.* glucose, fructose and sucrose) were at an initial concentration ≈30 g l^{-1}. The temperature applied was 30 °C and each fermentation point corresponded to a different flask that was individually collected. The following figures present the dissolved oxygen tension (DOT) and the specific oxygen consumption (q_{O_2}) along with fermentation time using glucose, fructose and sucrose as carbon source.



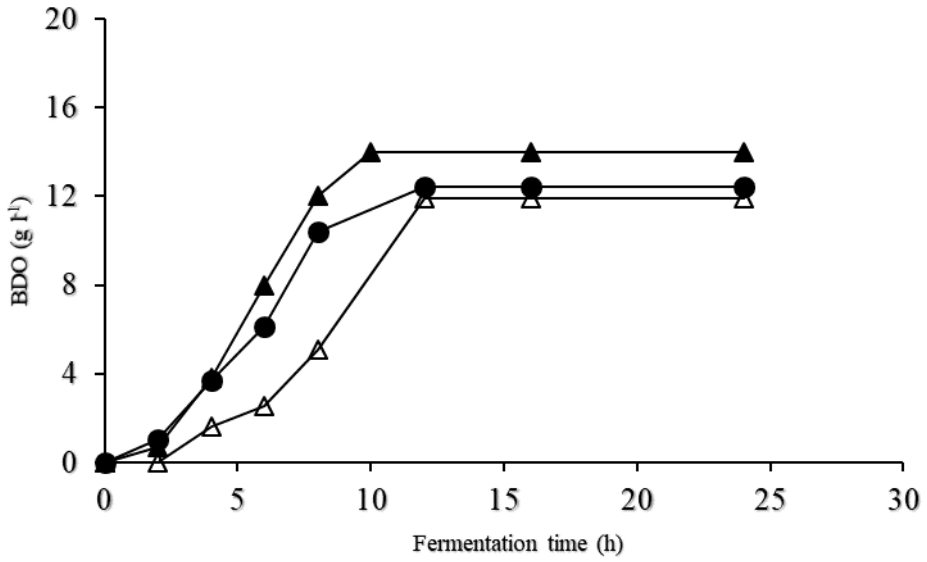
(a)



(b)

Figure 3.7. (a) Dissolved oxygen tension (DOT, % v/v) and (b) specific oxygen consumption rate (q_{O_2}, g(g.h)$^{-1}$) related with fermentation time during shake flask cultivation of *Klebsiella oxytoca* FMCC-197 on glucose (●), fructose (△) and sucrose (▲). Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0, initial glucose concentration ≈30 g l^{-1}.

As presented in the relevant figures, DOT (%, v/v) evolution as function of the fermentation time was similar during the fermentations using the three carbon sources; even at the early growth steps of the culture, DOT values decreased. The lowest value was noted after 16 hours of fermentation and it was ~45% v/v in the case of glucose and fructose. When sucrose was used as carbon source the lowest DOT value recorded was ~60% v/v. According to these results, it may be assumed that in all cases the fermentations were conducted under full aerobic conditions (Papanikolaou et al., 2004). On the other hand, the representation of the specific consumption rate of oxygen for the trials with the three different sugars employed as individual substrates at initial concentration adjusted at c. 30 g l^{-1}, demonstrates a significant respiratory activity for the strain at the first growth steps (q_{O_2}=0.40±0.05 g (g.h)$^{-1}$) that remarkably decreased at the late fermentation steps of the culture (q_{O_2}=0.07±0.02 g (g.h)$^{-1}$). BDO production occurred during both growth phases (*viz*. in both the culture steps of the increased and the decreased respiratory activity of the strain; see Figs 3.7.c.) demonstrating, therefore, that biosynthesis of BDO in *K. oxytoca* FMCC-197 may occur regardless of the physiological state of the culture as regards its respiratory activity.



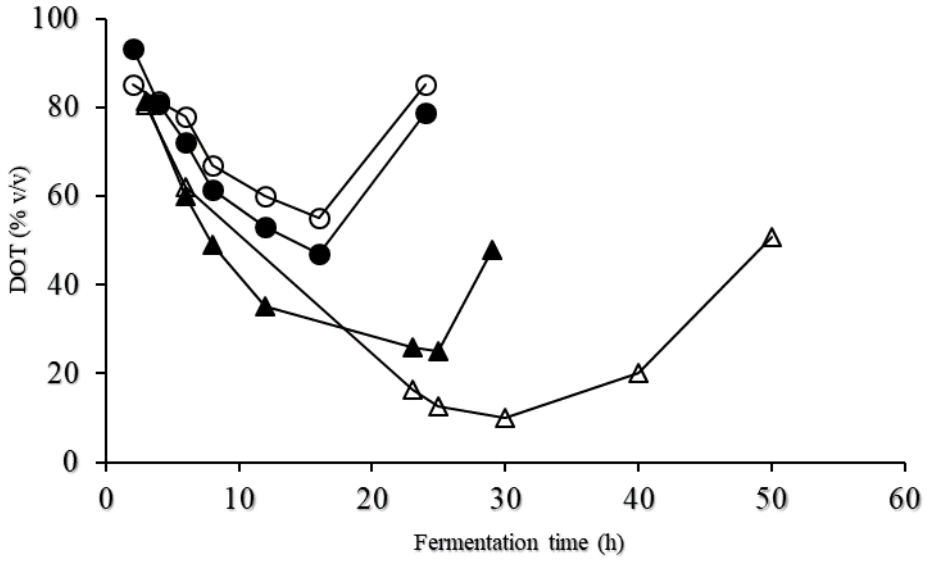
(c)

Figure 3.7. (c) 2,3-butanediol (BDO, g l^{-1}) production related with fermentation time during shake flask cultivation of *Klebsiella oxytoca* FMCC-197 on glucose (●), fructose (△) and sucrose (▲). Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0, initial glucose concentration ≈30 g l^{-1}.

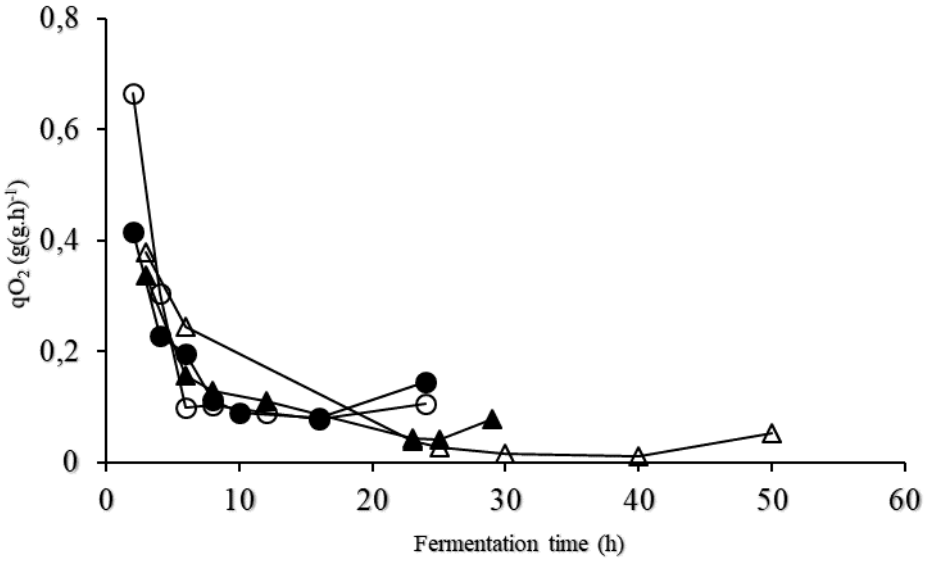
In the next step, it was desirable to focus on the impact of the initial sucrose concentration upon the DOT and the specific oxygen consumption rate (q_{O_2}) values during *K. oxytoca* FMCC-197 shake-flask experiments. Therefore, DOT and q_{O_2} values were determined for four different initial sucrose concentrations initially added into the medium (30, 60, 90 and 150 g l^{-1}) and the results are illustrated in Figs 3.8. (a and b). Figure 3.8.c. represents the kinetics of BDO production for the different initial concentrations of sucrose employed into the medium. As it has previously been seen in the trials performed with initial sugar concentration adjusted at 30 g l^{-1}, DOT values decreased at the first stages of the fermentation. On the other hand, the more the initial concentration of sucrose increased, the less the DOT value was observed into the flasks as the fermentations proceeded. However, it must be pointed out that in almost all trials, DO concentrations remained almost always in values >20-25% v/v, values that can be considered as the lower thresholds of concentrations for aerobic metabolism in several types of yeasts, fungi and bacteria (Metsoviti et al., 2011; Hagman et al., 2013; Sabra et al., 2017). Therefore, sufficient BDO production was carried out under aerobic conditions, although as previously stressed, this feature is not indeed a common feature for the bacteria that perform this type of conversion, since BDO bioconversion process is mostly considered as an “anaerobic” or “micro-aerobic” one (Ji et al., 2011; Zeng & Sabra, 2011). On the other hand, in the late fermentation steps and in the trial in which a very high initial sucrose concentration had been added into the medium (≈150 g l^{-1}), DOT values ≈10% v/v were detected,

suggesting that biosynthesis of BDO under micro-aerobic conditions occurred for the given period in the trial performed (i.e. between 25 and 35 h – see Fig. 3.8.a). It is worth mentioning, thus, that in the later case in which an indeed high initial sucrose concentration was employed (150 g l^{-1}) satisfying BDO production was achieved not only under aerobic conditions but even under oxygen-limited conditions.

On the other hand, the monitoring of q_{O_2} evolution during the fermentation carried out demonstrates that significant respiratory activity for the strain at the first growth steps (q_{O_2}ranging between 0.3 and 0.6 g $(g.h)^{-1}$) that very significantly decreased as the fermentation proceeded (specifically at the trials with initial sucrose concentrations adjusted at 90 and 150 g l^{-1}, the respiratory activity, as correlated with the achieved q_{O_2}values was indeed very low).

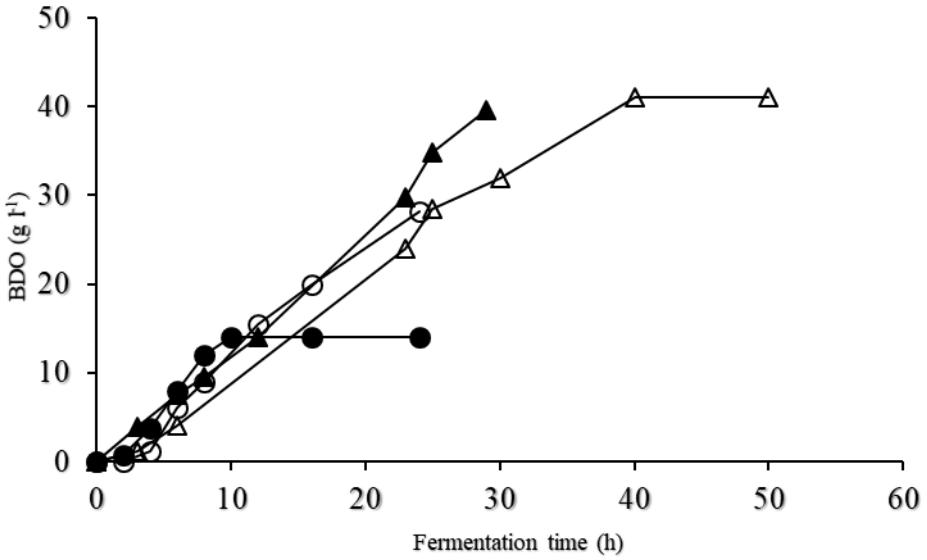


(a)



(b)

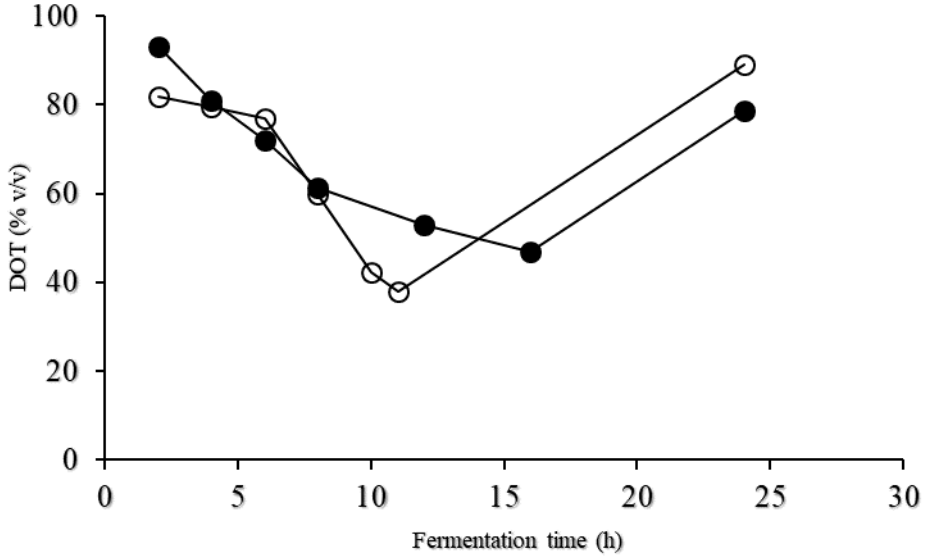
Figure 3.8. (a) Dissolved oxygen tension (DOT, % v/v) and (b) Oxygen consumption (q_{O_2}, $g(g.h)^{-1}$) related with fermentation time during shake flask cultivation of *Klebsiella oxytoca* FMCC-197 on sucrose using 30 (●), 60 (○), 90 (▲) and 150 (△) g l^{-1} initial concentration. Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0.



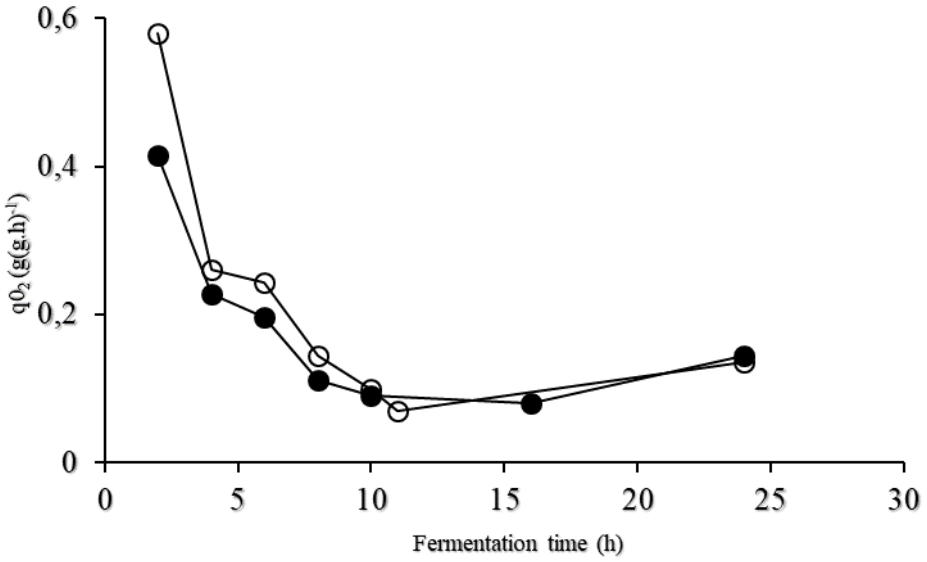
(c)

Figure 3.8. (c) 2,3-butanediol (BDO, g l^{-1}) production related with fermentation time during shake flask cultivation of *Klebsiella oxytoca* FMCC-197 on sucrose using 30 (●), 60 (○), 90 (▲) and 150 (△) g l^{-1} initial concentration. Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0.

Finally, in the last part of this chapter, the impact of the incubation temperature upon the DOT and the specific oxygen consumption rate (q_{O_2}) evolution during shake-flask experiments of *K. oxytoca* FMCC-197 was assessed. Therefore, a shake flask experiment at 37 °C using sucrose as carbon source was performed and the results obtained are compared to those at 30 °C (Figure 3.9. a and b). Figure 3.9.c. represents the kinetics of BDO production for the two different incubation temperatures employed into the medium). Similar observations as in the previous trials were seen; the DOT (%, v/v) decreased as function of the fermentation time, while it appears that the increase of the incubation temperature substantially decreased DOT values (at 37 °C, the lowest DO concentration value was *c*. 40% v/v). Likewise, remarkable respiratory activity, as demonstrated by the evolution of the specific oxygen consumption rate (q_{O_2}) was noted at the first growth steps (in any case higher for the temperature of 37 °C), drastically decreasing as the fermentation proceeded.

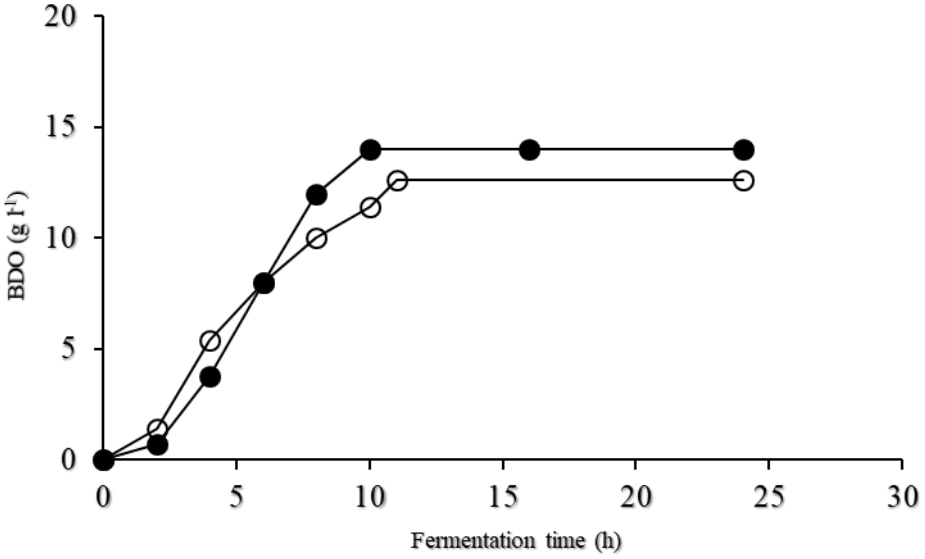


(a)



(b)

Figure 3.9. (a) Dissolved oxygen tension (DOT, % v/v) and (b) Oxygen consumption (q_{O_2}, g(g.h)$^{-1}$) related with fermentation time during shake flask cultivation of *Klebsiella oxytoca* FMCC-197 on sucrose at 30 (●) and 37 °C (○). Culture conditions: 180 rpm agitation rate, initial pH=7.0, initial glucose concentration ≈30 g l^{-1}.



(b)

Figure 3.9. (c) 2,3-butanediol (BDO, g l^{-1}) production related with fermentation time during shake flask cultivation of *Klebsiella oxytoca* FMCC-197 on sucrose at 30 (●) and 37 °C (○). Culture conditions: 180 rpm agitation rate, initial pH=7.0, initial glucose concentration ≈30 g l^{-1}.

3.3. Optimization of 2,3-butanediol (BDO) production in *Enterobacter* sp. FMCC-208 cultures using analytical-grade or low-cost carbohydrate-based substrates

3.3.1. Introduction

The strain *Enterobacter* sp. FMCC-208 was the second one which was selected for further investigation. From the preliminary results obtained through the previously performed screening process (see Table 3.1.), the strain *Enterobacter* sp. FMCC-208 was able to totally convert glucose and sucrose into BDO, reaching remarkable values of bioconversion yield and productivity rate. For instance, the whole substrate, 25.3 g l^{-1}, was totally consumed after 18 hours of fermentation and 11.2 g l^{-1} of BDO were produced. The final yield was 0.44 g g^{-1} while the productivity rate reached a value of 0.62 g l^{-1} h^{-1}.

Different parameters were studied during batch fermentations in order to optimize the BDO production bioprocess. For instance, the ability of the strain to grow in a wide range of substrate concentrations without any inhibition was studied. In a next approach, various individual carbon sources were used in order to determine the ability of the strain to grow and assimilate sugar substrates for BDO production. The range of pH values suitable for growth and BDO production was also investigated through four batch experiments in 2-l bioreactor.

The optimum values of temperature, substrate concentration and pH were applied in fed-batch bioreactor processes and fed-batch shake flask experiments that maximized BDO production. It should be stressed that non-aseptic trials have also been conducted in order to compare the growth and final product accumulation with the sterilized fermentations.

3.3.2. Initial trials under anaerobic and aerobic conditions demonstrating sugar and cane molasses assimilation and BDO production

During the first set of experiments, anaerobic fermentations in 1-l Duran bottles were carried out in order to investigate the potential of *Enterobacter* sp. FMCC-208 to convert sucrose and molasses into BDO (Table 3.6.). Commercial sucrose or cane molasses were employed as substrates in different initial concentrations and in all cases when the culture was stopped, the highest quantity of the sugar employed as substrate had been assimilated by the strain. In particular, three initial sugar concentrations were used, ~15, ~30, ~50 g l^{-1} in both cases. As it is shown in Table 3.7., the yield of BDO and Ace synthesized per g of sugar consumed ($Y_{BDO,Ace}$) was around 0.40 g g^{-1} in all cases, which is a relatively satisfactory value, corresponding to 80% of the maximum theoretical Y_{BDO} value (the maximum yield of BDO synthesized per unit of carbohydrate consumed is 0.50-0.53 g g^{-1} depending on the carbohydrate used as microbial substrate; for critical reviews see: Xiu & Zeng, 2008; Celinska & Grajek, 2009; Sabra et al., 2016). As for the volumetric productivity, the highest value (0.62 g l^{-1} h^{-1}) was noted when sucrose was used as the carbon source in an initial concentration of around 50 g l^{-1}.

In order to further assess the potential of *Enterobacter* sp. FMCC-208 to produce BDO, trials under aerobic conditions were performed in shake-flask experiments. Thus, two different experiments were conducted in which 30 and 60 g l^{-1} of commercial sucrose had been employed as substrate. Table 3.8. compares the final biomass production and product formation under anaerobic and aerobic conditions. As it is shown, although the bioconversion was satisfactory in both types of fermentations, $Y_{BDO,Ace}$ and BDO_{max} values were clearly higher during the aerobic trials; in fact, the conversion yield of BDO produced under aerobic conditions was very close to the maximum theoretical one (Celinska & Grajek, 2009), while the volumetric productivity value of around 1.20 g l^{-1} h^{-1}, was noted under the above-mentioned culture conditions. Considering that aerobic conditions led to higher productivity, all experiments that followed were conducted in shake flasks in order to determine the ability of the strain to assimilate various pentoses and hexoses along with the optimum values of temperature and pH. Interestingly therefore, besides *Klebsiella oxytoca* FMCC-197, the second microorganism that was selected to be further studied in the current investigation (*Enterobacter* sp. FMCC-208) presented equally noticeably higher BDO production under aerobic conditions compared to the anaerobic trials, and this is in contrast to the principal biochemical theory of BDO production process (Celinska & Grajek, 2009; Sabra et al., 2016).

Table 3.7. Cultures of *Enterobacter* sp. FMCC-208 on Duran bottles under anaerobic conditions. Representation of maximum biomass production, substrate consumption, final 2,3-butanediol concentration, conversion yield on sugars consumed and productivity in batch fermentations. Culture conditions: T=30 °C; agitation rate =180 rpm; initial pH=7.0. Each point is the mean value of two independent measurements.

Carbon source	Total sugars consumed ($g\ l^{-1}$)	X_{max} ($g\ l^{-1}$)	BDO ($g\ l^{-1}$)	Ace ($g\ l^{-1}$)	$Y_{BDO,Ace}$ ($g\ g^{-1}$)	$P_{BDO,Ace}$ ($g\ l^{-1}\ h^{-1}$)	Fermentation time (h)
Sucrose	15.5±1.0	2.2±0.2	5.8±0.1	0.9 ±0.2	0.43±0.01	0.48±0.01	14
	28.8±1.0	2.8±0.2	9.0±0.2	1.9±0.2	0.38±0.01	0.39±0.01	28
	53.0±2.1	4.0±1.5	19.0±1.2	2.0±0.3	0.40±0.02	0.62±0.02	34*
Molasses	16.0±0.3	3.2±0.5	6.0±0.2	0.5±0.1	0.41±0.01	0.27±0.01	24
	30.0±1.0	2.7±0.2	11.0±0.3	1.3±0.2	0.41±0.03	0.40±0.05	31
	49.9±2.0	2.9±0.3	18.0±1.0	1.0±0.3	0.38±0.02	0.38±0.03	50*

*Fermentations were extended after the indicated time and no more sugar has been consumed

Table 3.8. Comparison between maximum biomass production and 2,3-butanediol production by *Enterobacter* sp. FMCC-208 cultivated under anaerobic (Duran bottles) and aerobic (500-ml shake-flask experiments) conditions when commercial sucrose was employed as substrate. Culture conditions: T=30 °C; agitation rate =180 rpm; initial pH=7.0. Each point is the mean value of two independent measurements.

Fermentation mode	Total sugars consumed ($g\ l^{-1}$)	X_{max} ($g\ l^{-1}$)	BDO ($g\ l^{-1}$)	Ace ($g\ l^{-1}$)	$Y_{BDO,Ace}$ ($g\ g^{-1}$)	$P_{BDO,Ace}$ ($g\ l^{-1}\ h^{-1}$)	Fermentation time (h)
Anaerobic conditions	28.8±1.0	2.8±0.2	9.0±0.2	1.9±0.2	0.38±0.01	0.39±0.02	28
	53.0±2.1	4.0±1.5	19.0±1.2	2.0±0.3	0.40±0.02	0.62±0.02	34*
Aerobic conditions	36.9±1.2	5.3±0.1	12.0±1.1	1.2±0.3	0.36±0.02	1.20±0.10	11
	59.7±1.1	6.9±0.1	25.4±0.9	2.0±0.5	0.46±0.01	1.19±0.04	23

*Fermentation was extended after the indicated time with no more sugar having been consumed

3.3.3. Evaluation of the ability of the strain to assimilate different sugars

The current part of the investigation was further focused upon the ability of the strain *Enterobacter* sp. FMCC-208 to consume various pentoses and hexoses and simultaneously produce BDO. As previously indicated, several of these sugars are the principal components of various types of waste streams and residues (i.e. hemicelluloses-type hydrolysates, food-deriving wastewaters, etc), thus, potential significant biomass and BDO production during growth on various types of sugars could increase the "sustainability aspect" of the proposed bioconversions. Indeed, aerobic shake-flask fermentations were performed using different carbon sources at initial sugar

concentration of around 30 g l^{-1} and the obtained results are shown in Table 3.9.. As it is shown, the strain converted various monosaccharides into BDO, acetoin and other organic acids reaching high yields and productivities. Maximum volumetric productivity (>1.20 g $l^{-1}h^{-1}$) was achieved when glucose, fructose sucrose and mannose were used as carbon sources. Remarkably high values of yield of BDO and acetoin (Ace) observed per unit of sugar consumed ($Y_{BDO,Ace}$) (e.g. >0.47 g g^{-1}) were noted when hexoses (e.g. glucose, fructose, mannose and galactose) were added into the medium. Utilization of pentoses (i.e. arabinose and xylose) resulted in interesting substrate assimilation and BDO along with Ace production, but the conversion yield $Y_{BDO,Ace}$ was somehow lower than the one reported for the growth on hexoses (i.e. $Y_{BDO,Ace}$ value was ≈0.43 g g^{-1}) suggesting the non-utilization of the phospho-ketolase pathway concerning the pentoses break-down (Papanikolaou & Aggelis, 2011; 2019; Athenaki et al., 2018) by *Enterobacter* sp..

Table 3.9. Growth of *Enterobacter* sp. FMCC-208 in shake flasks using various sugars employed as sole carbon source at initial sugar concentration adjusted at 30 g l^{-1} under aerobic conditions. Representation of maximum biomass production, substrate consumption, final 2,3-butanediol, acetoin and other organic products concentration, conversion yield and productivity in batch fermentations under aerobic conditions using different carbon sources. Culture conditions: growth in 500-ml flasks filled with 100 ml, T=30°C; agitation rate =180 rpm; initial pH=7.0. Each point is the mean value of two independent measurements.

Carbon source	Substrate consumed (g l^{-1})	X_{max} (g l^{-1})	BDO (g l^{-1})	Ace (g l^{-1})	Eth (g l^{-1})	Suc (g l^{-1})	$Y_{BDO,Ace}$ (g g^{-1})	$P_{BDO,Ace}$ (g l^{-1} h^{-1})	Fermentation time (h)
Glucose	29.9±1.3	5.6±0.1	12.6±0.4	1.7±0.3	1.3±0.1	1.9±0.1	0.48±0.03	1.59±0.10	9
Fructose	29.4±0.4	6.3±0.5	12.8±0.3	1.4±0.1	1.0±0.1	1.0±0.1	0.48±0.01	1.42±0.03	10
Sucrose	36.9±1.2	5.3±0.3	12.0±1.1	1.2±0.3	1.5±0.2	2.0±0.3	0.36±0.02	1.20±0.05	11
Mannose	30.0±1.0	6.6±0.2	11.9±0.4	2.1±0.2	1.0±0.1	2.0±0.1	0.47±0.02	1.40±0.10	10
Galactose	28.8±0.3	4.0±0.2	11.9±0.4	1.9±0.1	1.1±0.1	1.9±0.1	0.48±0.01	0.99±0.03	14
Arabinose	27.4±0.5	3.3±0.3	10.5±0.5	1.3±0.3	0.9±0.1	1.7±0.2	0.43±0.01	0.84±0.03	14
Xylose	30.4±0.6	5.4±0.1	10.4±0.4	2.7±0.1	0.4±0.1	1.1±0.2	0.43±0.01	0.47±0.03	28
Molasses	32.5±1.0	5.6±0.2	11.9±0.4	2.2±0.2	0.8±0.1	1.4±0.3	0.44±0.02	1.01±0.02	14

3.3.4. Temperature effect on growth and BDO production during aerobic fermentations using sucrose as carbon source

Given that sucrose proved to be an efficient substrate for BDO production, it was decided to further optimize BDO production using this carbon source. Therefore, in a next step the bacterial growth and the metabolism related to the effect of the incubation temperature was studied by using commercial sucrose as starting material for the fermentations performed. 6 different incubation temperatures (i.e. T=25, 30, 34, 37, 40 and 42 °C) were evaluated in shake-flask cultures carried out using 20 and 40 g l^{-1} of initial sucrose added (Table 3.10.). As shown, the strain was capable of growing in a wide range of temperatures applied throughout the fermentation. Slight differences concerning the final biomass production amongst the different incubation temperatures was noted. The highest values concerning bioconversion yield ($Y_{BDO,Ace}$, in g g^{-1}) and volumetric productivity (in g l^{-1} h^{-1}) were recorded at T=37 °C in both initial sucrose concentrations applied into the culture medium. Therefore, all following experiments were conducted at T=37 °C.

Table 3.10. Temperature effect in total sugars consumption and 2,3-butanediol production, yield and productivity in shake-flask fermentations carried out with 20 g l^{-1} and 40 g l^{-1} of initial sucrose concentration by *Enterobacter* sp. FMCC-208. Culture conditions: growth in 500-ml flasks filled with 100 ml, agitation rate =180 rpm; initial pH=7.0. Each point is the mean value of two independent measurements.

T	Total sugars consumed	X_{max}	BDO	Ace	Eth	$Y_{BDO,Ace}$	$P_{BDO,Ace}$	Fermentation time
°C	(g l^{-1})	(g l^{-1})	(g l^{-1})	(g l^{-1})	(g l^{-1})	(g g^{-1})	(g $l^{-1}h^{-1}$)	(h)
25	19.5±0.6	4.0±0.1	6.0±0.4	1.0±0.1	1.6±0.3	0.36±0.03	0.78±0.05	9
	35.5±0.5	5.5±0.3	8.0±1.4	1.4±0.2	0.7±0.1	0.26±0.05	0.85±0.13	11
30	20.4±0.7	4.0±0.3	7.0±0.3	1.0±0.2	1.9±0.1	0.39±0.02	0.89±0.01	9
	36.9±1.2	5.3±0.1	12.0±1.1	1.2±0.3	1.5±0.2	0.36±0.02	1.20±0.10	11
34	20.5±0.9	4.5±0.2	6.0±0.2	1.8±0.1	1.7±0.3	0.38±0.01	0.98±0.01	8
	39.7±0.5	4.5±0.1	13.0±0.2	2.4±0.3	4.0±0.5	0.39±0.01	1.54±0.02	10
37	18.8±0.3	3.9±0.7	6.9±0.3	2.0±0.1	1.9±0.1	0.47±0.01	1.48±0.10	6
	46.1±0.5	4.1±0.1	16.0±0.6	2.8±1.0	5.0±0.2	0.41±0.02	2.35±0.01	8
40	20.5±0.2	3.2±0.1	6.8±0.1	1.5±0.2	1.6±0.1	0.40±0.01	1.04±0.02	8
	40.9±0.6	5.9±0.2	15.4±0.1	1.7±0.3	3.7±0.1	0.42±0.01	1.71±0.01	10
42	20.1±0.6	3.4±0.3	6.0±1.0	1.8±0.2	1.8±0.1	0.39±0.01	0.98±0.01	8
	39.6±0.6	5.7±0.1	14.8±0.5	2.0±0.1	4.2±0.2	0.42±0.01	1.68±0.06	10

3.3.5. Impact of sucrose concentration upon the microbial growth of *Enterobacter* sp. FMCC-208

Taking into consideration that the productivity was higher in the case of T=37 °C, experimental work was subsequently focused on the optimization of the bacterial growth using 10 different initial sucrose concentrations (*viz*. 5, 10, 15, 20, 40, 60, 80, 110, 130 and 150 g l^{-1}) in shake-flask experiments at T=37 °C. It was mostly interesting to evaluate the μ_{max} during growth on the several initial sucrose concentration media, and for this reason the curve of $\ln\left(X/X_0\right) = f(t)$ was fitted on the available experimental data within the early exponential growth phases of the trials. Table 3.11. presents the maximum specific growth rate during fermentations with different initial carbon source concentration. High values specific growth rate (>0.70 h^{-1}) were obtained when the initial substrate concentration was lower than 40 g l^{-1}. In higher initial sucrose concentrations employed, μ_{max} values decreased suggesting substrate inhibition exerted due to the relatively increased initial sucrose concentration added into the medium. Moreover, all other fermentation parameters are presented (Table 3.11.), and by taking into consideration the conversion yield of biomass produced per unit of substrate consumed ($Y_{X/S}$, g g^{-1}), that is a major kinetic parameter that should also be taken into consideration for the study of substrate inhibition phenomena (Papanikolaou et al., 2000; 2017; Krahe, 2003; Tchakouteu et al., 2015) it can be deduced that onset of substrate inhibition occurs in much lower initial sucrose concentrations imposed into the medium (i.e. *c.* 15 g l^{-1}), as compared to the rationale that takes into consideration the calculation of μ_{max}. On the other hand, the lag phase time that is another factor demonstrating possible substrate inhibition (Metsoviti et al., 2012b) seems to somehow increase to >4 h in indeed high initial sucrose concentrations (e.g. ≥130 g l^{-1}) imposed into the medium. Likewise, and despite the high sucrose concentrations (e.g. ≥80 g l^{-1} and up to 150 g l^{-1}) that were found in several of the shake-flask experiments carried out, sufficient sugar quantities have been assimilated, with the upper threshold of sucrose consumed for this type of culture configuration being ≈90 g l^{-1}. Finally, irrespective of the (sufficiently high in several trials) concentration of sucrose found into the medium, satisfactory BDO quantities (up to 40 g l^{-1} with simultaneous yield Y_{BDO}≈0.41-0.44 g g^{-1}) were produced.

Table 3.11. Growth of *Enterobacter* sp. FMCC-208 in shake flasks using various initial sucrose concentrations employed. Representation of the maximum specific growth rate of the strain in shake flask fermentations carried out at T=37 °C. Culture conditions: growth in 500-ml flasks filled with 100 ml, agitation rate =180 rpm; initial pH=7.0. Each point is the mean value of two independent measurements.

Initial sucrose concentration ($g\ l^{-1}$)	μ_{max} (h^{-1})	Sugar consumed ($g\ l^{-1}$)	X_{max} ($g\ l^{-1}$)	$Y_{X/S}$ ($g\ g^{-1}$)	BDO ($g\ l^{-1}$)	Lag phase duration (h)	Fermentation time (h)
≈5	0.86	5.8	2.3	0.40	2.0	0.5	4
≈10	0.85	9.0	3.6	0.40	4.2	0.5	5
≈15	0.73	15.8	3.9	0.25	7.1	1.0	6
≈20	0.83	19.0	3.9	0.21	7.0	1.5	6
≈40	0.45	46.5	4.1	0.09	16.0	1.5	8
≈60	0.21	60.8	7.0	0.12	24.0	1.5	23
≈80	0.27	79.2	6.5	0.08	37.0	1.5	26
≈110	0.19	90.0*	7.0	0.08	40.1	2.5	30*
≈130	0.17	88.7**	6.8	0.08	38.9	4.5	36**
≈150	0.10	93.0**	7.2	0.08	39.0	6.0	38**

*Initial sugars at 112.5 $g\ l^{-1}$, fermentation was extended after 30 h and no more sugar has been consumed

**Initial sugars at 136.5 $g\ l^{-1}$, fermentation was extended after 36 h and no more sugar has been consumed

*** Initial sugars at 155.0 $g\ l^{-1}$, fermentation was extended after 38 h and no more sugar has been consumed

3.3.6. Assimilation of sucrose and BDO production under non-aseptic conditions

In order to evaluate the ability of the strain to perform BDO bioprocess production in large-scale operations (Koutinas et al., 2016), it was interesting to carry out trials in media which had not been previously subjected to heat sterilization. Therefore, two shake-flask experiments were conducted in previously pasteurized media (T=80 °C for 15 min). Table 3.12. presents the substrate consumption along with BDO and Ace production. Moreover, as shown, during the flask experiment in which all of the fermentation points derived from one and the same flask, a yield of 0.41 g g^{-1} was reached. When each fermentation point was the mean value of two different flasks, the conversion yield obtained was slightly lower (=0.39 g g^{-1}). On the other hand, compared to the respective aseptic experiment, the volumetric productivity achieved during the culture in the previously pasteurized medium was somehow lower (2.35 against 1.22 g l^{-1} h^{-1}). Likewise, it should be stressed that a petri dish was inoculated at the end of the previously pasteurized experiment in order to detect any possible contamination. Indeed, after 15 h of cultivation, colonies of the strain *Enterobacter* sp. FMCC-208 were the only ones that were observed. This was based on both macroscopic observation of the colonies formed and microscopic observation of the cells obtained from the previously developed colonies.

Table 3.12. Cultures of *Enterobacter* sp. FMCC-208 in shake flasks performed in previously pasteurized media when initial sucrose concentration that had been adjusted at 40 g l^{-1}. Representation of maximum biomass production, substrate consumption, final 2,3-butanediol and acetoin concentration, conversion yield on sugar consumed and productivity in batch fermentations. Culture conditions: growth in 500-ml flasks filled with 100 ml, T=37 °C; agitation rate=180 rpm; initial pH=7.0. Each point is the mean value of two independent measurements.

Sugar consumed (g l^{-1})	X_{max} (g l^{-1})	BDO (g l^{-1})	Ace (g l^{-1})	Eth (g l^{-1})	$Y_{BDO,Ace}$ (g g^{-1})	$P_{BDO,Ace}$ (g l^{-1} h^{-1})	Fermentation time (h)
44.4±1.2[a]	5.0±0.5	16.3±0.5	2.0±0.5	2.3±0.2	0.41±0.02	1.22±0.04	15
44.0±1.0[b]	4.0±0.5	15.5±0.5	1.5±0.3	1.5±0.1	0.39±0.01	1.21±0.03	14

a: Samples taken from the same flask

b: Samples taken from different flasks

3.3.7. Influence of pH value in batch fermentations under aerobic conditions with sucrose employed as carbon source

In order to evaluate the optimum pH value for the bacterial growth and the production of BDO, four batch bioreactor experiments were carried out at constant pH values of 5.0, 6.0, 6.5 and 7.0. The strain presented a very restricted growth at a pH=5.0, and, therefore, very low sugar

quantities consumption has been observed during this trial. However, the bacterial growth was sufficient at higher pH values imposed into the medium. Table 3.13. presents the results from the three batch fermentations carried out at pH=6.0, 6.5 and 7.0 with an initial sucrose concentration adjusted at 40 g l^{-1}. Slight variation of the final biomass production was observed at the end of the three fermentations. As for the bioconversion of sugar into BDO+Ace, much better results concerning the final BDO+Ace concentration and the yield $Y_{BDO,Ace}$ were recorded at a medium pH value of 6.0 compared to the trials at 6.5 and 7.0. When the pH value was adjusted at 6.5 or 7.0, much higher volumetric productivities (i.e. 1.81 and 2.14 g $l^{-1}h^{-1}$) were achieved.

Table 3.13. Maximum biomass production of *Enterobacter* sp. FMCC-208, substrate consumption, final 2,3-butanediol and other organic products concentration, conversion yield and productivity in batch bioreactor fermentations in stable pH value under aerobic conditions using sucrose as carbon source, an initial sucrose concentration adjusted at 40 g l^{-1}. Culture conditions: T=37 °C; agitation rate =180 rpm; aeration 1 vvm; culture pH indicated in the table. Each point is the mean value of two independent measurements.

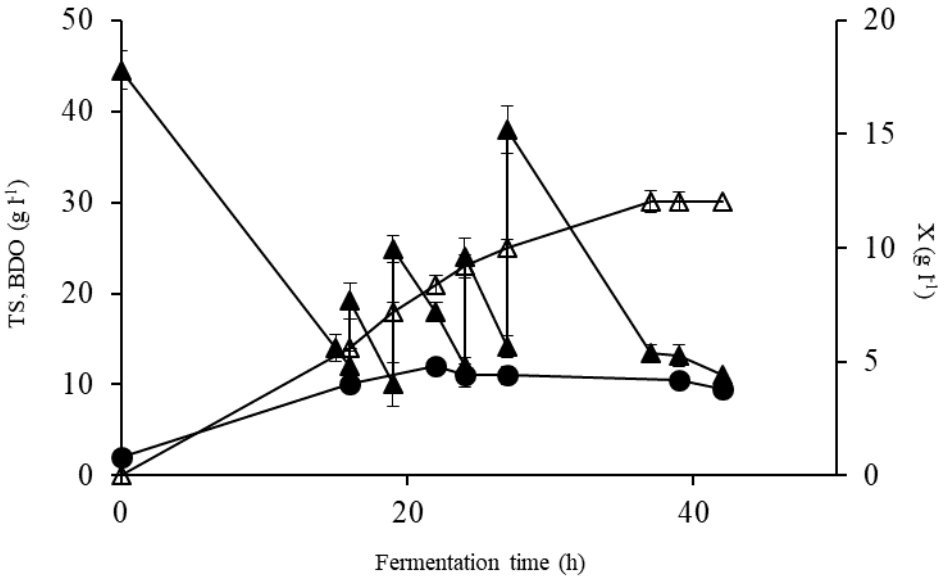
pH value	Total sugar consumed (g l^{-1})	X_{max} (g l^{-1})	BDO (g l^{-1})	Ace (g l^{-1})	Eth (g l^{-1})	Suc (g l^{-1})	$Y_{BDO,Ace}$ (g g^{-1})	$P_{BDO,Ace}$ (g l^{-1} h^{-1})	Fermentation time (h)
5.0	4.0±2.0	0.7±0.2	n.d.	n.d.	n.d.	n.d.	-	-	24
6.0	40.7±1.8	4.9±0.4	17.0±1.5	2.8±0.2	3.6±0.1	2.7±0.1	0.49±0.01	0.83±0.06	24
6.5	32.7±2.0	5.5±0.2	12.6±0.5	1.9±0.3	2.0±0.2	1.9±0.1	0.44±0.02	1.81±0.05	8
7.0	39.3±1.5	5.3±0.5	13.2±1.0	1.8±0.4	2.3±0.3	4.5±1.0	0.38±0.01	2.14±0.02	7

n.d.: Non-detected

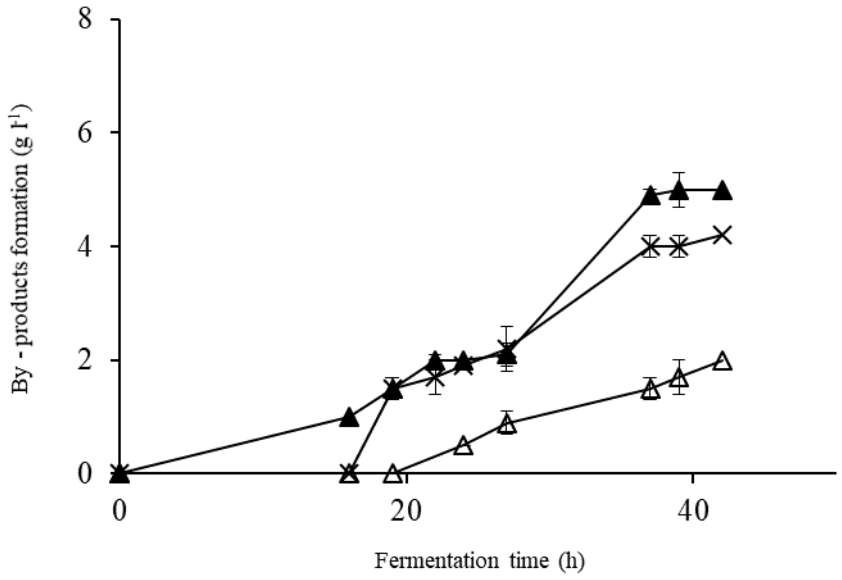
3.3.8. Fed-batch experiments for enhanced 2,3-butanediol production

The first set of fed-batch fermentations was carried out either under anaerobic or aerobic conditions. These trials were carried out at incubation temperature T=30 °C, since as it has previously been demonstrated, temperatures between 30 and 37 °C are favorable, concerning both the conversion yield of BDO produced per sugar consumed and the volumetric productivity of the process. As shown in Table 3.14. (and as it was expected through all of the previously mentioned results), BDO formation significantly increased under aerobic conditions. In particular, 30.1 g l^{-1} of BDO and 5.0 g l^{-1} of acetoin were accumulated into the growth medium at the end of the anaerobic fermentation. In contrast, at the end of the aerobic fermentation the remarkably high final BDO concentration of 90.3 g l^{-1} was achieved, while the final acetoin quantity produced was 10 g l^{-1}. DCW production was also remarkably higher in the case of aerobic conditions. The bioconversion yield (viz. $Y_{BDO,Ace}$ in g g^{-1}, that is the sum of 2,3-butanediol and acetoin produced divided by the quantity of total sugars consumed for the given fermentation point) was higher when aeration was

applied into the culture medium with a value of 0.43 g g^{-1}, while the yield obtained under anaerobic conditions was 0.39 g g^{-1}. As for the productivity, slight difference was observed in the case of aerobic and anaerobic fermentation, reaching a value of 0.84 g $l^{-1}h^{-1}$ and 0.90 g $l^{-1}h^{-1}$ respectively. It must be pointed out, that during the fed-batch bioreactor experiments performed, the "end of the culture" meant that the microorganism was virtually unable to further produce BDO and the substrate was converted to principally organic acids. Further incubation, thus, in both anaerobic and aerobic fed-batch experiments, was not taken into consideration, and the volumetric productivity was calculated as the sum of 2,3-butanediol and acetoin produced divided by the fermentation time at the given fermentation point in which the maximum amount of BDO+Ace was seen. Additionally, biomass concentration dramatically decreased due to the bacterial cell lysis. The kinetics of total sugars consumption as well as the production of BDO, other organic acids and DCW during anaerobic and aerobic fermentations are shown in Figures 3.10. and 3.11. respectively.

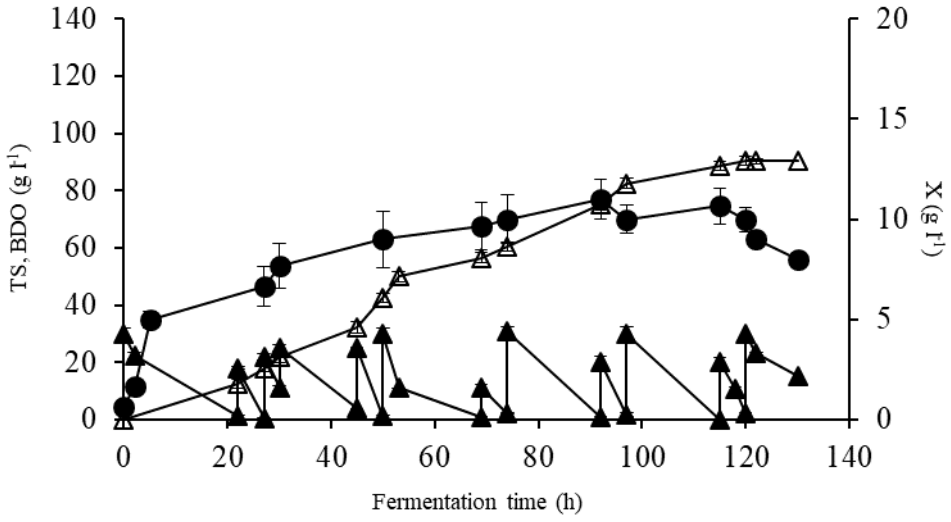


(a)



(b)

Figure 3.10. Kinetics of evolution of total sugars (TS, g l^{-1}) (▲), 2,3-butanediol (BDO, g l^{-1}) (△), biomass (X, g l^{-1}) (●) (a) and acetoin (Ace, g l^{-1}) (▲), lactic acid (Lac, g l^{-1}) (△) and ethanol (Eth, g l^{-1}) (×) (b) during growth of *Enterobacter* sp. FMCC-208 on molasses and sucrose in fed-batch bioreactor experiments. Culture conditions: anaerobic trial, 180 rpm agitation rate, pH fluctuating from 7.0 to 6.0, T=30 °C, growth on 2-l bioreactor. Each point is the mean value of two independent measurements.



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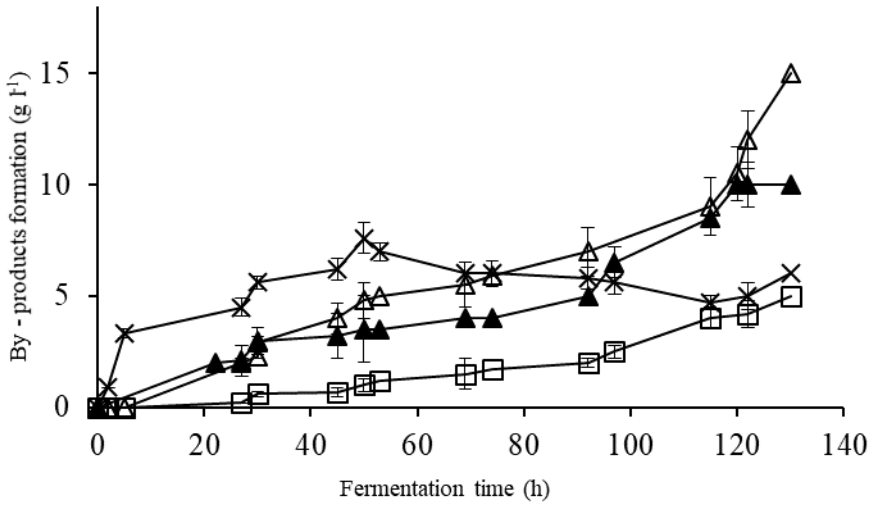
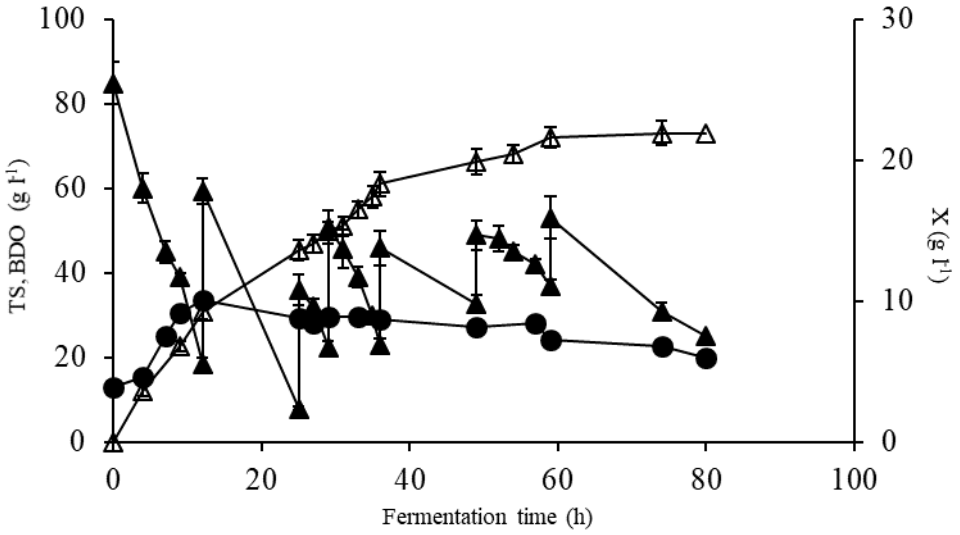


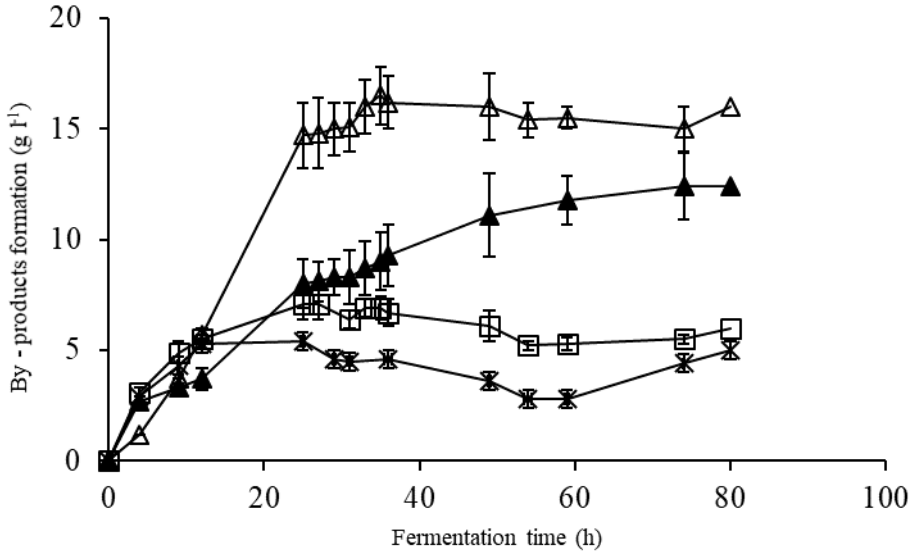
(b)

Figure 3.11. Kinetics of evolution of total sugars (TS, g l^{-1}) (▲), 2,3-butanediol (BDO, g l^{-1}) (△), biomass (X, g l^{-1}) (●) (a) and acetoin (Ace, g l^{-1}) (▲), lactic acid (Lac, g l^{-1}) (△) succinic acid (Suc, g l^{-1}) (□) and ethanol (Eth, g l^{-1}) (×) (b) during growth of *Enterobacter* sp. FMCC-208 on molasses and sucrose in fed-batch bioreactor experiments. Culture conditions: 1 vvm aeration, agitation rate from 180 to 400 rpm, pH fluctuating from 7.0 to 6.0, T=30 °C, growth on 2-l bioreactor. Each point is the mean value of two independent measurements.

As previously stated, the incubation temperature T=37 °C was also very favorable concerning the batch experiments carried out in shake flasks, therefore, a fed-batch experiment was conducted at this temperature under aerobic conditions, using molasses as initial carbon source while sucrose was added into the medium when needed. The kinetics of total sugars consumption as well as the production of BDO, acetoin and other organic acids and biomass accumulation during aerobic fed-batch fermentation at T=37 °C are shown in Figure 3.12. As shown, the final BDO production was 73.0 g l^{-1} while final acetoin accumulation into the medium was 12.4 g l^{-1}. The bioconversion yield was 0.45 g g^{-1} and the productivity rate was remarkably higher at 37 °C, reaching a value of 1.15 g $l^{-1}h^{-1}$.



(a)

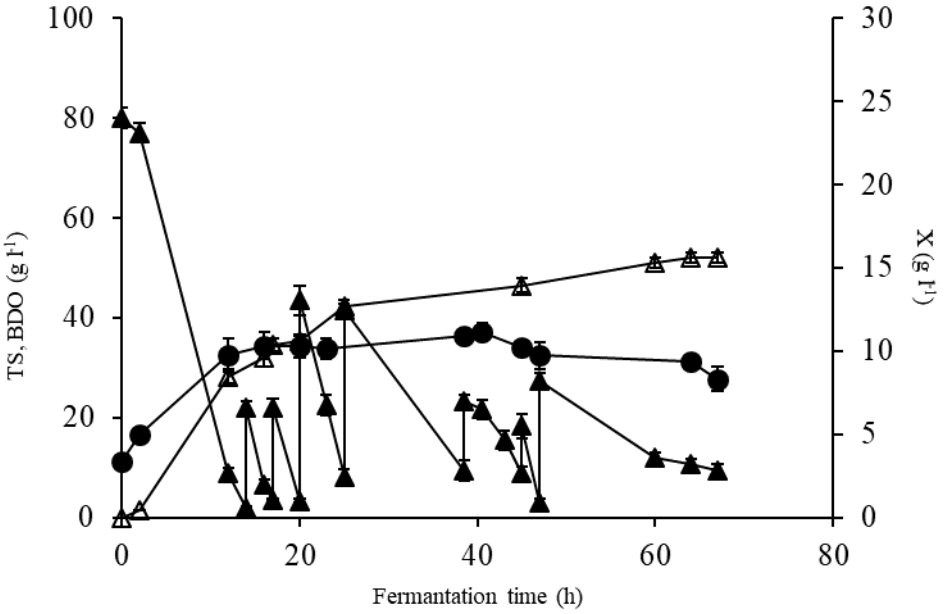


(b)

Figure 3.12. Kinetics of evolution of total sugars (TS, g l^{-1}) (▲), 2,3-butanediol (BDO, g l^{-1}) (△), biomass (X, g l^{-1}) (●) (a) and acetoin (Ace, g l^{-1}) (▲), lactic acid (Lac, g l^{-1}) (△), succinic acid (Suc, g l^{-1}) (□) and ethanol (Eth, g l^{-1}) (×) (b) during growth of *Enterobacter* sp. FMCC-208 on molasses and sucrose in fed-batch bioreactor experiments. Culture conditions: 1 vvm aeration, agitation rate from 180 to 400 rpm, pH fluctuating from 7.0 to 6.0, T=37 °C, growth on 2-l bioreactor. Each point is the mean value of two independent measurements.

Another fed-batch bioreactor experiment was conducted using molasses as the sole carbon source into the culture medium at T=37 °C. The aim was to identify the capability of the strain to grow and produce BDO with no substrate inhibition, using this sugar-processing residue as the only carbon source. As shown in Table 3.14. and Figure 3.13., although remarkable quantities of sugars had been assimilated, the final BDO concentration was considerably reduced when only molasses

were added into the culture medium as compared to the final product concentration in the case of the fed-batch experiment using molasses and pulses of sucrose. In particular, c. 230 g l^{-1} of total sugars derived from molasses were converted into 52 g l^{-1} of BDO and 8.7 g l^{-1} of acetoin while the final accumulation of the lactic acid was 33 g l^{-1}, the highest value detected among all the fed-batch experiments. As for the final bioconversion yield of molasses into BDO and acetoin was remarkably low, reaching a value of 0.26 g g^{-1} while the productivity rate value was 0.95 g $l^{-1}h^{-1}$.



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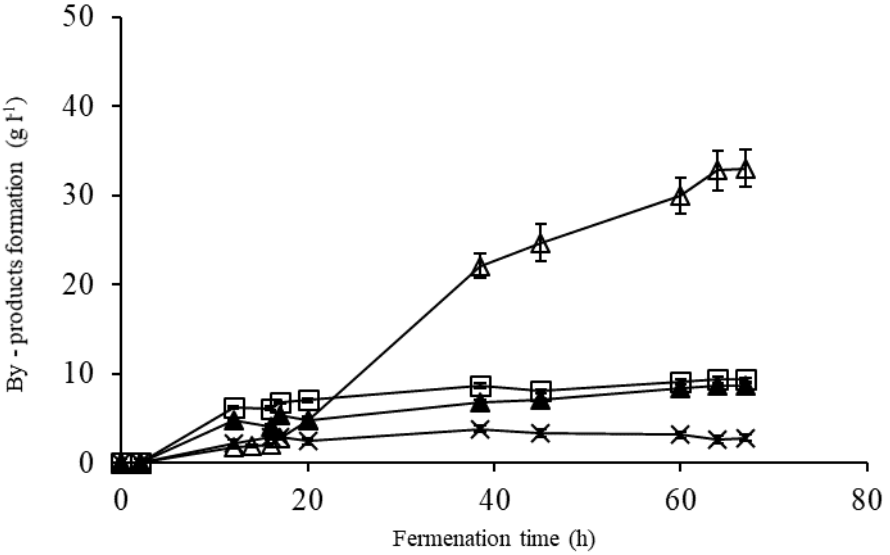


(b)

Figure 3.13. Kinetics of evolution of total sugars (TS, g l^{-1}) (▲), 2,3-butanediol (BDO, g l^{-1}) (△), biomass (X, g l^{-1}) (●) (a) and acetoin (Ace, g l^{-1}) (▲), lactic acid (Lac, g l^{-1}) (△), succinic acid (Suc, g l^{-1}) (□) and ethanol (Eth, g l^{-1}) (×) (b) during growth of *Enterobacter* sp. FMCC-208 on molasses in fed-batch bioreactor experiments. Culture conditions: 1 vvm aeration, agitation rate from 180 to 400 rpm, pH fluctuating from 7.0 to 6.0, T=37 °C, growth on 2-l bioreactor. Each point is the mean value of two independent measurements.

Two other fed-batch experiments using sucrose as the sole carbon source were performed in 2-l shake flasks (Table 3.14.). In the first shake-flask fed-batch experiment, the medium had been previously sterilized and at the end of the fermentation 74.2 g l^{-1} of BDO and 5.3 g l^{-1} of acetoin were produced with a simultaneous conversion yield of 0.43 g g^{-1}. The medium of the second shake-flask experiment had been previously pasteurized (T=80 °C for 15 min) and at the end of the fermentation 70.0 g l^{-1} of BDO and 5.0 g l^{-1} of acetoin were produced (yield =0.39 g g^{-1}). Table 3.14. shows the final substrate consumption along with growth and BDO production. At the end of the fermentation using pasteurized medium a petri dish was inoculated with the flask culture and after 24 h of incubation at T=30 °C, only a few colonies (<5%) presenting different morphology compared to that of the microorganism *Enterobacter* sp. FMCC-208 were seen. Microscopic observation of cells deriving from the "contaminant" colonies showed that the implicated contaminant microorganisms were Gram+ rods.

Table 3.14. Comparative values of maximum biomass production, substrate consumption, final 2,3-butanediol concentration, conversion yield and productivity in fed-batch trials of *Enterobacter* sp. FMCC-208 using molasses and sucrose as carbon source, under anaerobic and aerobic conditions. Culture conditions for the bioreactor experiments: T=30 °C or 37 °C; aeration rate=1 vvm (aerobic conditions); agitation rate: from 180 rpm to 400 rpm (aerobic conditions); agitation rate=180 rpm (anaerobic conditions); pH fluctuating from 7.0 to 6.0. Culture conditions for the shake-flask experiments: growth in 2-l flasks filled with 500 ml, T=37 °C; agitation rate: 180 rpm; pH fluctuating from 7.0 to 6.0.

Fermentation mode	Aeration mode	T °C	Total sugars consumed (g l^{-1})	X_{max} (g l^{-1})	BDO (g l^{-1})	Ace (g l^{-1})	Eth (g l^{-1})	Lac (g l^{-1})	Suc (g l^{-1})	$Y_{BDO,Ace}$ (g g^{-1})	$P_{BDO,Ace}$ (g l^{-1} h^{-1})	Fermentation time (h)
Bioreactor	Anaerobic	30	89.2±1.0[a]	4.8±1.2	30.1±1.1	5.0±0.3	4.0±1.0	1.7±0.3	0.0	0.39±0.01	0.90±0.05	39
	Aerobic	30	235.8±2.0[a]	11.0±1.0	90.3±1.5	10.0±0.5	7.0±1.0	12.0±1.3	4.2±0.6	0.43±0.05	0.84±0.05	120
	Aerobic	37	189.8±1.0[a]	10.1±2.1	73.0±2.9	12.4±1.5	4.4±0.1	15.0±1.0	5.5±0.2	0.45±0.02	1.15±0.05	74
	Aerobic	37	230.2±2.0[b]	10.1±2.1	52.0±1.1	8.7±0.2	2.8±0.1	33.0±2.1	9.3±0.2	0.26±0.02	0.95±0.10	64
Shake flask	Aerobic	37	185.0±3.2[c]	11.4±2.0	74.2±1.5	5.3±1.0	3.0±1.2	10.3±0.5	7.2±1.0	0.43±0.01	1.06±0.05	75
	Aerobic	37	190.0±3.2[d]	12.2±2.5	70.0±1.5	5.5±0.5	3.5±0.2	12.5±1.2	6.0±0.1	0.39±0.03	0.94±0.10	80

a: Molasses and sucrose as carbon source

b: Molasses as the sole carbon source

c: Sucrose as the sole carbon source; previously sterilized medium

d: Sucrose as the sole carbon source; previously pasteurized medium

3.3.9. Color removal occurring during molasses fermentation

Besides BDO production, trials performed on molasses employed as the sole carbon source were accompanied by non-negligible decolorization of the residue; therefore, anaerobic cultures performed in Duran bottles, were accompanied by a decolorization of the medium of 25%. On the other hand, aerobic growth occurring in shake-flask experiments, resulted in somehow higher decolorization of the medium (35%) (Fig. 3.14.). In both aerobic and anaerobic experiments performed, decolorization seemed to be a completely growth-associated process.

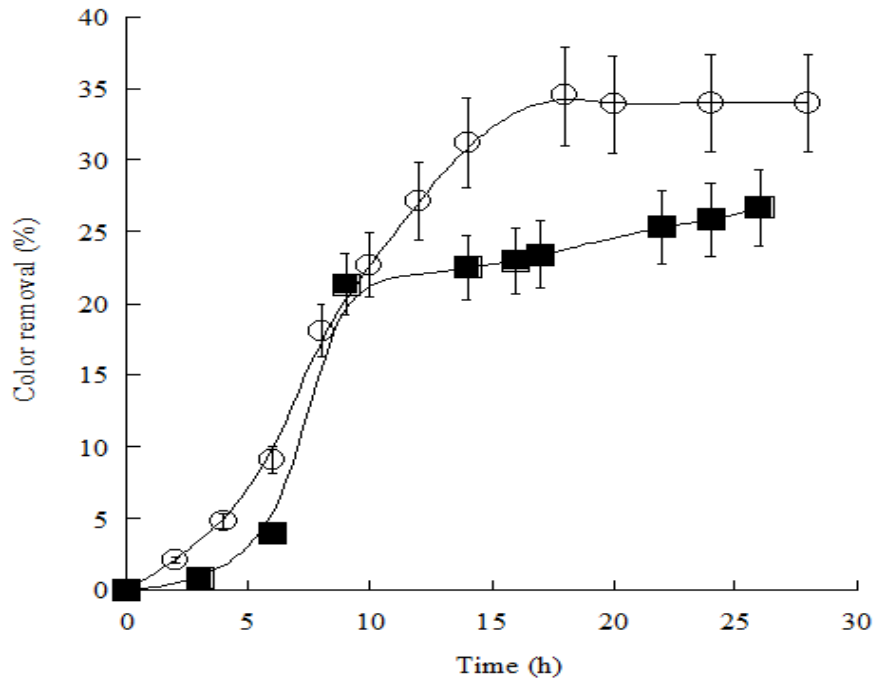


Figure 3.14. Color removal during growth of *Enterobacter* sp. FMCC-208 on molasses under anaerobic (■) and aerobic (○) conditions. Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0, initial total sugars concentration ≈30 g l^{-1}. Anaerobic experiments were conducted in Duran bottles and aerobic experiments on 500-ml shake flasks. Each point is the mean value of two independent measurements.

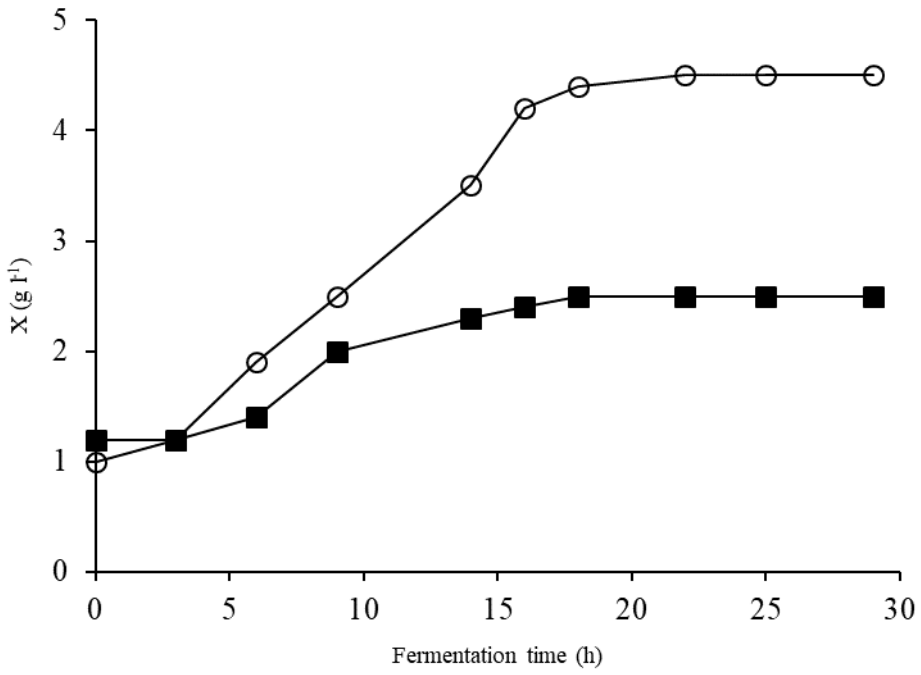
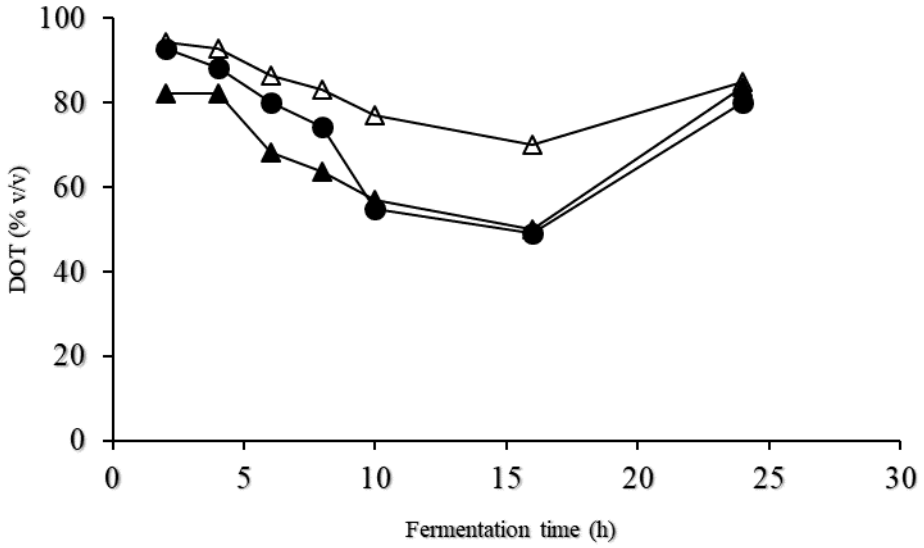


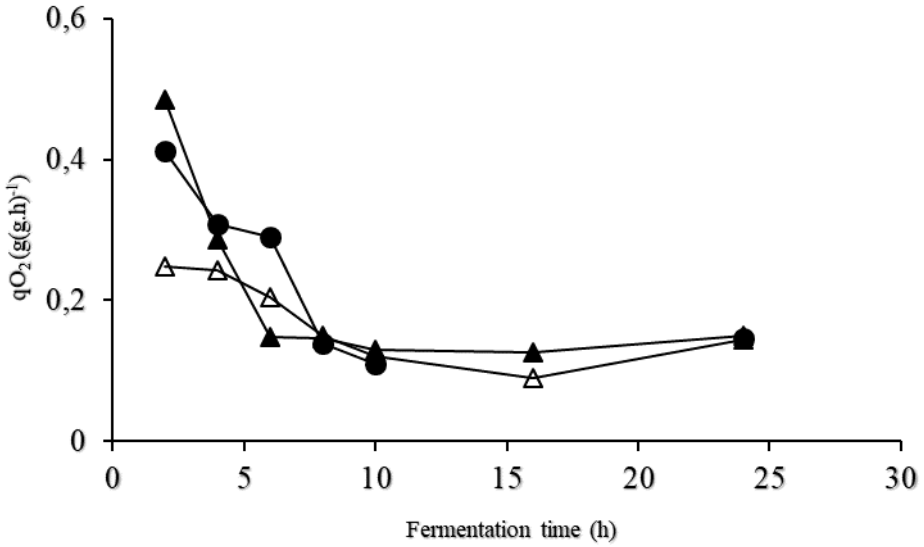
Figure 3.15. Growth of *Enterobacter* sp. FMCC-208 on molasses under anaerobic (■) and aerobic (○) conditions. Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0, initial total sugars concentration ≈30 g l^{-1}. Anaerobic experiments were conducted in Duran bottles and aerobic experiments on 500-ml shake flasks. Each point is the mean value of two independent measurements.

3.3.10. Effect of dissolved oxygen concentration upon the BDO production bioprocess carried out by *Enterobacter* sp.

As in the previous part of the manuscript where the impact of oxygen concentration was studied in relation to the production of BDO by *K. oxytoca*, in this last part of the current investigation, a similar series of trials was carried out using *Enterobacter* sp. FMCC-208. Therefore again, it was desirable to study the effect of the oxygen concentration and the oxygen uptake rate upon the performed bioprocess, during the shake-flask experiments carried out by the later microorganism. As previously the dissolved oxygen tension (DOT) and the specific oxygen consumption (q_{O_2}) evolution were initially studied in the first set of shake-flask trials, where glucose, fructose and sucrose were used at an initial concentration ≈30 g l^{-1} (T=30 °C) (Fig. 3.16. a; b).



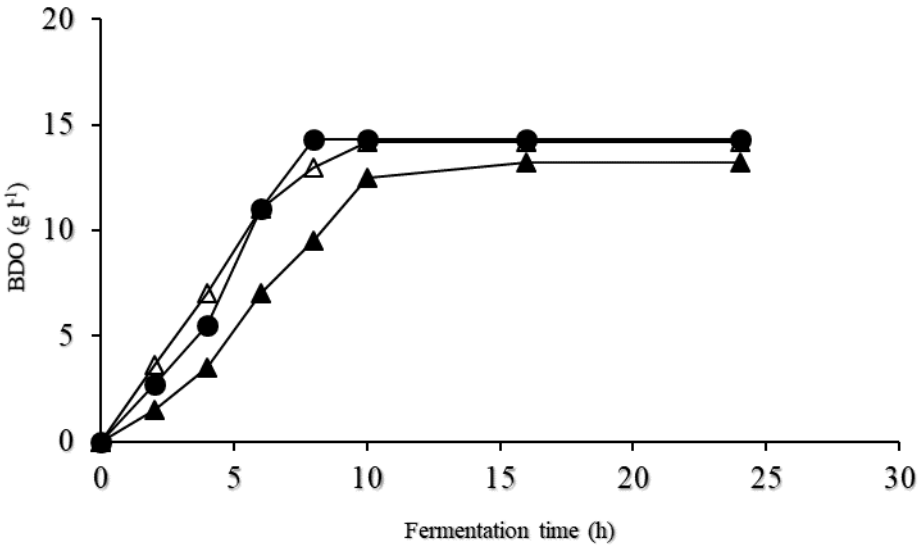
(a)



(b)

Figure 3.16. (a) Dissolved oxygen tension (DOT, % v/v) and (b) Oxygen consumption (q_{O_2}, g(g.h)$^{-1}$) related with fermentation time during shake flask cultivation of *Enterobacter* sp. FMCC-208 on glucose (●), fructose (△) and sucrose (▲). Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0, initial glucose concentration ≈30 g l^{-1}.

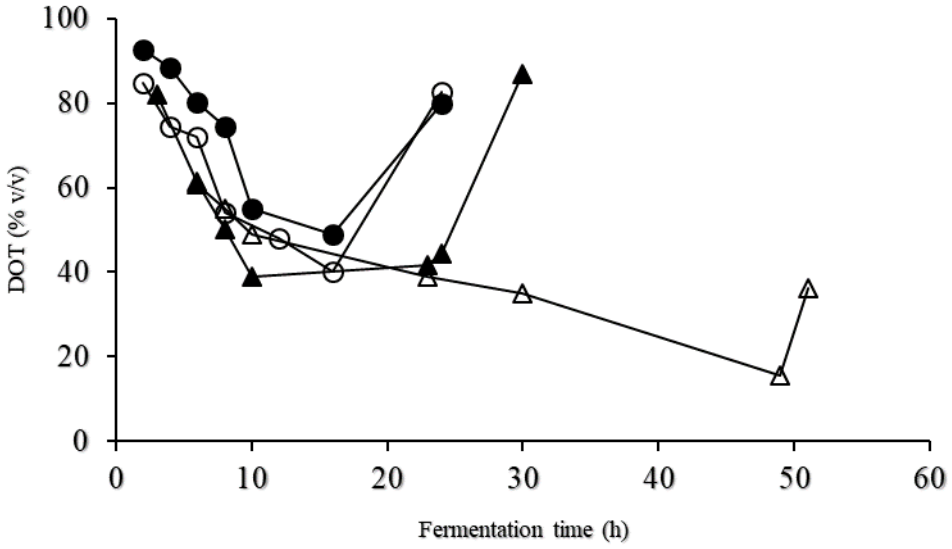
In full accordance with the results reported for *K. oxytoca*, DOT (%, v/v) evolution as function of the fermentation time was similar during the fermentations regardless of the individual sugar employed as substrate, and decreased even at the early growth steps of the culture. In any case though, DOT values remained always higher than ~60% v/v, indicating again that all trials were carried out under full aerobic conditions. However, the representation of the specific consumption rate of oxygen for the trials with the three different sugars (30 g l^{-1}) showed, as in the case of *K. oxytoca*, a significant respiratory activity for the strain *K. oxytoca* at the first growth steps (q_{O_2}=0.40±0.05 g (g.h)$^{-1}$) that remarkably decreased at the late fermentation steps of the culture (q_{O_2}=0.09±0.02 g (g.h)$^{-1}$). The results obtained are similar to those using the strain *K. oxytoca*, as the respiratory activity was q_{O_2}=0.40±0.05 g (g.h)$^{-1}$ and q_{O_2}=0.09±0.02 g (g.h)$^{-1}$), respectively. BDO production occurred during both growth phases (*viz.* in both the culture steps of the increased and the decreased respiratory activity of the strain; see Fig 3.16.c.) demonstrating that biosynthesis of BDO in *Enterobacter* sp. FMCC-208 occurred regardless of the physiological state of the microorganism concerning its respiratory activity.



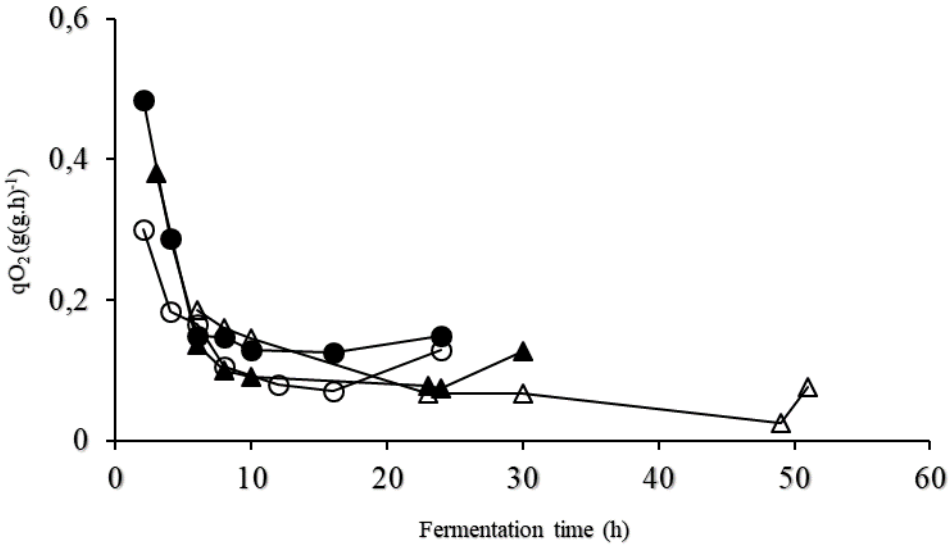
(c)

Figure 3.16. (c) 2,3-butanediol (BDO, g l^{-1}) production related with fermentation time during shake flask cultivation of *Enterobacter* sp. FMCC-208 on glucose (●), fructose (△) and sucrose (▲). Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0, initial glucose concentration ≈30 g l^{-1}.

Moreover, the impact of initial sucrose concentration upon the DOT and the specific oxygen consumption rate (q_{O_2}) evolution was studied during *Enterobacter* sp. FMCC-208 shake-flask experiments. Therefore, DOT and q_{O_2} values were determined for four different initial sucrose concentrations initially added into the medium (30, 60, 90 and 150 g l^{-1}) and the results are illustrated in Figs 3.17. (a and b; Fig. 3.17.c represents the kinetics of BDO production for the different initial concentrations of sucrose employed into the medium). DOT values decreased (in some cases remarkably) with sucrose concentration increase into the medium. However, in most cases DOT values were >20-25% v/v, therefore, as in the previous case of *K. oxytoca*, trials were not oxygen-limited (Hagman et al., 2013; Sabra et al., 2017). Moreover, as shown (Fig. 3.17.a) microaerobic conditions were achieved at 150 g l^{-1} after 40 hours of fermentation (DOT values ≈10% v/v), thus in the later case *Enterobacter* sp. FMCC-208 synthesized BDO under both aerobic and oxygen-limited conditions. From all the above-mentioned analysis therefore, it must be indicated again that satisfactory BDO production was observed in the flask experiments of *Enterobacter* sp. FMCC-208 under full aerobic conditions, although this is not the characteristic feature of the microorganisms that are carrying out this type of conversion (Ji et al., 2011; Zeng & Sabra, 2011). On the other hand, the monitoring of q_{O_2} evolution during the fermentation carried out demonstrated again, in accordance with the results achieved by *K. oxytoca*, that significant respiratory activity for the strain occurred at the first growth steps (q_{O_2}ranging between 0.3 and 0.5 g $(g.h)^{-1}$) that remarkably decreased as the fermentation proceeded (specifically at the trials with initial sucrose concentrations adjusted at 90 and 150 g l^{-1}, q_{O_2} value was 0.08 and 0.03 respectively).

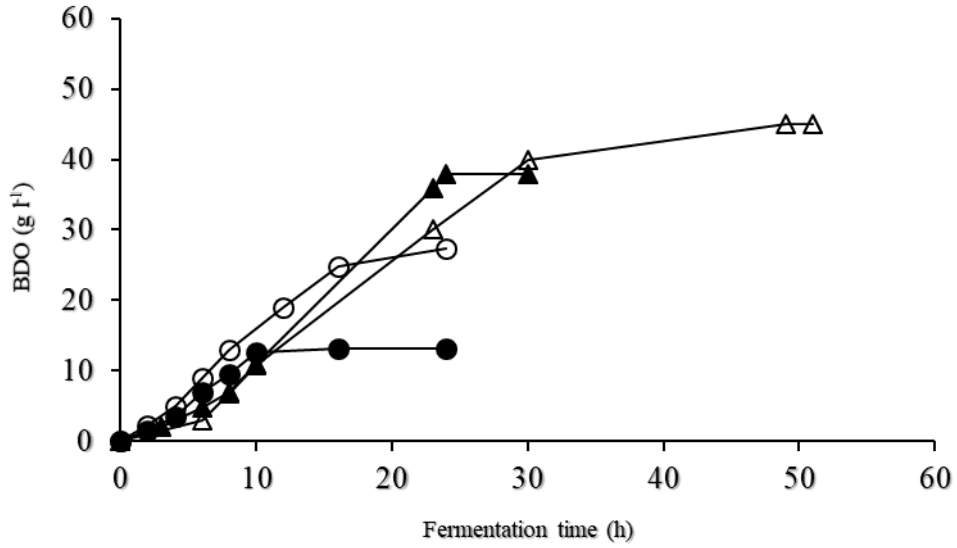


(a)



(b)

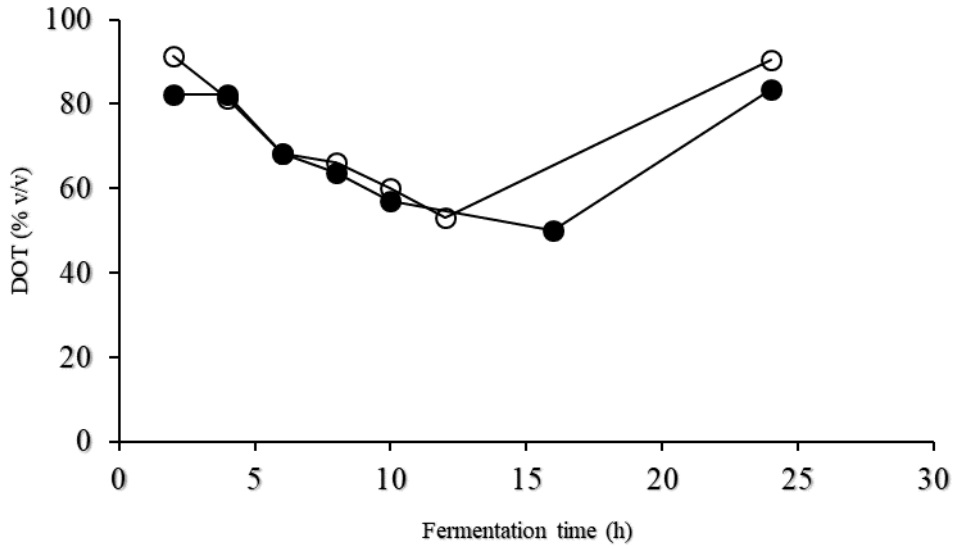
Figure 3.17. (a) Dissolved oxygen tension (DOT, % v/v) and (b) Oxygen consumption (q_{O_2}, $g(g.h)^{-1}$) related with fermentation time during shake flask cultivation of *Enterobacter* sp. FMCC-208 on sucrose using 30 (●), 60 (○), 90 (▲) and 150 (△) g l^{-1} initial concentration. Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0.



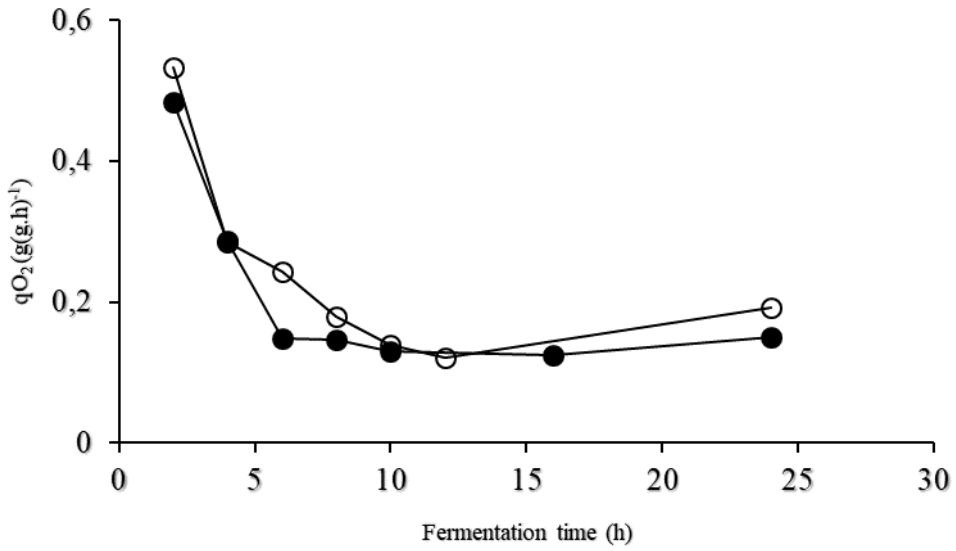
(c)

Figure 3.17. (c) 2,3-butanediol (BDO, g l^{-1}) production related with fermentation time during shake flask cultivation of *Enterobacter* sp. FMCC-208 on sucrose using 30 (●), 60 (○), 90 (▲) and 150 (△) g l^{-1} initial concentration. Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0.

Finally, an experiment at 37 °C was performed using sucrose as carbon source and Figure 3.18. compares the results obtained with the one at 30 °C. Similar observations as in the previous trials were seen; the DOT (%, v/v) decreased as function of the fermentation time, while it appears that the increase of the incubation temperature very slightly decreased DOT, in disagreement with the results reported by *K. oxytoca*. Likewise, as previously, remarkable respiratory activity, as demonstrated by the evolution of the specific oxygen consumption rate (q_{O_2}) was noted at the first growth steps decreasing as the fermentation proceeded.

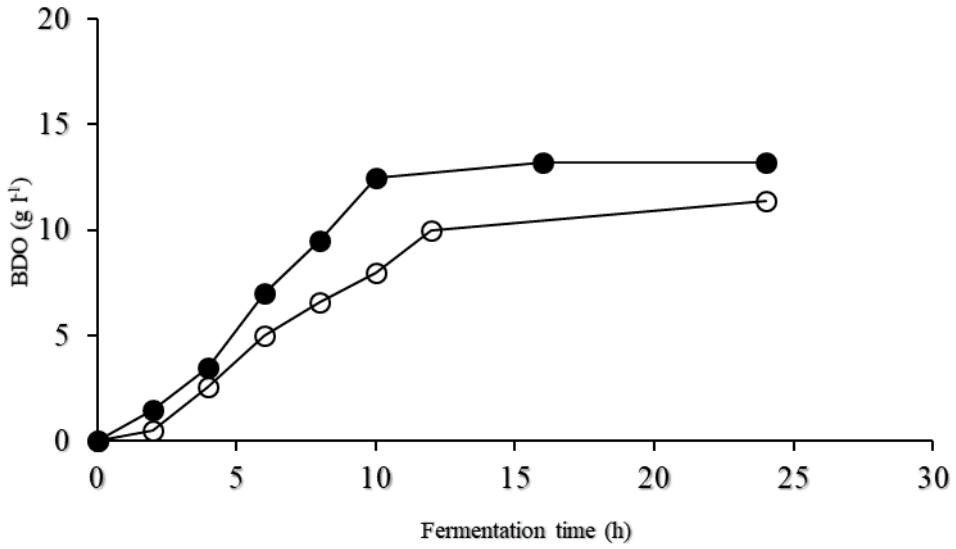


(a)



(b)

Figure 3.18. (a) Dissolved oxygen tension (DOT, % v/v) and (b) Oxygen consumption (q_{O_2}, $g(g.h)^{-1}$) related with fermentation time during shake flask cultivation of *Enterobacter* sp. FMCC-208 on sucrose at 30 (●) and 37 °C (○). Culture conditions: 180 rpm agitation rate, initial pH=7.0, initial glucose concentration ≈30 g l^{-1}.



(c)

Figure 3.18. (c) 2,3-butanediol (BDO, g l^{-1}) production related with fermentation time during shake flask cultivation of *Enterobacter* sp. FMCC-208 on sucrose at 30 (●) and 37 °C (○). Culture conditions: 180 rpm agitation rate, initial pH=7.0, initial glucose concentration ≈30 g l^{-1}.

4. Discussion

The significant expansion of the agricultural production, the subsequent numerous agro-industrial applications that occur and the current way of life of the western societies itself, have led to the enormous generation of low- or negative-cost crude solid, semi-solid or liquid materials that are difficult to treat and valorize (Peters, 2007; Philippoussis, 2010; Sarris et al., 2018). Specifically, food wastes constitute a major environmental, economic and social problem; in developed countries, food of *c.* 100 kg (220 lb) per person per year is wasted, only at the consumption stage, without taking into consideration the losses carried out in the food-processing units (Gustavsson et al., 2011). Flour-rich waste streams, solid simple waste sugars (e.g. waste sucrose or glucose generated in the confectionary industries), waste breads and waste-waters containing high concentrations of sugars (i.e. glucose, fructose, etc) constitute a major source of pollution within the EU. Amongst the seven most important food-processing by-products in terms of production capacities within the EU27, in three ones (namely 1) grain mill products; 2) sugars and relevant products; 3) bread, fresh pastry goods and cakes) mostly composed of sucrose and glucose, very high quantities are annually generated with evident negative effects for the humans and the environment (Koutinas et al., 2014). The majority of these waste streams are currently used as animal feed, fertilizers, and substrates for composting or vermi-composting processes or are land-filled (Koutinas et al., 2014; Lin et al., 2014).

The last years and within the frame of the circular economy, a “new” trend concerning the management – treatment of the various organic residues has gained noticeable interest, namely the utilization of agro-industrial residues as substrates of various types of microorganisms in several types of fermentation configurations, in order for the production of added-value bio-products that can be used in the chemical and food industries to occur (Lin et al. 2014). Every year huge quantities of agro-food and industrial residue streams which are rich in lignin, cellulose and other carbohydrates are produced. As a result, the research has been focusing to all aspects of the development of economically efficient and environmentally friendly and sustainable ways of the utilization of the above-mentioned wastes and residues for the production of added-value products *via* microbial technology (i.e. genetic engineering and systems biology studies in order to “construct” robust microbial strains with “desired” properties, fermentation technology studies in order to perform process optimization, bioprocess modeling and process simulation, etc) (Koutinas et al., 2014; Lin et al., 2014). Therefore, important aspects on the Industrial Biotechnology in order to proceed to production of added-value dedicated metabolites with the aid of microbial fermentations refer to: (1) the isolation and identification of microorganisms that are potentially capable to produce in high concentrations the requested microbial metabolites; (2) the identification of culture conditions for improving the production of the aimed metabolites; (3) the subsequent production of the targeted metabolites using highly productive culture configurations and

fermentation strategies; (4) the simultaneous production of (non-antagonistic) microbial compounds (if possible), with the targeted metabolites; (5) The combination (if possible) of various wastes and by-products as microbial carbon sources for the proposed production schemes; (6) the potential production of the targeted metabolites under non-aseptic conditions; (7) the production of the desired metabolites in pilot-scale operations (Papanikolaou & Aggelis, 2011; 2019; Koutinas et al., 2014; Sarris et al., 2016; 2018).

The employment of low-cost substrates for the production of various added-value metabolites with the aid of Industrial Microbiology (i.e. microbial oils, polysaccharides, organic acids, diols, etc) is of crucial importance for reducing the cost of the implicated bioprocesses and simultaneously exempting the environment from highly polluted wastes that can be generated through the various agro-industrial activities (Huang et al., 2013; Lin et al., 2014; Qin et al., 2017; Papanikolaou & Aggelis, 2019). A large variety of zero or even negative cost raw hydrophilic materials have been considered and used as feedstocks for the sustainable production of biochemicals through the use of the microbial and the biochemical technology; research concerning the utilization of these raw materials has mainly focused on the utilization of: 1) crude glycerol (by-product of several agro-industrial processes like the one of the creation of biodiesel); 2) wastewaters or side-products of food-processing facilities that contain variable quantities of sugars (i.e. cheese-whey, various fractions of molasses, solid waste streams containing carbohydrates like i.e. waste sugars and waste bread, expired juices, olive-mill wastewaters, wastewaters deriving from confectionary facilities, etc); 3) low-molecular weight organic acid resources, equally deriving from several agro-industrial and agro-chemical processes; 4) lignocellulosic sugars or hydrolysates or wastewaters containing these types of sugars (Huang et al., 2013; Koutinas et al., 2014; Qin et al., 2017; Sarris et al., 2016; 2018). It is noted that the removal of non-toxic and non-hazardous wastes material deriving from food-processing facilities (i.e. flour-rich waste streams, waste breads, etc), costs c. 0.4-0.7 US $ per kg of waste, and currently occurs mostly in order these materials to be subjected to composting or vermi-composting processes (Papanikolaou & Aggelis 2010; Athenaki et al., 2018), therefore establishment of a bio-refinery scheme in order for valorization of these wastes with simultaneous production of various metabolites with a plethora of applications in the Food Technology and the Chemical Industry (like i.e. 2,3-butanediol – BDO and acetoin – Ace) to occur, could be of significant importance for the implicated food-processing facilities.

As indicated in the previous chapters, BDO, an added-value platform chemical compound that can be synthesized through microbial fermentations conducted when various low-cost materials are employed as substrates, presents important applications in agro-food, pharmaceutical and chemical industries (Zeng & Sabra, 2011; Sabra et al., 2016). Therefore, methyl-ethyl-ketone, the dehydration product of BDO can be used as an excellent organic solvent for resins and lacquers,

while it can also find applications as liquid fuel having a higher heat of combustion than that of ethanol (Xiu & Zeng, 2008; Ji et al., 2010; 2011; Zeng & Sabra, 2011). BDO can also be converted into 1,3-butadiene, which is used as a basic chemical compound for the production of synthetic rubbers, polyesters and polyurethanes, it can also be dehydrated to form 3-buten-2-ol, or it can be dehydrogenated to form acetoin and diacetyl, compounds presenting importance for the food industry and technology (Maddox, 1996; Sabra et al., 2016). Likewise, BDO can be ketalized with acetone to produce a "tetramethyl" compound, which is a potential gasoline blending agent similar to the commonly used methyl tert-butyl ether (MTBE) (Ji et al., 2010; Sabra et al., 2016).

Strains which are able to produce BDO in significant quantities belong but are not limited to the genera *Klebsiella*, *Enterobacter*, *Bacillus* and *Serratia* (Lee & Maddox, 1986; Maddox, 1996; 2008; Nakashimada et al., 1998; Soltys et al., 2001; Syu, 2001; Marwoto et al., 2004; Ma et al., 2009; Cheng et al., 2010). Various strains of *Lactobacillus* and *Lactococcus* have also been reported capable to present the potential of synthesizing BDO in remarkable concentrations (Celinska & Grajek, 2009). An important drawback related with the use of the principal BDO-producing microorganisms *K. pneumoniae* and *S. marcescens* (Maddox, 2008; Zeng & Sabra, 2011; Sabra et al., 2016), refers to the fact that several strains of these species are (important) opportunistic pathogens (Narashisma Rao et al., 1998) and, therefore, potential scale-up of the bioprocess using the above-mentioned species is not an obvious task (Li et al., 2013).

Concerning substrates used in the concerned bioprocess, a variety of monosaccharides, both hexoses and pentoses, or glycerol, can be converted into BDO (Lee & Maddox, 1984; 1986; Syu, 2001; Maddox, 2008; Celinska & Grajek, 2009; Li et al., 2013; Cho et al., 2015). Sugar-based materials (i.e. analytical-grade sugars, commercial sugars, industrially produced feedstocks i.e. very high polarity sucrose from sugarcane mills or sugar-based effluents) are the principal carbon sources amenable for the synthesis of BDO. This metabolite can be produced *via* microbial bioconversion using predominantly bacterial strains (see previously noticed bacterial species and genera implicated), although recent research has shown that yeasts and marine microalgae can also be used as BDO producers (Hon-Nami et al., 2006; Nan et al., 2014). As indicated in the previous chapters, all of the above-mentioned substrates are initially converted to pyruvate before generation of major products of the microbial catabolism. From glucose or glycerol, pyruvate is formed *via* the Embden-Meyerhof pathway (glycolysis). In contrast, the production of pyruvate from pentoses must proceed *via* a combination of the pentose phosphate, the phospho-ketolase and the Embden-Meyerhof pathways (Jansen & Tsao, 1983; Athenaki et al., 2018; Papanikolaou & Aggelis, 2019).

Satisfactory BDO concentrations achieved in the various sugar-based fermentations reported are within the range of 70-90 g l^{-1}, while the maximum theoretical conversion yield of BDO produced per unit of sugar consumed, depended on the nature of the sugar employed (i.e. sucrose,

maltose or dextrins presents a slightly higher maximum theoretical yield compared with the simple polysaccharides) is 0.50-0.53 g g^{-1} (for reviews see: Maddox, 2008; Xiu & Zeng, 2008; Celinska & Grajek, 2009; Zeng & Sabra, 2011; Sabra et al., 2016). In addition to BDO, a mixture of acetate, lactate, formate, succinate, acetoin, and ethanol are also produced through the mixed acid – BDO fermentation pathway (Magee & Kosaric, 1987; Koutinas et al., 2014), while when glycerol is employed as substrate, 1,3-propanediol can also be generated as final product, specifically if anaerobic conditions are imposed (Zeng & Sabra, 2011; Cho et al, 2015; Sabra et al., 2016). Finally, factors influencing BDO production from several types of bacterial strains can be either nutritional ones (like the concentration and the nature of carbon substrate, nitrogen source, etc) as well as factors related with the implicated culture conditions i.e. aeration, temperature and pH (Xiu & Zeng, 2008; Celinska & Grajek, 2009; Ji et al., 2011).

The present investigation refers to the utilization of low-cost (i.e. cost of commercial sucrose is c. 0.1 $/kg, while to cost of molasses is even lower) and abundant raw materials (commercial sucrose and its principal by-product molasses are produced in very high and continuously increasing quantities worldwide) in order for BDO and Ace) to be produced. Various newly isolated, food-derived and potentially non-pathogenic strains (Narashisma Rao et al., 1998) were assessed for their potential to convert mainly glucose and sucrose into BDO during initial anaerobic cultivations in Duran bottles. Therefore nine bacterial strains which belong to the family of Enterobacteriaceae, were initially screened. The strains employed were the following ones: *E. ludwigii* FMCC-204, *E. aerogenes* FMCC-9, *E. aerogenes* FMCC-10, *C. freundii* FMCC-207, *K. oxytoca* FMCC-197, *C. freundii* FMCC-8, *C. farmeri* FMCC-5, *C. farmeri* FMCC-7 and *Enterobacter* sp. FMCC-208. As presented in Table 3.1., six strains, namely *E. ludwigii* FMCC-204, *E. aerogenes* FMCC-9, *E. aerogenes* FMCC-10, *C. freundii* FMCC-207, *K. oxytoca* FMCC-197 and *Enterobacter* sp. FMCC-208, have shown great ability to consume glucose and sucrose and produce BDO in promising yields (>0.40 g g^{-1}) and productivity rates. On the other hand, three strains, namely *C. freundii* FMCC-8, *C. farmeri* FMCC-5 and *C. farmeri* FMCC-7 could only consume glucose, no BDO production was detected into the culture medium and acetic acid was the only metabolite produced at a final concentration lower than 10 g l^{-1}. The three bacterial strains could not assimilate sucrose and slight biomass production was noted at the end of the fermentation.

Two strains, namely *K. oxytoca* FMCC-197 and *Enterobacter* sp. FMCC-208, were proven to be the optimal candidates for BDO production, as they combined remarkable bioconversion yield and productivity rate, for several carbon sources used (most studies were carried out with commercial sucrose and molasses used individually or as blends) and they were selected for further investigation.

Preliminary batch experiments under anaerobic and aerobic conditions using commercial sucrose and molasses as carbon source at relatively low initial total sugar concentrations imposed into the medium revealed the ability both studied strains (*K. oxytoca* FMCC-197 and *Enterobacter* sp. FMCC-208) to produce BDO. On the other hand, shake-flask experiments, in which oxygen was found in sufficient quantities into the medium during all growth phases – at least in sucrose concentrations up to 90 g l^{-1}, dissolved oxygen tension (DOT) values were always ≥25% v/v while only at initial concentration of *c.* 150 g l^{-1} there could have been a potential growth in some growth phases under micro-aerobic conditions – led to higher productivity (both as regards absolute BDO values into the medium in g l^{-1} and relative values of BDO synthesized per unit of sugar consumed in g g^{-1}) compared to the anaerobic trials. In general, an important parameter related to BDO production is considered to be the oxygen supply, while BDO is considered to be a product the metabolism of which is linked to the anaerobic or micro-aerobic metabolism (Celinska & Grajek, 2009; Zeng & Sabra, 2011; Dai et al., 2015). The physiological feature observed in the current investigation, meaning, in fact, the synthesis of BDO (and Ace) under sufficient aerobic conditions, can be considered as somehow unusual result. However, in a relatively restricted number studies, aeration has shown to (significantly in several cases) enhance BDO synthesis (Maddox, 1996; Petrov and Petrova, 2010; Metsoviti et al., 2012a), whereas in some cases indeed intensive aeration strategy (i.e. 2.2. vvm of air supply in the bioreactor) maximized BDO biosynthesis (Petrov & Petrova, 2010). Nevertheless, the general biochemical consideration and theory related to BDO biosynthesis, indicates that the oxidative pathway, where $NADH_2$ co-factors are recycled through the respiratory chain, does not favor the above-mentioned process, favoring the formation of bacterial dry cell weight production to the detriment of BDO biosynthesis (Zeng & Sabra, 2011; Sabra et al., 2016). During most shake-flask and aerobic bioreactor experiments carried out in the current investigation, DOT values were always ≥25%, v/v, while during the BDO productive phase these values were 30%, v/v. These values correspond to sufficient aerobic conditions in the fermentation (Krahe, 2003; Metsoviti et al., 2011; Hagman et al., 2013; Papanikolaou et al., 2017). Within the above-mentioned range of DOT values (*viz*. 20-30%, v/v), certainly dry cell weight production is enhanced when compared to the respective anaerobic trials, whereas in this DOT range, potentially a satisfactory intra-cellular pool of $NADH_2$ could always have been maintained to be used through acetoin dehydrogenase and 2,3-butanediol dehydrogenase reactions, enhancing, thus, 2,3-butanediol and acetoin production (Maddox, 1996; Celinska & Grajek, 2009; Zeng & Sabra, 2011). A similar trend in which higher BDO production was noted in shake-flask experiments (presenting, in any case, DOT values ≥30%, v/v) compared to Duran anaerobic bottles, has already been reported during growth on waste glycerol by another potentially non-pathogenic newly isolated *E. aerogenes* strain (strain FMCC-10, equally isolated from several types of food-

stuffs) (Doulgeraki et al., 2011; Metsoviti et al., 2012a). Likewise, DOT values of *c*. 20%, v/v, throughout the BDO productive phase of *B. licheniformis* DSM 8785 have been considered as optimum ones in order to maximize BDO production in fed-batch bioreactor glucose-based experiments (BDO_{max}≈125 g l^{-1}; Y_{BDO}=0.37 g g^{-1}) (Jurchescu, 2013). In contrast, intensively high oxygen supply in most cases seems to prevent BDO production due to rapid and irreversible inactivation of α-acetolactate synthase and the subsequent metabolic shift towards DCW production instead of BDO biosynthesis (Celinska & Grajek, 2009; Zeng & Sabra, 2011; Koutinas et al., 2014). In any case, it must be pointed out that the potential of BDO production by strains cultivated under sufficient aerobic conditions like *K. oxytoca* FMCC-197 and *Enterobacter* sp. FMCC-208 presents interest and merits of more detailed research in the future.

Given that the main food industry waste fraction consists of numerous carbohydrates, the ability of the studied strains to convert various types of sugars into BDO and Ace presents remarkable interest, since these types of sugar supplements are found in several types of lignocellulosic or food-waste hydrolysates (Lin et al., 2014; Athenaki et al., 2018). Specifically, galactose can be found in cheese whey. Moreover, glucose and fructose are found in several types of food waste streams and residues like inulin-, starchy- or waste bread-hydrolysates, glucose-based wastewaters, or fruit and vegetable wastes which are rich in the above-mentioned hexoses. Therefore, for both microorganisms studied, a set of shake-flask experiments using different individual sugars as substrates such as glucose, fructose, mannose, arabinose, galactose and xylose was performed. A combination of high yield and productivity was achieved when hexoses were added into the medium, while pentoses were also converted into BDO in satisfying, but lower, yields. Despite the somehow lower concentrations obtained during growth on pentoses (i.e. xylose and arabinose) the interest on the bioconversion of these types of sugars into 2,3-butanediol and acetoin is obvious, since these types of sugar supplements are found in several types of lignocellulosic hydrolysates or in wastewaters deriving from the paper processing units (i.e. spent sulfite liquor) (Koutinas et al., 2014; Lin et al., 2014; Athenaki et al., 2018). Likewise, glucose and fructose fermentation into BDO, the conversion of which indicated indeed high $Y_{BDO,Ace}$ values, presents remarkable interest since these types of sugars are found in several types of food-processing waste streams and residues or (of course only for the case of glucose) derive from (the abundant) cellulose hydrolysis (Lin et al., 2014). On the other hand, given that pentoses break-down involves two different pathways (i.e. the pentose-phosphate pathway and the phospho-ketolase pathway) while phospho-ketolase pathway is more efficient than the one of phosphate-pentose (and equally more efficient compared to the typical EMP pathway of hexoses; see: Athenaki et al. 2018) it may be assumed that mostly the pentose-phosphate pathway is employed in both *Enterobacter* sp. FMCC-208 and *K. oxytoca* FMCC-197 strains.

The study further focused on the determination of various parameters in order to achieve the highest BDO production in the minimum time for both *K. oxytoca* FMCC-197 and *Enterobacter* sp. FMCC-208. The temperature effect on BDO production was tested and the optimum results were obtained at temperatures ranging between 30 °C and 37 °C. More specifically, for *Enterobacter* sp. FMCC-208, at T=37 °C, conversion of sucrose into BDO and Ace in shake-flask trials reached the productivity of *c.* 2.4 g l^{-1} h^{-1}, which is amongst the highest ones reported in the international literature (Celinska & Grajek, 2009; Zeng & Sabra, 2011; Jung et al., 2013; Li et al., 2013). The following step was to investigate the range of the initial sucrose imposed, and as it was anticipated, substrate inhibition occurred at the somehow elevated initial concentrations into the medium. The threshold of sucrose inhibition was different when various kinetic parameters like μ_{max}, $Y_{X/S}$, or lag phase time were taken into consideration for both microorganisms, but, in any case, what was indeed interesting referred to the fact that enhanced sucrose assimilation and significant BDO production were both reported even when indeed high sugar quantities (i.e. concentrations up to 150 g l^{-1}) were found into the medium. Similar "resistance" upon enhanced sugar concentrations into the medium has been reported for a scarce number of BDO producing microorganisms like *E. aerogenes* DSM 30053 and *Bacillus licheniformis* DSM 8785 (Zeng et al., 1991; Jurchescu, 2013; Jurchescu et al., 2013), but in the later case microorganisms deriving from culture collections and no food-derived new isolates (as in the current investigation) were employed as microbial cell factories.

Fed-batch bioreactor experiments under optimized (aerobic) conditions were conducted for both microorganisms; the final product synthesis were for *Enterobacter* sp. FMCC-208 90.3 g l^{-1} of BDO and 10 g l^{-1} of acetoin (BDO+Ace=100.3 g l^{-1}) with a conversion yield of product synthesized per total sugar consumed $Y_{BDO,Ace}$≈0.43 g g^{-1}. For *K. oxytoca* FMCC-197 the respective values were 101.1 g l^{-1} of BDO and 14.2 g l^{-1} of Ace (BDO+Ace=115.3 g l^{-1}), with concomitant conversion yield $Y_{BDO,Ace}$=0.40 g g^{-1}. These values are amongst the very satisfactory ones reported in the international literature as regards BDO+Ace production by wild-type bacterial strains.

Enterobacter sp. strains have been intensively studied in relation to their potential of BDO (and Ace) production and in several cases, production of 2,3-butanediol higher than the one reported by *Enterobacter* sp. FMCC-208 was obtained. For instance, the wild-type *E. aerogenes* DSM 30053 deriving from official culture collection (*viz.* DSM) successfully converted glucose into BDO with very high final concentration (*c.* 110 g l^{-1}) during fed-batch experiments (Zeng et al., 1991). The mutated strain *E. cloacae* CGMCC 6053 produced very high BDO+Ace quantities (i.e. up to *c.* 100 g l^{-1}) during growth on sugarcane molasses that were freshly prepared and used for fermentation without previous heat sterilization, in fed-batch bioreactor micro-aerobic trials (Dai et al., 2015). Moreover, *E. cloacae* strain SDM was systematically metabolically engineered to

construct a cell factory for the production of enantio-pure (2R,3R)-2,3-butanediol and, indeed, when a lignocellulosic hydrolysate was used as substrate, 119.4 g l^{-1} of (2R,3R)-BDO (purity>96.0%) were obtained in fed-batch bioreactor experiments (Li et al., 2015). The cultivation of a genetically engineered *E. aerogenes* strain (in which sucrose regulator – ScrR had been disrupted from the genomic DNA) on agro-industrial residues (sugarcane molasses) during a fed-batch experiments, has led to the production of 98.7 g l^{-1} of BDO at 36 h, reaching one of the highest volumetric productivities referred in the literature *viz.* 2.74 g l^{-1} h^{-1} (Jung et al., 2013). Another genetic engineering approach of *E. aerogenes* in which deletion of lactate dehydrogenase occurred (creation of the strain EMY-01 – KCTC ΔldhA) resulted in tremendous BDO production and volumetric productivity (*c.* 118 g l^{-1} and 2.19 g l^{-1} h^{-1}) in fed-batch glucose-based bioreactor experiments (Jung et al., 2012). Finally, alleviation of carbon catabolite repression through genetic modifications in *E. aerogenes* resulted in the creation of the robust strain EMY-70S that efficiently utilized sugarcane molasses producing very high BDO concentrations (up to 140 g l^{-1}), equally in fed-batch bioreactor experiments (Jung et al., 2015). Concluding, in various cases in which *Enterobacter* sp. strains have been used in order for BDO production to be carried out, very high BDO concentrations (in several cases higher than that obtained by *Enterobacter* sp. FMCC-208) have been achieved. However, contrary to the newly isolated and deriving from food-stuffs strain used in the current investigation, in many of the previously mentioned cases, *Enterobacter* sp. strains derive either from official culture collections (i.e. DSM) or are mutated / genetically engineered ones (i.e. CGMCC 6053, SDM, EMY-01, etc).

Klebsiella sp. strains are also characterized by remarkable ability of BDO production in high yields on various substrates; for instance, the strain *K. oxytoca* M1 (Cho et al., 2013) was able to successfully convert various carbon sources into BDO with the maximum yield and productivity obtained, 0.32 g g^{-1} and 0.40 g $l^{-1}h^{-1}$ respectively, when glucose had been used as substrate. Further process optimization, led to tremendous BDO+Ace production in fed-batch glucose-based cultures (BDO_{max}=118.5 g l^{-1}, Ace_{max}=42.1 g l^{-1}, $Y_{BDO,Ace}$=0.46 g g^{-1}) (Cho et al., 2015a). Non-negligible productivities reported so far were noted when *K. pneumoniae* CICC 10011 was cultivated on glucose (Qin et al., 2006). The final BDO production was 52.4 g l^{-1} while the productivity was 1.0-1.5 g $l^{-1}h^{-1}$. A recent research focused on BDO production using sugarcane hydrolysate by the strain *K. pneumoniae* CGMCC 1.9131 (Song et al., 2012). The maximum yield was 0.43 g g^{-1} and the maximum productivity was 1.45 g $l^{-1}h^{-1}$. Other sugar-based raw materials (i.e. Jerusalem artichoke tubers) have been considered as potential substrates for BDO production with a maximum production of 80.5 g l^{-1} reported (Li et al., 2010). In another case, a *K. oxytoca* strain produced the very high quantity of 130 g l^{-1} of BDO during fed-batch bioreactor fermentation (Ji et al., 2010). A *K. oxytoca* mutant (strain NBRF4) was used during another fed-batch cultivation, producing 34.2 g

l^{-1} of BDO without organic acid production in 70 h (Han et al., 2013). Finally, using crude glycerol, residue deriving from biodiesel production, the genetically engineered *K. oxytoca* M3 in which *pduC* (encoding glycerol dehydratase large subunit) and *ldhA* (encoding lactate dehydrogenase) genes were deleted in order to maximize the pathway glycerol ⟶ 2,3-butanediol, converted glycerol into BDO with very high final concentration and yield (BDO_{max}=131.5 g l^{-1}, yield =0.44 g per g of glycerol consumed) (Cho et al. 2015b). Recently, *B. licheniformis* (GRAS microorganism), cultivated in shake-flask fed-batch experiments, was reported to produce a BDO_{max} quantity that was very high (=144.7 g l^{-1}, productivity =1.14 g $l^{-1}h^{-1}$; Jurchescu et al. 2013). Another newly isolated GRAS strain *B. licheniformis* strain (strain 10-1-A) produced very high BDO quantities (up to *c*. 116 g l^{-1}) when glucose was used as substrate with a simultaneous high conversion yield of 0.47 g per g of glucose consumed and an excellent volumetric productivity of 2.4 g $l^{-1}h^{-1}$, in fed-batch bioreactor trials (Li et al., 2013). A synopsis of the results reported in the literature and their comparison with the current investigation concerning BDO and Ace production by *K. oxytoca* strains is found in Table 3.15., and by *Enterobacter* sp. strains in Table 3.16..

Table 3.15. BDO production by different bacterial strains of the *Klebsiella* species

Strain	Substrate	BDO (g l^{-1})	Yield (g g^{-1})	Fermentation mode	Reference
Klebsiella oxytoca ME-UD-3	Glucose	130	0.48	Fed-batch/ Bioreactor	Ji et al. (2010)
Klebsiella oxytoca DSM 3539	Molasses	118.0	0.42	Repeated batch	Afschar et al. (1991)
Klebsiella oxytoca M1	Glucose	19.0	0.32	Batch/ Shake flasks	Cho et al. (2013)
	Xylose	17.1	0.28		
	Galactose	15.1	0.25		
	Fructose	18.2	0.29		
Klebsiella oxytoca NBRF4	Glucose	34.2	0.35	Fed-batch/ Bioreactor	Han et al. (2013)
Klebsiella oxytoca M1	Glucose	118.5 (+42.1 Ace)	0.46	Fed-batch/ Bioreactor	Cho et al. (2015a)
Klebsiella oxytoca M3	Crude glycerol	131.5	0.44	Fed-batch/ Bioreactor	Cho et al. (2015b)
Klebsiella pneumoniae CICC 10011	Glucose	52.4	0.38	Batch/ Shake flasks	Qin et al. (2006)
K. pneumoniae SDM	Corn steep liquor	151	0.48	Fed-batch/ Bioreactor	Ma et al. (2009)
K. pneumoniae SDM	Corncob molasses	78.9	0.41	Fed-batch/ Bioreactor	Wang et al. (2010)
Klebsiella pneumoniae CGMCC 1.9131	Glucose	20.93	0.38	Batch/ Shake flasks	Song et al. (2012)
	Xylose	11.1	0.49		
	Sugarcane acid hydrolysate	17.35	0.43		
	Sugarcene alkali	14.53	0.43		

	hydrolysate				
K. pneumoniae G31	Glycerol	70	0.39	Fed-batch/ Bioreactor	Petrov and Petrova (2010)
K. pneumoniae CICC 10781	Cheese whey powder	57.6	0.40	Fed-batch/ Biore-actor	Guo et al. (2017)
Klebsiella oxytoca FMCC-197	Glucose	10.9+1.5*	0.45	Batch/ Shake flasks	Current study
	Fructose	10.7+1.2*	0.42		
	Mannose	9.8+0.9*	0.45		
	Xylose	4.2+0.2*	0.30		
	Arabinose	7.7+0.5*	0.47		
	Galactose	10.7+1.1*	0.41		
	Molasses+Sucrose	101.1+14.2*	0.40	Fed-batch/ Bioreactor	
	Molasses	35.1+8.0*	0.38		
	Sucrose	51.0+9.0*	0.41	Fed-batch/Shake flask	

*BDO+Ace production

Table 3.16. BDO production by different bacterial strains of the *Enterobacter* species

Strain	Substrate	BDO ($g\ l^{-1}$)	Yield ($g\ g^{-1}$)	Fermentation mode	Reference
Enterobacter cloacae NRRL B-23289	Glucose	18.6	0.37	Batch/ Shake flasks	Saha et al. (1999)
	Xylose	18.9	0.38		
	Galactose	18.9	0.38		
	Mannose	18.6	0.37		
	Arabinose	21.7	0.43		
Enterobacter cloacae CGMCC 605	Sugarcane molasses	90.8 (+8.7 Ace)*	0.39	Fed-batch/ Bioreactor	Dai et al. (2015)
Enterobacter cloacae CGMCC 605	Glucose	110.9 (+8.8 Ace)*	0.42	Fed-batch/ Bioreactor	Dai et al. (2015)
Enterobacter cloacae SDM	Corn stover	119.4	0.47	Fed-batch/ Bioreactor	Li et al. (2015)
Enterobacter aerogenes DSM 30053	Glucose	c. 110	0.48	Fed-batch/ Bioreactor	Zeng et al. (1991)
Enterobacter aerogenes EMY-01 (KCTC ΔldhA)	Glucose	118.1	c. 0.46	Fed-batch/ Bioreactor	Jung et al. (2012)
Enterobacter aerogenes KCTC 2190-EMY68	Sugarcane molasses	98.7	0.37	Fed-batch/ Bioreactor	Jung et al. (2013)
Enterobacter aerogenes EMY-70SP	Sugarcane molasses	129.4	0.39	Fed-batch/ Bioreactor	Jung et al. (2015)
Enterobacter aerogenes EMY-70S	Sugarcane molasses	140.0	0.39	Fed-batch/ Bioreactor	Jung et al. (2015)
Enterobacter ludwigii FMCC-204	Fruit extract	18.2	0.36	Batch/Shake flasks	Liakou et al. (2017)
		50.1	0.40	Fed-batch /Bioreactor	
	Glucose	12.6+1.7*	0.48		

Enterobacter sp. FMCC-208	Fructose	12.8+1.4*	0.48	Batch/ Shake flasks	Current study
	Sucrose	12.0+0.3*	0.36		
	Mannose	11.9+2.1*	0.47		
	Galactose	11.9+1.9*	0.48		
	Arabinose	10.5+0.3*	0.43		
	Xylose	10.4+0.1*	0.43		
	Molasses+Sucrose	90.3+10*	0.43	Fed-batch/ Bioreactor	
	Molasses	73.0+12.4*	0.45		
	Sucrose	74.2+5.3*	0.43	Fed-batch/ Shake flask	

*BDO+Ace production

Both implicated strains which were used during this research have proven to be therefore promising BDO and Ace producers as they were able to consume sucrose, molasses and other carbon sources, reaching high yields and productivity rates. In general, some principal BDO-producing microorganisms are characterized as opportunistic pathogens (Li et al., 2013), therefore, obviously, it is of interest to identify natural wild-type, food-deriving and potentially non-pathogenic microorganisms like *Enterobacter* sp. FMCC-208 and *K. oxytoca* FMCC-197 capable to produce in remarkable quantities metabolites of added value (Celinska & Grajek, 2009; Koutinas et al., 2014).

Moreover, during growth of both strains on molasses employed as carbon source, the microorganisms besides production of the requested metabolites (BDO and Ace) performed non-negligible decolorization of the culture medium under both anaerobic and aerobic conditions. For instance, anaerobic cultures performed in Duran bottles using the strain *K. oxytoca* FMCC-197, were accompanied by a decolorization of the medium of 40%. Aerobic growth occurring in shake-flask experiments resulted in higher decolorization of the medium 50%. As for the strain *Enterobacter* sp. FMCC-208, anaerobic cultures performed in Duran bottles, were accompanied by a decolorization of the medium of 25%. Aerobic growth occurring in shake-flask experiments resulted in higher decolorization of the medium 35%. In all cases the decolorization of molasses seemed to be a completely growth-associated process.

The results obtained suggest that molasses wastewaters deriving after a potential BDO fermentation by *K. oxytoca* FMCC-197 and *Enterobacter* sp. FMCC-208 could have been considered as already partially treated wastewaters before their safe disposal. The disposal of wastewaters deriving from several types of molasses fermentations (i.e. production of citric acid,

single-cell protein or ethanol) constitute a major source of soil and aquatic pollution, since melanoïdines, phenolic compounds, etc that are contained in these residues are highly resistant to microbial attack and conventional biological processes such as activated sludge treatment are insufficient in removing these components (Dahiya et al., 2001). Given that several types of bacteria are known for their ability to break down phenolic compounds and melanoïdines (Dahiya et al., 2001; Metsoviti et al., 2011), optimization of molasses decolorization by these strains seems to be an interesting topic that needs to be studied in the future.

Various reports in literature suggest utilization of bacteria, yeast (Tondee et al., 2008; Sarris et al., 2014; 2017; 2019), and fungal strains (Miranda et al., 1996; Tsioulpas et al., 2003) for decolorization of molasses, molasses-containing wastewaters (MWWs), melanoïdines and related types of wastewaters (i.e. olive-mill wastewaters). Excellent review-articles summarizing the process of decolorization of several of the above-mentioned residues with the aid of various microbial strains have recently appeared (Sarris et al., 2018). Specifically, the fungi *Cunninghamella echinulata* and *Mortierella isabellina* were grown on molasses, showing non-negligible substrate decolorization up to ~75% for *C. echinulata* (after 400 h of culture) and ~20% for *M. isabellina* (200 h after inoculation) simultaneously with the production of lipid-rich fungal biomass (Chatzifragkou et al., 2010). Moreover, waste molasses was used as growth medium for *Leuconostoc mesenteroides* to produce bacteriocin, and simultaneous decolorization of up to ~27% of this residue was performed by the same species together with the requested bacteriocin biosynthesis (Metsoviti et al., 2011). Ohmomo et al. (1985) used the fungus *Coriolus versicolour* Ps4a for the decolorization of melanoïdines and achieved a decolorization rate of up to ~80%. Ohmomo et al. (1988) performed trials with *Aspergillus oryzae* strain Y-2-32 which absorbed in its mycelia low molecular weight melanoïdines. *A. niger*, used by Miranda et al. (1996) led to 83% decolorization of MWWs. Raghukumar and Rivonkar (2001) studied the decolorization of molasses spent wash by white-rot fungus *Flavodon flavus*, isolated from a marine habitat, that was able to quickly degrade the high molecular weight fraction. Tondee et al. (2008) cultivated the yeast strain *Issatchenkia orientalis* No SF9-246 (isolated from rotten banana) in a malt extract-glucose-peptone broth containing melanoïdines, and a decolorization rate of 60.2% was obtained within 7 days.

Enterobacter sp. FMCC-208 and *K. oxytoca* FMCC-197 strains have shown great ability to grow and produce BDO even under non-aseptic conditions, a feature presenting obvious interest for a potential scale-up of the process. To our knowledge, in a restricted number of reports efficient BDO production occurred under non-aseptic conditions (i.e. production of 100 g l^{-1} of BDO+Ace by the mutated strain *E. cloacae* CGMCC 6053; see: Dai et al., 2015). In order to provide cost-competitive alternatives, it is imperative to develop efficient, cost-saving, and robust fermentation processes. Non-aseptic fermentation processes offer several benefits compared to “sterile”

fermentation, including elimination of sterility, reduced maintenance requirements, relatively simple bioreactor design, and simplified operations carried out during the bioconversion step (Chen and Wan, 2017). Thus, cost effectiveness of non-aseptic fermentation makes it a practical platform for low cost, large volume production of biofuels and bulk chemicals. Likewise, non-aseptic production of metabolites has attracted significant interest in the Industrial Microbiology. Therefore, slightly earlier or more recent reports have indicated non-aseptic production of ethanol (Sarris et al., 2009; 2013; 2014), citric acid (Sarris et al., 2017), 1,3-propanediol (Chatzifragkou et al., 2011), microbial lipid (Santamauro et al., 2014; Moustogianni et al, 2015; Tchakouteu et al., 2017) and also other metabolites (Chen and Wan, 2017) by various types of microorganisms (yeasts, fungi and bacteria) cultivated in several types fermentation configurations and carbon sources.

As indicated in the previous paragraphs, both *K. oxytoca* FMCC-197 and *Enterobacter* sp. FMCC-208 presented significant BDO production in fed-batch experiments, in which blends or molasses and commercial sucrose were employed as substrates, with the achieved results being competitive to those using various genetically modified bacteria which belong to the genus *Klebsiella* and *Enterobacter*, as presented in Tables 3.15 and 3.16.. It is worth mentioning that both strains revealed lower BDO and Ace synthesis when molasses were employed as the sole carbon source into the medium while lactic acid production was increased, especially in the case of *Enterobacter* sp. FMCC-208. Constant addition of molasses into the environment of the reactor that is due to the fed-batch system employed might result in the continuous addition of inhibitors (i.e. phenolic compounds like phenol, *m*-cresol, etc, melanoïdines and potentially other recalcitrant compounds; see i.e.: Dahiya et al., 2001; Lin et al., 2014), that could potentially favor the activity of lactate dehydrogenase or could decrease the activity of α-acetolactate synthase, and thus could provoke a metabolic shift towards the synthesis of lactic acid to the detriment of BDO and Ace production (if the above-mentioned events happened, loss of the carbon flow from the pathway sugar ⟶ 2,3-butanediol in favor to that of sugar ⟶ lactic acid would occur). On the other hand, comparison between the shake-flask / Duran-bottle experiments in which either molasses or sucrose were used as microbial substrates in similar and relatively low initial total sugar concentrations employed, did not reveal seriously lower BDO production in the molasses-based media compared to trials performed on sucrose. In some cases too, molasses fermentation resulted in a higher $Y_{BDO,Ace}$ value than that achieved on sucrose. Therefore, the previously developed rationale in which the constant addition of molasses (and, thus, inhibitors) could be the main reason for the metabolic shift towards lactic acid production might be reasonable.

Concluding, it should again be stressed that the current agro-industrial expansion has led to an excessive production of various low- or negative-cost agro-industrial by-products and residues.

Carbohydrates (mono- or poly-saccharides) are the main constituents of biomass and occur as cell wall and storage carbohydrates, glycoconjugates and transportation carbohydrates (Peters, 2007; Sarris et al., 2018). Low cost carbon sources such as industrial (not analytical-grade) sugars directly deriving from sugar refinery plants, as well as wastes or by-products such as molasses, can be a beneficial option for microbial fermentations. Molasses are the main by-product deriving from sugar manufacturing process. Due to the presence of fermentative sugars in significant concentrations, this substance has be utilized as substrate in a remarkable number of microbial conversions, especially in ethanol production, and to lesser extent in other fermentations (e g production of citric acid, microbial lipid, etc) since it is considered as one of the cheapest sugar-based feedstocks (Peters, 2007; Sarris et al., 2018). *K. oxytoca* FMCC-197 and *Enterobacter* sp. FMCC-208, these newly isolated and originated from food-stuffs microorganisms can be considered as promising microbial cell factories, amenable to convert several types of sugar-based renewable materials (i.e. commercial sucrose, sugarcane molasses, etc) into BDO and Ace at satisfactory concentrations, conversion yields and volumetric productivities, performing in several cases in previously non-thermally sterilized media, while growth on molasses can be accompanied by partial decolorization of the residue.

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Table 3.6. Comparative values of maximum biomass production, substrate consumption, final 2,3-butanediol concentration, conversion yield and productivity in fed-batch trials of *Klebsiella oxytoca* FMCC-197 using molasses and sucrose as carbon source, under anaerobic and aerobic conditions. Culture conditions for the bioreactor experiments: T=30 °C or 37 °C; aeration rate=1 vvm (aerobic conditions); agitation rate: from 180 rpm to 400 rpm (aerobic conditions); agitation rate=180 rpm (anaerobic conditions); pH fluctuating from 7.0 to 6.0. Culture conditions for the shake-flask experiments: growth in 2-l flasks filled with 500 ml, T=30 °C; agitation rate: 180 rpm; pH fluctuating from 7.0 to 6.0.

Table 3.7. Cultures of *Enterobacter* sp. FMCC-208 on Duran bottles under anaerobic conditions. Representation of maximum biomass production, substrate consumption, final 2,3-butanediol concentration, conversion yield on sugars consumed and productivity in batch fermentations. Culture conditions: T=30 °C; agitation rate =180 rpm; initial pH=7.0. Each point is the mean value of two independent measurements.

Table 3.8. Comparison between maximum biomass production and 2,3-butanediol production by *Enterobacter* sp. FMCC-208 cultivated under anaerobic (Duran bottles) and aerobic (500-ml shake-flask experiments) conditions when commercial sucrose was employed as substrate. Culture conditions: T=30 °C; agitation rate =180 rpm; initial pH=7.0. Each point is the mean value of two independent measurements.

Table 3.9. Growth of *Enterobacter* sp. FMCC-208 in shake flasks using various sugars employed as sole carbon source at initial sugar concentration adjusted at 30 g l^{-1} under aerobic conditions. Representation of maximum biomass production, substrate consumption, final 2,3-butanediol, acetoin and other organic products concentration, conversion yield and productivity in batch fermentations under aerobic conditions using different carbon sources. Culture conditions: growth in 500-ml flasks filled with 100 ml, T=30°C; agitation rate =180 rpm; initial pH=7.0. Each point is the mean value of two independent measurements.

Table 3.10. Temperature effect in total sugars consumption and 2,3-butanediol production, yield and productivity in shake-flask fermentations carried out with c. 20 g l^{-1} and 40 g l^{-1} of initial sucrose concentration by *Enterobacter* sp. FMCC-208. Culture conditions: growth in 500-ml flasks filled with 100 ml, agitation rate =180 rpm; initial pH=7.0. Each point is the mean value of two independent measurements.

Table 3.11. Growth of *Enterobacter* sp. FMCC-208 in shake flasks using various initial sucrose concentrations employed. Representation of the maximum specific growth rate of the strain in shake flask fermentations carried out at T=37 °C. Culture conditions: growth in 500-ml flasks filled with 100 ml, agitation rate =180 rpm; initial pH=7.0. Each point is the mean value of two independent measurements.

Table 3.12. Cultures of *Enterobacter* sp. FMCC-208 in shake flasks performed in previously pasteurized media when initial sucrose concentration that had been adjusted at 40 g l^{-1}. Representation of maximum biomass production, substrate consumption, final 2,3-butanediol and acetoin concentration, conversion yield on sugar consumed and productivity in batch fermentations. Culture conditions: growth in 500-ml flasks filled with 100 ml, T=37 °C; agitation rate=180 rpm; initial pH=7.0. Each point is the mean value of two independent measurements.

Table 3.13. Maximum biomass production of *Enterobacter* sp. FMCC-208, substrate consumption, final 2,3-butanediol and other organic products concentration, conversion yield and productivity in batch bioreactor fermentations in stable pH value under aerobic conditions using sucrose as carbon source, an initial sucrose concentration adjusted at 40 g l^{-1}. Culture conditions: T=37 °C; agitation rate =180 rpm; aeration 1 vvm; culture pH indicated in the table. Each point is the mean value of two independent measurements.

Table 3.14. Comparative values of maximum biomass production, substrate consumption, final 2,3-butanediol concentration, conversion yield and productivity in fed-batch trials of *Enterobacter* sp. FMCC-208 using molasses and sucrose as carbon source, under anaerobic and aerobic conditions. Culture conditions for the bioreactor experiments: T=30 °C or 37 °C; aeration rate=1 vvm (aerobic conditions); agitation rate: from 180 rpm to 400 rpm (aerobic conditions); agitation rate=180 rpm (anaerobic conditions); pH fluctuating from 7.0 to 6.0. Culture conditions for the shake-flask experiments: growth in 2-l flasks filled with 500 ml, T=37 °C; agitation rate: 180 rpm; pH fluctuating from 7.0 to 6.0.

Table 3.15. BDO production by different bacterial strains of the *Klebsiella* species.

Table 3.16. BDO production by different bacterial strains of the *Enterobacter* species.

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(a) and acetoin (Ace, g l^{-1}) (▲), lactic acid (Lac, g l^{-1}) (△) and ethanol (Eth, g l^{-1}) (×) (b) during growth of *Klebsiella oxytoca* FMCC-197 on molasses and sucrose in fed-batch bioreactor experiments. Culture conditions: 1 vvm aeration, agitation rate from 180 to 400 rpm, pH fluctuating from 7.0 to 6.0, T=30 °C, growth on 2-l bioreactor. Each point is the mean value of two independent measurements.

Figure 3.2. (c) Representation of 2,3-butanediol and acetoin production (BDO+Ace; g l^{-1}) per total sugars consumed (TS; g l^{-1}) during fed-batch bioreactor culture of *Klebsiella oxytoca* FMCC-197 on molasses and sucrose. Culture conditions: 1 vvm aeration, agitation rate from 180 to 400 rpm, pH fluctuating from 7.0 to 6.0, T=30 °C, growth on 2-l bioreactor. Each point is the mean value of two independent measurements.

Figure 3.3. Kinetics of evolution of total sugars (TS, g l^{-1}) (▲), 2,3-butanediol (BDO, g l^{-1}) (△), biomass (X, g l^{-1}) (●) (a) and acetoin (Ace, g l^{-1}) (▲), lactic acid (Lac, g l^{-1}) (△) and ethanol (Eth, g l^{-1}) (×) (b) during growth of *Klebsiella oxytoca* FMCC-197 on molasses in fed-batch bioreactor experiments. Culture conditions: 1 vvm aeration, agitation rate from 180 to 400 rpm, pH fluctuating from 7.0 to 6.0, T=30 °C, growth on 2-l bioreactor. Each point is the mean value of two independent measurements.

Figure 3.4. Kinetics of evolution of total sugars (TS, g l^{-1}) (▲), 2,3-butanediol (BDO, g l^{-1}) (△), biomass (X, g l^{-1}) (●) (a) and acetoin (Ace, g l^{-1}) (▲), lactic acid (Lac, g l^{-1}) (△) and ethanol (Eth, g l^{-1}) (×) (b) during growth of *Klebsiella oxytoca* FMCC-197 on molasses and sucrose in fed-batch bioreactor experiments. Culture conditions: 1 vvm aeration, agitation rate from 180 to 400 rpm, pH fluctuating from 7.0 to 6.0, T=37 °C, growth on 2-l bioreactor. Each point is the mean value of two independent measurements.

Figure 3.5. Color removal during growth of *Klebsiella oxytoca* FMCC-197 on molasses under anaerobic (■) and aerobic (○) conditions. Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0, initial total sugars concentration ≈30 g l^{-1}. Anaerobic experiments were conducted in Duran bottles and aerobic experiments on 500-ml shake flasks. Each point is the mean value of two independent measurements.

Figure 3.6. Growth of *Klebsiella oxytoca* FMCC-197 on molasses under anaerobic (■) and aerobic (○) conditions. Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0, initial total sugars concentration ≈30 g l^{-1}. Anaerobic experiments were conducted in Duran bottles and aerobic experiments on 500-ml shake flasks. Each point is the mean value of two independent measurements.

Figure 3.7. (a) Dissolved oxygen tension (DOT, % v/v) and (b) Oxygen consumption (q_{O_2}, g(g.h)$^{-1}$) related with fermentation time during shake flask cultivation of *Klebsiella oxytoca* FMCC-197 on glucose (●), fructose (△) and sucrose (▲). Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0, initial glucose concentration ≈30 g l^{-1}.

Figure 3.7. (c) 2,3-butanediol (BDO, g l^{-1}) production related with fermentation time during shake flask cultivation of *Klebsiella oxytoca* FMCC-197 on glucose (●), fructose (△) and sucrose (▲). Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0, initial glucose concentration ≈30 g l^{-1}.

Figure 3.8. (a) Dissolved oxygen tension (DOT, % v/v) and (b) Oxygen consumption (q_{O_2}, $g(g.h)^{-1}$) related with fermentation time during shake flask cultivation of *Klebsiella oxytoca* FMCC-197 on sucrose using 30 (●), 60 (○), 90 (▲) and 150 (△) g l^{-1} initial concentration. Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0.

Figure 3.8. (c) 2,3-butanediol (BDO, g l^{-1}) production related with fermentation time during shake flask cultivation of *Klebsiella oxytoca* FMCC-197 on sucrose using 30 (●), 60 (○), 90 (▲) and 150 (△) g l^{-1} initial concentration. Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0.

Figure 3.9. (a) Dissolved oxygen tension (DOT, % v/v) and (b) Oxygen consumption (q_{O_2}, $g(g.h)^{-1}$) related with fermentation time during shake flask cultivation of *Klebsiella oxytoca* FMCC-197 on sucrose at 30 (●) and 37 °C (○). Culture conditions: 180 rpm agitation rate, initial pH=7.0, initial glucose concentration ≈30 g l^{-1}.

Figure 3.9. (c) 2,3-butanediol (BDO, g l^{-1}) production related with fermentation time during shake flask cultivation of *Klebsiella oxytoca* FMCC-197 on sucrose at 30 (●) and 37 °C (○). Culture conditions: 180 rpm agitation rate, initial pH=7.0, initial glucose concentration ≈30 g l^{-1}.

Figure 3.10. Kinetics of evolution of total sugars (TS, g l^{-1}) (▲), 2,3-butanediol (BDO, g l^{-1}) (△), biomass (X, g l^{-1}) (●) (a) and acetoin (Ace, g l^{-1}) (▲), lactic acid (Lac, g l^{-1}) (△) and ethanol (Eth, g l^{-1}) (×) (b) during growth of *Enterobacter* sp. FMCC-208 on molasses and sucrose in fed-batch bioreactor experiments. Culture conditions: anaerobic trial, 180 rpm agitation rate, pH fluctuating from 7.0 to 6.0, T=30 °C, growth on 2-l bioreactor. Each point is the mean value of two independent measurements.

Figure 3.11. Kinetics of evolution of total sugars (TS, g l^{-1}) (▲), 2,3-butanediol (BDO, g l^{-1}) (△), biomass (X, g l^{-1}) (●) (a) and acetoin (Ace, g l^{-1}) (▲), lactic acid (Lac, g l^{-1}) (△) succinic acid (Suc, g l^{-1}) (□) and ethanol (Eth, g l^{-1}) (×) (b) during growth of *Enterobacter* sp. FMCC-208 on molasses and sucrose in fed-batch bioreactor experiments. Culture conditions: 1 vvm aeration, agitation rate from 180 to 400 rpm, pH fluctuating from 7.0 to 6.0, T=30 °C, growth on 2-l bioreactor. Each point is the mean value of two independent measurements.

Figure 3.12. Kinetics of evolution of total sugars (TS, g l^{-1}) (▲), 2,3-butanediol (BDO, g l^{-1}) (△), biomass (X, g l^{-1}) (●) (a) and acetoin (Ace, g l^{-1}) (▲), lactic acid (Lac, g l^{-1}) (△), succinic acid (Suc, g l^{-1}) (□) and ethanol (Eth, g l^{-1}) (×) (b) during growth of *Enterobacter* sp. FMCC-208 on molasses and sucrose in fed-batch bioreactor experiments. Culture conditions: 1 vvm aeration, agitation rate from 180 to 400 rpm, pH fluctuating from 7.0 to 6.0, T=37 °C, growth on 2-l bioreactor. Each point is the mean value of two independent measurements.

Figure 3.13. Kinetics of evolution of total sugars (TS, g l^{-1}) (▲), 2,3-butanediol (BDO, g l^{-1}) (△), biomass (X, g l^{-1}) (●) (a) and acetoin (Ace, g l^{-1}) (▲), lactic acid (Lac, g l^{-1}) (△), succinic acid (Suc, g l^{-1}) (□) and ethanol (Eth, g l^{-1}) (×) (b) during growth of *Enterobacter* sp. FMCC-208 on molasses in fed-batch bioreactor experiments. Culture conditions: 1 vvm aeration, agitation rate from 180 to 400 rpm, pH fluctuating from 7.0 to 6.0, T=37 °C, growth on 2-l bioreactor. Each point is the mean value of two independent measurements.

Figure 3.14. Color removal during growth of *Enterobacter* sp. FMCC-208 on molasses under anaerobic (■) and aerobic (○) conditions. Culture conditions: *T*=30 °C, 180 rpm agitation rate, initial pH=7.0, initial total sugars concentration ≈30 g l^{-1}. Anaerobic experiments were conducted in Duran bottles and aerobic experiments on 500-ml shake flasks. Each point is the mean value of two independent measurements.

Figure 3.15. Growth of *Enterobacter* sp. FMCC-208 on molasses under anaerobic (■) and aerobic (○) conditions. Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0, initial total sugars concentration ≈30 g l^{-1}. Anaerobic experiments were conducted in Duran bottles and aerobic experiments on 500-ml shake flasks. Each point is the mean value of two independent measurements.

Figure 3.16. (a) Dissolved oxygen tension (DOT, % v/v) and (b) Oxygen consumption (q_{O_2}, $g(g.h)^{-1}$) related with fermentation time during shake flask cultivation of *Enterobacter* sp. FMCC-208 on glucose (●), fructose (△) and sucrose (▲). Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0, initial glucose concentration ≈30 g l^{-1}.

Figure 3.16. (c) 2,3-butanediol (BDO, g l^{-1}) production related with fermentation time during shake flask cultivation of *Enterobacter* sp. FMCC-208 on glucose (●), fructose (△) and sucrose (▲). Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0, initial glucose concentration ≈30 g l^{-1}.

Figure 3.17. (a) Dissolved oxygen tension (DOT, % v/v) and (b) Oxygen consumption (q_{O_2}, $g(g.h)^{-1}$) related with fermentation time during shake flask cultivation of *Enterobacter* sp. FMCC-208 on sucrose using 30 (●), 60 (○), 90 (▲) and 150 (△) g l^{-1} initial concentration. Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0.

Figure 3.17. (c) 2,3-butanediol (BDO, g l^{-1}) production related with fermentation time during shake flask cultivation of *Enterobacter* sp. FMCC-208 on sucrose using 30 (●), 60 (○), 90 (▲) and 150 (△) g l^{-1} initial concentration. Culture conditions: T=30 °C, 180 rpm agitation rate, initial pH=7.0.

Figure 3.18. (a) Dissolved oxygen tension (DOT, % v/v) and (b) Oxygen consumption (q_{O_2}, $g(g.h)^{-1}$) related with fermentation time during shake flask cultivation of *Enterobacter* sp. FMCC-208 on sucrose at 30 (●) and 37 °C (○). Culture conditions: 180 rpm agitation rate, initial pH=7.0, initial glucose concentration ≈30 g l^{-1}.

Figure 3.18. (c) 2,3-butanediol (BDO, g l^{-1}) production related with fermentation time during shake flask cultivation of *Enterobacter* sp. FMCC-208 on sucrose at 30 (●) and 37 °C (○). Culture conditions: 180 rpm agitation rate, initial pH=7.0, initial glucose concentration ≈30 g l^{-1}.

List of publications in peer reviewed journals

1. **Palaiogeorgou, A. M.**, Papanikolaou, S., de Castro, A. M., Freire, D. M. G., Kookos, I. K., & Koutinas, A. A., A newly isolated *Enterobacter* sp. strain produces 2,3-butanediol during its cultivation on low-cost carbohydrate-based substrates. *FEMS Microbiology Letters* 2019, 366:fny280.

2. Liakou, V., Pateraki, C., **Palaiogeorgou, A. M.**, Kopsahelis, N., de Castro, A. M., Freire, D. M. G., Nychas, G. J. E., Papanikolaou, S., Koutinas, A. A., Valorisation of fruit and vegetable waste from open markets for the production of 2,3-butanediol. *Food and Bioproducts Processing* 2018, 108:27–36.

Conference poster presentations

1. **Παλαιογεώργου Α.Μ.,** Λιάκου Β., Κουτίνας Α., Παπανικολάου Σ., Μελέτη της ικανότητας βιομετατροπής της σουκρόζης σε 2,3-βουτανοδιόλη από βακτηριακά στελέχη και βελτιστοποίηση της παραγωγής 2,3-βουτανοδιόλης στο στέλεχος *Klebsiella oxytoca* FMCC-197 σε ημι-συνεχείς καλλιέργειες. 6ο Συνέδριο Μικροβιόκοσμος 2015, Εθνικό Ιδρυμα Ερευνών.

2. Liakou, V., Pateraki, C., **Palaiogeorgou, A. M.**, Papanikolaou, S., Koutinas, A. A. 2,3-Butanediol production from fruit and vegetable waste streams. 11th International Conference on Renewable Resources & Biorefineries 2015, York, United Kingdom.

3. **Palaiogeorgou A. M.**, Papanikolaou S., Koutinas A., Optimization of biotechnological production or 2,3-butanediol using a newly isolated *Enterobacter* sp. strain. 13th International Conference on Renewable Resources & Biorefineries 2017, Wroclaw, Poland.

4. **Palaiogeorgou A. M.**, Liakou V., Delopoulos M. E. I., Koutinas A., Papanikolaou S., Screening of bacterial strains capable of converting sucrose into 2,3-butanediol and optimization of 2,3-butanediol production by *Klebsiella oxytoca* FMCC-197. 13th International Conference on Renewable Resources & Biorefineries 2017, Wroclaw, Poland.