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«Βιοτεχνολογική επεξεργασία υποστρωμάτων με βάση τα υγρά απόβλητα ελαιουργίας: παραγωγή προϊόντων προστιθέμενης αξίας με χρήση στελεχών των ζυμών *Yarrowia lipolytica* και *Saccharomyces cerevisiae*»

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AND HUMAN NUTRITION

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"Biotechnological treatment of olive mill wastewaters-based media: production of added-value compounds with the use of strains of yeasts *Yarrowia lipolytica* and *Saccharomyces cerevisiae*"

PhD THESIS

DIMITRIOS SARRIS

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Στους γονείς μου Γιάννη και Χρυσούλα,
στην αδερφή μου Κική.

Η «δυσκολία» δεν είναι εμπόδιο. Είναι νοοτροπία.

Ο χρόνος είναι φως…

ΕΥΧΑΡΙΣΤΙΕΣ

Το πιο «*δύσκολο*» κομμάτι αυτής της συναρπαστικής διαδρομής είναι αυτό των ευχαριστιών. Τόσα συναισθήματα σχεδόν ακατόρθωτο να εκφραστούν με λόγια και μάλιστα σε τόσο λίγο χώρο. Αξίζει όμως μια προσπάθεια… Όλο αυτόν τον καιρό, στο Εργαστήριο Μικροβιολογίας και Βιοτεχνολογίας Τροφίμων του Τμήματος Επιστήμης Τροφίμων και Διατροφής του Ανθρώπου του Γεωπονικού Πανεπιστημίου Αθηνών, συνεργάστηκα με πολλούς ανθρώπους σε άριστο κλίμα. Ο καθένας από αυτούς ξεχωριστά και όλοι μαζί συνέβαλαν στην ολοκλήρωση αυτού του έργου σε όλα τα επίπεδα. Ας με συγχωρέσει όποιος άθελά μου παραλήφθηκε.

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Ένα ιδιαίτερο ευχαριστώ από εδώ, έστω και έτσι, στη Μαρία Γαλιώτου-Παναγιώτου που δεν είναι πια κοντά μας. Μου έκανε την τιμή να με δεχθεί ως υποψήφιο διδάκτορα στην ομάδα της. Κέρδισε το σεβασμό μου με την αγάπη και την επιμονή της για αυτό που έκανε. Απέδειξε περίτρανα, όντας παράδειγμα προς μίμηση, πως δεν υπάρχει τίποτα και κανένας που μπορεί να σε σταματήσει από αυτό που ονειρεύεσαι να κάνεις.

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Δε θα ξεχάσω ποτέ και πάντοτε θα ευχαριστώ τους φοιτητές μου από το Τ.Ε.Ι. Θεσσαλίας. Κατά τη διάρκεια της διδασκαλίας μου εκεί, με έμαθαν πώς να μαθαίνω και μου υπέδειξαν έναν πολύ ωραίο τρόπο για να αντιλαμβάνομαι ακόμα πιο καθαρά την κατάσταση και από τη μεριά του «άλλου». Ιδιαίτερα ευχαριστώ στους Θοδωρή Λαμπρόπουλο και Ευθύμη Τσαλίκη. Η υποστήριξή τους σε ζόρικες στιγμές δεν ανταποδίδεται.

Οι γονείς μου με έφεραν στο φως και με ανέθρεψαν με θυσίες ουσιαστικές, βάζοντας πάντοτε το δικό τους θέλω, τις δικές τους ανάγκες, πολύ χαμηλά προκειμένου να πάρω την απαραίτητη ώθηση. Η νοοτροπία που με δίδαξε ο πατέρας μου περί αυθεντικής αξιοπρέπειας και η φράση της μάνας μου «Πλούσιος δεν είναι κείνος που έχει πολλά χρήματα, αλλά κείνος που δεν τα έχει ανάγκη», χαράζουν τη διαδρομή μου εντός και εκτός επιστήμης. Ελπίζω και εύχομαι καθημερινά να ανταποκρίνομαι στις προσδοκίες τους για να τους ανταποδώσω (πράξη που μου ζητούν να μην κάνω ποτέ) έστω στο ελάχιστο, τα όσα μου προσφέρουν. Η αδελφή μου Κική, με σπάνια ευαισθησία, με ένιωσε αληθινά και αυτό αρκούσε. Σε αυτούς αποδίδω την αφιέρωση αυτής της διατριβής.

«*Μόνο με την καρδιά βλέπεις καλά…την ουσία τα μάτια δεν την βλέπουν…*». Η Μαρίνα ήταν από τους ελάχιστους ανθρώπους, αν όχι η μοναδική, που ενώ είδε μονάχα την κορυφή του παγόβουνου, συνέλαβε αμέσως τι κρύβεται από κάτω καθώς επίσης και το πραγματικό εγχείρημα. Σιωπηλή, υπομονετική, συγκαταβατική. Ήταν πάντα, μα πάντα εκεί. Στα πιο «*δύσκολα*» και στα πιο ουσιαστικά. Με αγάπη. Δεν έχω λόγο να της πω ευχαριστώ γιατί γνωρίζει πολύ καλά πως δε χρειάζεται ποτέ να πούμε «ευχαριστώ» ο ένας στον άλλο.

Δημήτρης Σαρρής
Δεκέμβριος 2013

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ABSTRACT

The ability of two yeast species, *Yarrowia lipolytica* [strains ACA-YC 5028, ACA-YC 5033 and W29 (ATCC 20460)] and *Saccharomyces cerevisiae* (strain MAK-1), to simultaneously bioremediate (reduce phenolic content and color) olive mill wastewater (OMW)-based media and produce (high-) added value products (yeast biomass, citric acid, ethanol, cellular lipids) was assessed at the present study, which is divided in four main parts: In the first part of this work, the ability of three *Yarrowia lipolytica* strains to grow on and convert glucose-enriched OMWs into microbial mass, cellular lipids and citric acid in aseptic shake-flask cultures was assessed. Decolorization (~63%) and removal of phenolic compounds (~34% *w/w*) occurred. In nitrogen-limited cultures citric acid in non-negligible quantities was produced [maximum citric acid (Cit_{max}) ~18.1 g L^{-1}; total citric acid yield on glucose consumed ($Y_{Cit/Glc}$) ~0.51 g g^{-1}] but adaptation of cultures to media supplemented with OMWs reduced the final citric acid quantity and conversion yield values achieved. In contrast, the accumulation of cellular lipids was favored by OMWs addition compared to the control experiment (no OMWs addition). On the other hand, in carbon-limited cultures, insignificant amounts of citric acid were produced (as excpected) whereas, despite the presence of inhibitory compounds into the medium, biomass production [maximum biomass (X_{max}) ~13.0 g L^{-1}; dry cell weight yield on glucose consumed ($Y_{X/Glc}$) ~0.45 g g^{-1}] was enhanced with the addition of OMWs into the synthetic medium, as compared with the control experiment (no OMW addition). Fatty acid analysis of total cellular lipids produced demonstrated that for all strains, cultures in media supplemented with OMWs favored the biosynthesis of cellular lipids that contained increased concentrations of cellular oleic acid.

In the second part of this thesis, the ability of a selected *Y. lipolytica* strain that in the previous part had produced significant quantities of citric acid irrespective of the addition of OMWs into the medium, namely ACA-YC 5033, to grow on glucose-enriched OMWs was further studied. Higher quantities of OMWs as compared with the first part of the work were added, trials were also performed in pasteurized media (besides aseptic cultures), while equally batch bioreactor experiments were performed. Decolorization (~58%) and remarkable removal of phenolic compounds [up to 51% *w/w*, at the trial with initial phenolic compounds concentration (Ph_0) ~5.50 g L^{-1}] occurred. Such high value of phenolic compounds removal from the fermentation medium that occurred in the above-mentioned fermentation, was amongst the highest ones reported so far in the international literature concerning growth of yeasts on phenol-containing residues. In nitrogen-limited flask fermentations (in which Cit_{max}~19.0 g L^{-1}; $Y_{Cit/Glc}$~0.74 g g^{-1}), dry cell weight concentration was reduced proportionally to the phenolic content but the addition of OMWs, very

interestingly, stimulated proportionally reserve lipid accumulation process [maximum total lipid (L_{max}) ~1.0 g L^{-1}; total lipid yield in biomass ($Y_{L/X}$) ~0.27 g g^{-1}] comparing to control experiments, suggesting that OMWs seemed to be a "lipogenic" medium. The overall maximum total citric acid concentration achieved (Cit_{max}~47.0 g L^{-1}; $Y_{Cit/Glc}$~0.67 g g^{-1}) occurred in the trial with the highest commercial sugar supplementation of OMW-based media (initial glucose concentration, Glc_0, ~80.0 g L^{-1}). On the other hand, cultures performed at high phenol content media (Ph_0~4.50 and 5.50 g L^{-1}) clearly inhibited the growth of the microorganism, but surprisingly enough lipid accumulation seemed to be stimulated by the addition of OMWs at these ratios. In carbon-limited fermentations, biomass production was enhanced by OMW addition. In the aspect of a potential scale-up of the technology and in order to reduce the cost of the proposed bioprocess, shake-flask and batch bioreactor experiments were performed in a previously pasteurized medium; comparing aseptic and pasteurized shake-flasks cultures, no significant differences were observed in kinetics (for both biomass and lipid production) while the assimilation rate of glucose (in g L^{-1} h^{-1}) seemed to be linear for both experiments, with glucose consumption rate being higher in the aseptic than the in pasteurized cultures. On the contrary reduction of citric acid production was observed in the pasteurized trial by both means of Cit_{max} and $Y_{Cit/Glc}$ values. Comparing aseptic shake-flask and the respective aseptic bioreactor fermentations of OMW-based media (that presented almost equal Glc_0 and Ph_0 concentrations), biomass and lipid production were insignificantly enhanced in bioreactor trials whereas the strain reached its kinetics plateau earlier in shake-flasks than in bioreactor cultures. Glucose consumption rate was higher in the shake-flask cultures. Concerning citric acid production, it seemed to decrease in the bioreactor cultures (by both means of Cit_{max} and $Y_{Cit/Glc}$ values).

At the third part of the manuscript, the ability of *Saccharomyces cerevisiae* strain MAK-1 to grow on and convert glucose-enriched OMWs into biomass, cellular lipids and ethanol in aseptic and non-aseptic shake-flask and batch bioreactor cultures was assessed. In general, aseptic and non-aseptic processes demonstrated similar kinetic results. Decolorization (~63%) and phenol removal (~34% *w/w*) from OMWs was achieved. In aseptic shake-flask cultures, enrichment with OMWs increased ethanol and biomass production. Batch-bioreactor trials performed showed higher ethanol [maximum ethanol concentration ($EtOH_{max}$) ~52.0 g L^{-1}; ethanol yield on glucose ($Y_{EtOH/Glc}$) ~0.46 g g^{-1}] and lower biomass quantities compared with the respective shake-flask experiments. Moreover, OMWs addition in batch-bioreactor trials significantly enhanced biomass production while it did not remarkably affect ethanol biosynthesis. Fatty acid analysis of cellular lipids demonstrated that in OMW-based media, cellular lipids contained increased concentrations of oleic

and linoleic acid in accordance with the repective trials of the first part of this study when *Y. lipolytica* strains were used.

At the fourth part of this thesis, the ability of *Saccharomyces cerevisiae* strain MAK-1 to grow on and convert blends of OMWs and molasses into biomass and ethanol under non-aseptic shake-flask and batch bioreactor cultures was assessed. OMWs were used as simultaneous substrate and process water of the fermentations employed and molasses were used as low-cost substrate to supplement already existing OMWs sugar content for the enhancement of added value compounds production. The rationale of the utilization of OMW and molasses blends was to study the effect of these mixtures of residues upon the physiological and kinetic behavior of the strain, since in a potential scale-up of the process, OMWs could be used as tap water substitute for molasses dilution. This is the first time in the international literature in which such types of blends are used in a fermentation process. Decolorization (~60%) and removal of phenolic compounds (~28% *w/w*) occurred. Under aerated conditions in shake-flask cultures, adaptation of cultures to molasses media supplemented with OMWs did not significantly decrease ethanol and biomass production. Under similar aerated bioreactor cultures biomass production (X_{max}~5.7 g L^{-1}; yield of dry cell weight per total sugars consumed ($Y_{X/TS}$) ~0.07 g g^{-1}) was reduced whereas ethanol production ($EtOH_{max}$~42.0 g L^{-1}; $Y_{EtOH/TS}$~0.49 g g^{-1}) significantly increased as compared with the flask cultures. Comparing aerobic with non-aerated bioreactor experiments, biomass production showed some slight decrease whereas ethanol production slightly increased in the latter case.

The yeast strains tested in this study could be regarded as possible candidates for simultaneous OMWs remediation and production of (added-) value compounds, in some cases under completely non-aseptic conditions.

ΠΕΡΙΛΗΨΗ

Στην παρούσα μελέτη, η οποία αποτελείται από τέσσερα μέρη, διερευνήθηκε η ικανότητα δύο ειδών ζυμομυκήτων, του *Yarrowia lipolytica* [στελέχη ACA-YC 5028, ACA-YC 5033 και W29 (ATCC 20460)] και του *Saccharomyces cerevisiae* (στέλεχος MAK-1) να αυξηθούν σε υποστρώματα με βάση τα υγρά απόβλητα ελαιουργίας (ΥΑΕ) ώστε να παραχθούν πολύτιμοι μεταβολίτες (βιομάζα, αιθανόλη, κιτρικό οξύ και κυτταρικά λιπίδια) χρήσιμοι στη Βιομηχανική Βιοτεχνολογία και την Τεχνολογία Τροφίμων και ταυτοχρόνως να μειωθεί η ρυπογόνος δύναμη του αποβλήτου μέσω της μείωσης της συγκέντρωσης των φαινολικών ενώσεων αλλά και του χρώματός του.

Στο πρώτο μέρος, διερευνήθηκε η ικανότητα τριών στελεχών της ζύμης *Y. lipolytica* να αυξηθούν σε υποστρώματα με βάση τα ΥΑΕ υπό ασηπτικές συνθήκες σε ανακινούμενες φιάλες, ώστε να παραχθεί βιομάζα, κιτρικό οξύ και κυτταρικά λιπίδια υπό συνθήκες περιοριστικές σε άζωτο [αρχική συγκέντρωσης γλυκόζης (Glc_0) ~35.0 g L^{-1}, αρχικός λόγος άνθρακα/αζώτου (C/N) ~85] και υπό συνθήκες περιοριστικές σε άνθρακα (Glc_0~28.0 g L^{-1}, C/N~13). Έγινε προσθήκη ΥΑΕ στο συνθετικό μέσο σε διάφορες αναλογίες ώστε η αρχική συγκέντρωση φαινολικών ενώσεων (Ph_0) να είναι (σε g L^{-1}): 0.00 (πείραμα αναφοράς χωρίς προσθήκη ΥΑΕ στο συνθετικό μέσο), ~1.15 και ~1.55. Σημειώθηκε αποχρωματισμός (εύρος μέγιστων τιμών 45-63%) και μείωση των φαινολικών ενώσεων (εύρος μέγιστων τιμών 13-34% *w/w*) στο μέσο τόσο στις ζυμώσεις υπό συνθήκες περιοριστικές σε άζωτο όσο και στις ζυμώσεις υπό συνθήκες περιοριστικές σε άνθρακα. Το ποσοστό μείωσης του χρώματος και μείωσης των φαινολικών ενώσεων του μέσου έδειξε να αυξάνεται με την προσθήκη ΥΑΕ (εξαιρώντας το στέλεχος ACA-YC 5033). Στις καλλιέργειες υπό συνθήκες περιοριστικές σε άζωτο παράχθηκε σε ικανοποιητικές ποσότητες συνολικό κιτρικό οξύ [π.χ. μέγιστη συγκέντρωση κιτρικού οξέος (Cit_{max}) ~18.1 g L^{-1}, με αντίστοιχο συντελεστή απόδοσης κιτρικού οξέος προς αναλωθείσα γλυκόζη ($Y_{Cit/Glc}$) ~0.51 g g^{-1} για το στέλεχος *Y. lipolytica* ACA-YC 5033 σε υποστρώματα εμπλουτισμένα με ΥΑΕ]. Το ισοκιτρικό οξύ παρουσίασε μια ποσότητα της τάξης του 5 – 8% *w/w* του συνολικού κιτρικού οξέος που παράχθηκε, ανεξαρτήτα από το στέλεχος που χρησιμοποιήθηκε. Η προσθήκη ΥΑΕ οδήγησε σε σοβαρή μείωση της παραγωγής του κιτρικου οξέος (τόσο σε απόλυτες τιμές συγκέντρωσης όσο και σε τιμές του συντελεστή απόδοσης) ειδικά για το στέλεχος W29. Αντίθετα, η παραγωγή των κυτταρικών λιπιδιών ευνοήθηκε με την προσθήκηΥΑΕ συγκρίνοντας με το πείραμα αναφοράς (χωρίς προσθήκη ΥΑΕ) κυρίως για το στέλεχος W29 [μέγιστη συγκέντρωση ολικού λίπους (L_{max}) ~1.9 g L^{-1}, λίπος επί ξηρής μάζας ($Y_{L/X}$) ~0.28 g g^{-1}], υποδεικνύοντας πως η προσθήκη ΥΑΕ ευνόησε τη διαδικασία συσσώρευσης λίπους. Αξίζει να σημειωθεί πως στις περισσότερες ζυμώσεις υπό συνθήκες περιοριστικές σε άζωτο, παρουσιάστηκε μερική μείωση της συγκέντρωσης του

παραγόμενου λίπους (υπό την έννοια τόσο των τιμών L_{max} όσο και των τιμών $Y_{L/X}$) στην αρχή της στατικής φάσης αύξησης του μικροοργανισμού, ακριβώς πριν ξεκινήσει σε αυξημένες συγκεντρώσεις η έκκριση κιτρικού οξέος στο μέσο. Στις ζυμώσεις υπό συνθήκες περιοριστικές σε άνθρακα δεν παράχθηκε κιτρικό οξύ (όπως άλλωστε αναμενόταν) και παρά την ύπαρξη παρεμποδιστικών παραγόντων (π.χ. φαινολικών συστατικών), η παραγωγή της βιομάζας [μέγιστη συγκέντρωση βιομάζας (X_{max}) ~13.0 g L^{-1}, συντελεστής απόδοσης βιομάζας ($Y_{X/Glc}$) ~0.45 g g^{-1}] ενισχύθηκε με την προσθήκη YAE στο συνθετικό μέσο. Υπήρξε σημαντικά υψηλότερη παραγωγή βιομάζας στις ζυμώσεις υπό συνθήκες περιοριστικές σε άνθρακα από ότι στις ζυμώσεις υπό συνθήκες περιοριστικές σε άζωτο όπως επίσης και ο ρυθμός κατανάλωσης της γλυκόζης ήταν μεγαλύτερος στις ζυμώσεις υπό συνθήκες περιοριστικές σε άνθρακα. Αναφορικά με την παραγωγή ολικού λίπους, στις περισσότερες ζυμώσεις υπό συνθήκες περιοριστικές σε άνθρακα το λίπος επί ξηρής μάζας μετρήθηκε στο εύρος 5-12% *w/w* επί ξηρής ουσίας, υποδεικνύοντας πως δεν υπήρξε αξιοσημείωτη συσσώρευση κυτταρικων λιπιδίων υπό αυτές τις συνθήκες. Η ανάλυση των λιπαρών οξέων των κυτταρικών λιπιδίων που παράχθηκαν, έδειξε ότι η προσαρμογή όλων των στελεχών της ζύμης στα υποστρώματα με βάση τα YAE έστρεψε το μεταβολισμό προς τη σύνθεση κυτταρικών λιπιδίων που περιείχαν αυξημένες συγκεντρώσεις κυτταρικού ελαϊκού οξέος.

Δεδομένης της δυναμικής του στελέχους ACA-YC 5033 να παράγει σε υψηλές ποσότητες κιτρικό οξύ ανεξάρτητα από την προσθήκη YAE στο συνθετικό μέσο, στο δεύτερο μέρος της διατριβής, μελετήθηκε η ικανότητα του στελέχους αυτού να αυξηθεί σε υποστρώματα με βάση τα YAE (επιπλέον σε ανακινούμενες φιάλες υπό συνθήκες παστερίωσης και σε βιοαντιδραστήρα υπό συνθήκες ασηπτικές αλλά και υπό συνθήκες παστερίωσης) σε υψηλές αρχικές συγκεντρώσεις YAE (και συνεπώς φαινολικών συστατικών) στο μέσο της καλλιέργειας. Σημειώθηκε αποχρωματισμός (~58%) και αξιοσημείωτη μείωση των φαινολικών ενώσεων [~51% *w/w*, τιμή που σημειώθηκε ανέλπιστα στο πείραμα με αρχική συγκέντρωση φαινολικών ενώσεων (Ph_0) ~5.50 g L^{-1}]. Η τόσο υψηλή τιμή μείωσης των φαινολικών ενώσεων του μέσου ζύμωσης που σημειώθηκε, ανήκει στις υψηλότερες τιμές που έχουν αναφερθεί ως τώρα στη διεθνή βιβλιογραφία αναφορικά με την αύξηση ζυμών σε υπολείμματα που περιέχουν φαινολικές ενώσεις.

Στις καλλιέργειες υπό συνθήκες περιοριστικές σε άζωτο (στις οποίες Cit_{max}~19.0 g L^{-1}, $Y_{Cit/Glc}$~0.74 g g^{-1}), η συγκέντρωση της X_{max} μειώθηκε αναλογικά με την περιεκτικότητα σε φαινόλες, με την προσθήκη YAE στο μέσο. Αξιοσημείωτο είναι πως η προσθήκη YAE ενεργοποίησε τη διαδικασία συσσώρευσης λιπιδίων (L_{max}~1.0 g L^{-1}, $Y_{L/X}$~0.27 g g^{-1}) εν συγκρίσει με το πείραμα αναφοράς, υποδεικνύοντας πως τα YAE δείχνουν να αποτελούν ένα «ελαιοσυσσωρευτικό» υπόστρωμα. Η υψηλότερη συγκέντρωση κιτρικού οξέος που σημειώθηκε σε όλη τη μελέτη (Cit_{max}~47.0 g L^{-1}, $Y_{Cit/Glc}$~0.67 g g^{-1}), προέκυψε στη ζύμωση με την υψηλότερη

προσθήκη εμπορικής γλυκόζης (Glc_0~80.0 g L^{-1}). Στα πειράματα με την υψηλότερη συγκέντρωση φαινολικών ενώσεων (Ph_0~4.50 and ~5.50 g L^{-1}) παρουσιάστηκε σημαντική αναστολή της αύξησης του μικροοργανισμού αλλά η παραγωγή των κυτταρικών λιπιδίων ενεργοποιήθηκε με την προσθήκη YAE σε αυτές τις αναλογίες. Σε συνθήκες περιοριστικές σε άνθρακα, η παραγωγή βιομάζας ενισχύθηκε από την προσθήκη YAE. Συγκρίνοντας τις καλλιέργειες σε ανακινούμενες φιάλες υπό ασηπτικές συνθήκες και συνθήκες παστερίωσης σημειώθηκαν μη αξιοσημείωτες αλλαγές όσον αφορά στην κινητική του μικροοργανισμού (τόσο στην παραγωγή βιομάζας οσο και στην παραγωγή ολικού λίπους) ενώ ο ρυθμός κατανάλωσης της γλυκόζης ήταν γραμμικός και στις δύο περιπτώσεις με υψηλότερο αυτόν στις ζυμώσεις υπό ασηπτικές συνθήκες από ότι στις ζυμώσεις υπό συνθήκες παστερίωσης. Αντίθετα, παρατηρήθηκε μείωση της παραγωγής κιτρικού οξέος στις ζυμώσεις υπό συνθήκες παστερίωσης υπό την έννοια τόσο των απόλυτων τιμών Cit_{max} όσο και των σχετικών τιμών $Y_{Cit/Glc}$. Συγκρίνοντας τα πειράματα σε ανακινούμενες φιάλες υπό ασηπτικές συνθήκες με τα αντίστοιχα πειράματα σε βιοαντιδραστήρα (που παρουσίασαν παρόμοιες συγκεντρώσεις Glc_0 και Ph_0), παρατηρήθηκε μη-αξιοσημείωτη βελτίωση στην παραγωγή βιομάζας και λιπιδιών ενώ το στέλεχος παρουσίασε νωρίτερα τη στατική του φάση στις φιάλες από ότι στο βιοαντιδραστήρα. Ο ρυθμός κατανάλωσης της γλυκόζης ήταν υψηλότερος στις ζυμώσεις σε φιάλες. Η παραγωγή κιτρικού οξέος μειώθηκε στις ζυμώσεις σε βιοαντιδραστήρα (υπό την έννοια τόσο των τιμών Cit_{max} όσο και των τιμών $Y_{Cit/Glc}$).

Στο τρίτο μέρος, διερευνήθηκε η ικανότητα της ζύμης *S. cerevisiae* στέλεχος MAK-1 να αυξηθεί σε υποστρώματα με βάση τα YAE ώστε να παραχθεί βιομάζα, αιθανόλη και κυτταρικά λιπίδια σε ανακινούμενες φιάλες και σε βιοαντιδραστήρα υπό ασηπτικές αλλά και μη ασηπτικές συνθήκες (σε όλες τις περιπτώσεις η σύγκριση ασηπτικών και μη ασηπτικών συνθηκών παρουσίασε παρόμοια κινητική). Στις ζυμώσεις σε ανακινούμενες φιάλες υπό ασηπτικές αλλά και μη ασηπτικές συνθήκες, έγινε προσθήκη YAE στο συνθετικό μέσο σε διάφορες αναλογίες ώστε η Ph_0 να είναι (σε g L^{-1}): 0.00 (πείραμα αναφοράς χωρίς προσθήκη YAE στο συνθετικό μέσο), ~1.20, ~2.00 και~2.90. Για κάθε Ph_0 προστέθηκε στο μέσο ποσότητα γλυκόζης ώστε η αρχική συγκέντρωσή αυτής να είναι (σε g L^{-1}): ~40.0, ~55.0 και ~75.0 . Τόσο η μέγιστη συγκέντρωση αρχικής γλυκόζης που επιλέχθηκε, όσο και οι αρχικές συγκεντρώσεις φαινολικών ενώσεων, αναφέρονται στις αντίστοιχες συγκεντρώσεις που μπορεί να βρεθούν σε τυπικά YAE παραγόμενα από διάφορες μεθόδους πίεσης του ελαιοκάρπου. Στις καλλιέργειες με ανακινούμενες φιάλες υπό ασηπτικές συνθήκες, η προσθήκη YAE αύξησε θεαματικά την παραγωγή βιομάζας παρά το γεγονός ότι, όπως αναφέρθηκε, τα YAE περιέχουν ανασταλτικούς παράγοντες. Η υψηλότερη τιμή X_{max} που σημειώθηκε ήταν ~19.0 g L^{-1} στο πείραμα με Glc_0~75.0 g L^{-1} και Ph_0~2.90 g L^{-1} (ενώ X_{max}~11.0 g L^{-1} σημειώθηκε στο αντίστοιχο πείραμα αναφοράς). Επίσης, όπως αναμενόταν, με την

αύξηση της αρχικής συγκέντρωσης γλυκόζης στο μέσο, παρατηρήθηκε και αύξηση της παραγωγής της βιομάζας. Σημαντικό σημείο αποτελεί το γεγονός ότι μετά την ολική κατανάλωση της γλυκόζης του μέσου, η αιθανόλη που είχε παραχθεί άρχισε να καταναλώνεται προς νέα παραγωγή βιομάζας και σε αρκετές περιπτώσεις δε, παρατηρούταν η ανακατανάλωση όλης της ποσότητας της προηγούμενης συντιθέμενης αιθανόλης. Η μέγιστη συγκέντρωση αιθανόλης ($EtOH_{max}$) που παράχθηκε στις ζυμώσεις σε ανακινούμενες φιάλες ήταν ~26.0 g L^{-1} με συντελεστή απόδοσης αιθανόλης ($Y_{EtOH/Glc}$) ~0.36 g g^{-1} στο πείραμα με Glc_0~75.0 g L^{-1} και Ph_0~2.90 g L^{-1} ενώ ο υψηλότερος συντελεστής απόδοσης αιθανόλης ήταν ~0.47 g g^{-1} (με αντίστοιχη μέγιστη συγκέντρωση αιθανόλης ~17.0 g L^{-1}) στο πείραμα με Glc_0~40.0 g L^{-1} και Ph_0~2.00 g L^{-1}. Η προσθήκη YAE στο συνθετικό μέσο έως μία συγκεκριμένη αναλογία ευνόησε την παραγωγή της αιθανόλης. Λαμβάνοντας υπ'όψιν το συντελεστή απόδοσης αιθανόλης, σημειώνεται πως σημαντικά υψηλότερη τιμή αυτού παρατηρήθηκε στη ζύμωση με Ph_0~2.0 g L^{-1}, συνθήκες οι οποίες επιλέχθηκαν για προσαρμογή της πειραματικής διαδικασίας σε ζυμώσεις σε βιοαντιδραστήρα. Συγκρίνοντας τις ζυμώσεις σε φιάλες υπό ασηπτικές συνθήκες (Glc_0~40.0 g L^{-1}, Ph_0~0.00 g L^{-1} και Glc_0~75.0 g L^{-1}, Ph_0~2.90 g L^{-1}) με τις αντίστοιχες υπό μη ασηπτικές συνθήκες, σημειώνεται πως δεν παρατηρήθηκε διαφορά τόσο στην παραγωγή βιομάζας όσο και στην παραγωγή αιθανόλης. Αναφορικά με τις ζυμώσεις που πραγματοποιήθηκαν σε βιοαντιδραστήρα έγινε σύγκριση πειράματος αναφοράς (ζύμωση χωρίς προσθήκη YAE) υπό ασηπτικές και υπό μη ασηπτικές συνθήκες χωρίς να παρατηρηθεί αξιοσημείωτη διαφορά στην κινητική του μικροοργανισμού. Ακολούθως, μελετήθηκε η επίδραση του αερισμού (0.0, 0.5, 1.0 και 1.5 vvm) της καλλιέργειας και δεν παρατηρήθηκε αξιοσημείωτη διαφορά στην κινητική. Λόγω των παραπάνω παρατηρήσεων επιλέχθηκαν (για λόγους μείωσης κόστους) οι μη-ασηπτικές συνθήκες χωρίς αερισμό για τη συνέχεια των πειραμάτων με την προσθήκη στο μέσο YAE, γεγονός το οποίο οδήγησε σε αξιοσημείωτη αύξηση της βιομάζας χωρίς όμως να επηρεάσει τη βιοσύνθεση της αιθανόλης. Σημειώνεται επίσης πως, οι ζυμώσεις που πραγματοποιήθηκαν σε βιοαντιδραστήρα παρουσίασαν υψηλότερες ποσότητες αιθανόλης και χαμηλότερες βιομάζας εν συγκρίσει με τα αντίστοιχα πειράματα των ανακινούμενων φιαλών. Τέλος, προκειμένου να αυξηθεί η συγκέντρωση της παραγόμενης αιθανόλης πραγματοποιήθηκε ζύμωση σε βιοαντιδραστήρα υπό μη ασηπτικές συνθήκες χωρίς αερισμό με αρχική συγκέντρωση γλυκόζης ~115.0 g L^{-1} και αρχική συγκέντρωση φαινολικών ενώσεων ~2.90 g L^{-1} με $EtOH_{max}$~52.0 g L^{-1} ($Y_{EtOH/Glc}$~0.46 g g^{-1}). Σημειώθηκε αξιοσημείωτος αποχρωματισμός (~63%) και μείωση των φαινολικών ενώσεων (~34% *w/w*) στο μέσο. Για μια δεδομένη αρχική συγκέντρωση γλυκόζης, αναφορικά με τις ζυμώσεις σε ανακινούμενες φιάλες, το ποσοστό μείωσης του χρώματος και μείωσης των φαινολικών ενώσεων του μέσου αυξήθηκε με την προσθήκη YAE. Συγκρίνοντας τις τιμές αποχρωματισμού και μείωσης

φαινολικών ενώσεων στα πειράματα σε φιάλες υπό μη ασηπτικές συνθήκες με αυτές των αντίστοιχων ζυμώσεων σε βιοαντιδραστήρα υπό μη ασηπτικές συνθήκες, συμπεραίνεται ότι ήταν παρόμοιες. Η ανάλυση των λιπαρών οξέων των κυτταρικών λιπιδίων (παράχθηκαν σε ποσότητα μικρότερη του 10% *w/w* επί ξηρής ουσίας), έδειξε ότι στα υποστρώματα με βάση τα ΥΑΕ τα κυτταρικά λιπίδια περιείχαν αυξημένες συγκεντρώσεις ελαϊκού και λινελαϊκού οξέος, κάτι που βρίσκεται σε συμφωνία με τα αντίστοιχα πειράματα του πρώτου μέρους αυτής της μελέτης όπου μελετήθηκαν τα κυτταρικά λιπίδια του μικροοργανισμού *Y. lipolytica*.

Στο τέταρτο μέρος, ερευνήθηκε η ικανότητα της ζύμης *S. cerevisiae* στέλεχος MAK-1 να αυξηθεί σε μίγματα ΥΑΕ και μελάσας, ώστε να παραχθεί βιομάζα και αιθανόλη σε ανακινούμενες φιάλες και βιοαντιδραστήρα υπό αερόβιες και αναερόβιες, μη ασηπτικές συνθήκες. Τα ΥΑΕ χρησιμοποιήθηκαν ταυτοχρόνως τόσο ως υπόστρωμα της ζύμωσης όσο και ως νερό της διεργασίας και η μελάσα ως χαμηλού κόστους υπόστρωμα για τον εμπλουτισμό των ήδη υπαρχόντων σακχάρων των ΥΑΕ προς την ενίσχυση της παραγωγής προϊόντων προστιθέμενης αξίας. Η λογική της χρήσης μιγμάτων ΥΑΕ και μελάσας ήταν η μελέτη της επίδρασης των μιγμάτων αυτών στη φυσιολογία και την συμπεριφορά της κινητικής του στελέχους, καθώς σε ενδεχόμενη κλιμάκωση μεγέθους των ζυμώσεων, τα ΥΑΕ θα μπορούσαν να αντικαταστήσουν το νερό που χρησιμοποιείται για την αραίωση της μελάσας στη διαδικασία βιοτεχνολογικής επεξεργασίας και αξιοποίησής της. Πραγματοποιήθηκαν προκαταρτικά πειράματα σε υποστρώματα μελάσας αραιωμένα με νερό [αρχική συγκέντρωση ολικών σακχάρων (TS_0) ~100.0 g L^{-1}, χωρίς την προσθήκη ΥΑΕ] υπό αερόβιες ασηπτικές συνθήκες σε ανακινούμενες φιάλες για να αξιολογηθεί η προσθήκη διαλύματος μεταλλικών αλάτων (η σύσταση της μελάσας περιλαμβάνει μη αμελητέα ποσότητα μεταλλικών στοιχείων). Η προσθήκη μεταλλικών αλάτων στο μέσο επηρέασε αρνητικά την παραγωγή της βιομάζας και της αιθανόλης, επομένως η πρακτική αυτή εξαιρέθηκε από τη συνέχεια των πειραμάτων. Κατόπιν, ζυμώσεις με μίγματα μελάσας και ΥΑΕ (προσθήκη 10% *v/v*) σε ανακινούμενες φιάλες υπό ασηπτικές συνθήκες συγκρίθηκαν με αντίστοιχες καλλιέργειες υπό μη ασηπτικές συνθήκες. Η συγκέντρωση της παραγόμενης αιθανόλης ήταν ελαφρώς υψηλότερη και η συγκέντρωση της βιομάζας χαμηλότερη στις ζυμώσεις υπό ασηπτικές συνθήκες. Τα προαναφερθέντα πειράματα υπό μη ασηπτικές συνθήκες θεωρήθηκαν ικανοποιητικά γεγονός το οποίο οδήγησε στην εφαρμογή μη ασηπτικών συνθηκών στις υπόλοιπες ζυμώσεις. Στη συνέχεια εκτελέστηκαν πειράματα σε ανακινούμενες φιάλες υπό μη ασηπτικές συνθήκες με μίγματα μελάσας και ΥΑΕ. Η αρχική συγκέντρωση ολικών σακχάρων ήταν ~100.0 g L^{-1} και η προσθήκη των ΥΑΕ έγινε στις εξής αναλογίες (% *v/v*): 0, 10, 20, 30, 40, 50. Η παραγωγή της βιομάζας δεν επηρεάστηκε σοβαρά από την προσθήκη ΥΑΕ στο μέσο, αλλά οι μέγιστες τιμές X_{max} και του συντελεστή απόδοσης της παραγόμενης βιομάζας προς αναλωθέντα συνολικά σάκχαρα $Y_{X/TS}$

παράχθηκαν στην καλλιέργεια χωρίς προσθήκη YAE. Η ποσότητα των ολικών σακχάρων που έμεινε ακατανάλωτη αυξήθηκε με την προσθήκη YAE. Αναφορικά με τη βιοσύνθεση της αιθανόλης, η προσθήκη YAE στο μίγμα επηρέασε αρνητικά την παραγωγή της εν συγκρίσει με το πείραμα χωρίς την προσθήκη YAE (μόνο μελάσα). Η προσθήκη YAE σε αναλογία 20% *v/v* έδωσε ικανοποιητικά αποτελέσματα – αναφορικά με τη βιοσύνθεση τόσο βιομάζας όσο και αιθανόλης – οπότε και επιλέχθηκε ως συνθήκη για εφαρμογή σε ζυμώσεις σε βιοαντιδραστήρες υπό μη ασηπτικές συνθήκες. Αρχικά μελετήθηκε η επιρρόη του αερισμού (0.0 και 1.2 vvm) της καλλιέργειας. Παρατηρήθηκε μη αξιοσημείωτη ελάττωση στην παραγωγή της βιομάζας αλλά και αύξηση στη βιοσύνθεση της αιθανόλης στις καλλιέργειες υπό μη αερισμό. Συγκρινόμενες και οι δύο προαναφερθείσες συνθήκες με το αντίστοιχο πείραμα στις ανακινούμενες φιάλες, σημειώνεται πως η παραγωγή της βιομάζας μειώθηκε εμφανώς και η παραγωγή της αιθανόλης αυξήθηκε στις ζυμώσεις που πραγματοποιήθηκαν στο βιοαντιδραστήρα. Επίσης στις καλλιέργειες που πραγματοποιήθηκαν στις φιάλες, μετά την κατανάλωση όλης της ποσότητας των ολικών σακχάρων του μέσου, η αιθανόλη που είχε παραχθεί άρχισε να καταναλώνεται προς νέα παραγωγή βιομάζας. Λαμβάνοντας υπ' όψιν πως στις καλλιέργειες που πραγματοποιήθηκαν στο βιοαντιδραστήρα υπό αναερόβιες, μη ασηπτικές συνθήκες παράχθηκε ελαφρώς υψηλότερη ποσοτητα αιθανόλης, η σειρά πειραμάτων που ακολούθησε πραγματοποιήθηκε χωρίς αερισμό. Επομένως μελετήθηκαν ζυμώσεις στο βιοαντιδραστήρα υπό μη ασηπτικές συνθήκες χωρίς αερισμό με προσθήκη YAE σε σταθερή αναλογία (20% *v/v*) και αυξανόμενη συγκέντρωση ολικών σακχάρων (μέσω της προσθήκης αυξανόμενης ποσότητας μελάσας) σε (g L^{-1}): ~100.0, ~135.0, ~150.0, ~200.0. Η αύξηση της ποσότητας της μελάσας επηρέασε αρνητικά την αύξηση του μικροοργανισμού και την αφομοίωση των σακχάρων με αποτέλεσμα στο πείραμα με TS_0~200.0 g L^{-1} να μην παρατηρηθεί καθόλου αύξηση του μικροοργανισμού. Αναφορικά με τη βιοσύνθεση της αιθανόλης, η προσθήκη μελάσας στο μέσο ευνόησε την παραγωγή της αναλογικά έως την καλλιέργεια με TS_0~135.0 g L^{-1}. Πέραν αυτής της συγκέντρωσης η παραγωγή της αιθανόλης επηρεάστηκε αρνητικά (π.χ. καθόλου αιθανόλη δεν παράχθηκε στο πείραμα με TS_0~200.0 g L^{-1}). Σημειώθηκε αποχρωματισμός (εύρος μέγιστων τιμών 28-60%) και μείωση των φαινολικών ενώσεων (εύρος μέγιστων τιμών 12-26% *w/w*). Συγκρίνοντας τις τιμές των ποσοστών αποχρωματισμού και μείωσης φαινολικών ενώσεων στα πειράματα σε φιάλες υπό μη ασηπτικές συνθήκες με αυτές των αντίστοιχων ζυμώσεων σε βιοαντιδραστήρα υπό μη ασηπτικές συνθήκες, συμπεραίνεται ότι ήταν παρόμοιες.

Τα στελέχη των ζυμομυκήτων που μελετήθηκαν, μπορούν να θεωρηθούν ως υποψήφιοι μικροοργανισμοί για την ταυτόχρονη απορρύπανση των YAE και την παραγωγή προϊόντων υψηλής προστιθέμενης αξίας χρήσιμων στη βιομηχανία των τροφίμων, σε ορισμένες περιπτώσεις υπό μη ασηπτικές συνθήκες.

1. INTRODUCTION

1.1. Olive Mill Wastewaters (OMWs)

Olive mill wastewaters (OMW) are the principal waste stream that derives from the olive fruit processing by mechanical means, for olive oil production. These residues are considered to be one of the strongest and most difficult to treat agro-industrial effluents (Lanciotti *et al.*, 2005). The overall annual production of OMWs is estimated to be over $3x10^7$ m^3 (Sayadi and Ellouz, 1995; Benitez *et al.*, 1997; Mantzavinos and Kalogerakis, 2005; Massadeh and Modallal, 2008). In Greece, the production of these residues is estimated to be up to $1.5x10^6$ m^3 (Tsioulpas *et al.*, 2002). In these wastewaters, biological oxygen demand (BOD) and chemical oxygen demand (COD) concentration values can be 200-400 times higher than in typical municipal sewage (Lanciotti *et al.*, 2005; Di Serio *et al.*, 2008). The aesthetic degradation due to their strong odor and dark color as also their high organic load have a direct impact on the environment. On the other hand, an indeed major problem that renders as as very difficult task the treatment of OMWs refers to the fact that in many Mediterranean countries including Greece, OMWs are produced in large quantities in a quite short period of time with the production of olive oil (and, thus, the generation of OMWs) being performed in scattered low-size facilities (Arvanitoyiannis and Kassaveti, 2008).

1.1.1. Volume and composition of OMWs

The olive oil industry is characterized by its great environmental impact due to the production of a highly polluted wastewater and/or solid residue, olive skin and stone (olive husk). As mentioned above, the overall annual production of OMWs is estimated to be over 3×10^7 m^3 (Mantzavinos and Kalogerakis, 2005; Massadeh and Modallal, 2008). In Greece, the production of these residues is estimated to be up to $1.5x10^6$ m^3 (Tsioulpas *et al.*, 2002). The yield in olive oil and in wastes from olive oil industries depending on the production process, is depicted in Table 1.1.. The OMW is generally characterized by various special features, such as intensive violet-dark brown up to black color, strong specific olive oil smell, high degree of organic pollution, pH between 3 and 6 (slightly acid), high electrical conductivity, high content of polyphenols (0.5 – 24.0 g L^{-1}) and high content of solid matter (Μπαλατσούρας, 1999; Niaounakis and Halvadakis, 2006).

The volume and composition of OMWs depends on the olive fruit variety, the maturity of the olives, the olive's water content, the soil, the harvesting time, the presence of pesticides and fertilizers, the climatic conditions during growth, the storage time before processing, the conditions and the technology implicated for the extraction of oil, the availability of water in the industry (as in some regions as various Greek islands, in Spain and Northern Africa there is lack in water) and the special treatment of raw material in each industry (Bambalov *et al.*, 1989; Hamdi,1993;

Μπαλατσούρας, 1999; Crognale *et al.*, 2006; Niaounakis and Halvadakis, 2006; Amaral *et al.*, 2008; Di Serio *et al.*, 2008).

Table 1. 1. Inputs and outputs from olive oil industry

Production process	Inputs	Outputs
Traditional process (pressing)	Olives (1 ton)	Oil (200 kg)
	Wash water (0.10-0.12 m^3)	Solid waste (400 kg)
	Energy (40-63 kWh)	Wastewater (600 kg)
Three-phase process	Olives (1 ton)	Oil (200 kg)
	Wash water (0.10-0.12 m^3)	Solid waste (500-600 kg)
	Fresh water for decanter (0.50-1.0 m^3)	Wastewater (800-950 kg)
	Water to polish the impure oil (10 kg)	-
	Energy (<90-117 kWh)	-
Two-phase process	Olives (1 ton)	Oil (200 kg)
	Wash water (0.10-0.12 m^3)	Solid waste (500-600 kg)
	Energy (<90-117 kWh)	Wastewater (800-950 kg)

(Arvanitoyiannis and Kassaveti, 2008)

The OMW organic fraction is principally composed by sugars, cellulose and pectin, (poly-) phenolic compounds, simple phenolic acids, phenolic alcohols, polyalcohols, various amino acids, proteins, organic acids and residual oil (Lanciotti *et al.*, 2005; Mantzavinos and Kalogerakis, 2005; Crognale *et al.*, 2006; Amaral *et al.*, 2008). Regarding the mineral fraction, all minerals are presented in various fresh OMW samples (Hamdi, 1993; Sayadi and Ellouz, 1995; Benitez *et al.* 1997; Mantzavinos and Kalogerakis, 2004; Ayed *et al.*, 2005; Di Serio *et al.*, 2008). In some cases, OMWs deriving from press extraction systems, besides phenolic compounds, contain reducing carbohydrates (principally glucose) in very high quantities (i.e. >70 g L^{-1}) (Crognale *et al.*, 2006) that also pose significant problems related with their treatment. Likewise, Amaral *et al.* (2008)

proposed that besides the aforementioned reasons, O.M.Ws toxicity may be attributed to low pH, and high solids and fat content. Phenolic and fatty compounds, included in O.M.Ws, may inhibit the growth of several types of microorganisms and stop conventional secondary and anaerobic treatment in municipal treatment plants (Amaral *et al.*, 2008). Such compounds cannot be biodegraded easily (Tsioulpas *et al.*, 2002; Aggelis *et al.*, 2003; Lanciotti *et al.*, 2005; Paraskeva and Diamadopoulos, 2006; Crognale *et al.*, 2006; Di Serio *et al.*, 2008; Papanikolaou *et al.*, 2008a). The characteristic parameters and composition of OMW are presented in Table 1.2.. The OMW dark color and phytotoxic and antimicrobial effects have been attributed to the phenolic compounds (presence of polymeric phenols that display a lignin-like structure and constitute most resistant fraction of OMW) that are found in various concentrations in the residue (Sayadi and Ellouz, 1992; Kissi *et al.*, 2001; Tsioulpas *et al.*, 2002; Aggelis *et al.*, 2003; Ahmadi *et al.*, 2006; D'Annibale *et al.*, 2006; Amaral *et al.*, 2008; Dermeche *et al.*, 2013).

Phenolic compounds include many organic substances having as common characteristic an aromatic ring with one or more substituted hydroxyl groups and a functional side-chain. Natural phenolic compounds include simple molecules, such as phenolic acids and highly polymerized compounds such as tannins. The most common forms of phenolic compounds are conjugated with various sugar molecules (mono-, di-, or oligosaccharides), organic acids and lipids or even with other phenols linked to hydroxyl groups or (less frequently) to aromatic carbon atoms. Phenolic compounds are quantitatively and qualitatively abundant in OMWs including various phenolic acids (cinnamic acid, p-coumaric acid, caffeic acid, ferulic acid, vanillic acid, gallic acid, syringic acid, sinapic acid, homovallinic acid, 4-hydroxyphenyl acetic acid), secoiridoids and derivatives (oleuropein, demethyloleuropein, verbascoside, ligstroside, tyrosol, hydroxytyrosol, 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde) and flavonoids (luteolin, luteolin 7-O-glucoside, luteolin 7-O-glucoside, luteolin 4'-O-glucoside, rutin, quercetin). During the olive oil extraction process the major fraction of these compounds shifts from olive pulp to OMWs. This phenomenon is the main obstacle to the biological detoxification of OMWs. The variability in the phenolic contents of OMWs from various origins complicates significantly their treatments by bioconversion because of the fact that some consortia of microorganisms that can efficiently treat some OMW can be inhibited by one another. It should be stressed out that the valorization of some phenolic compounds appears to be relatively uncertain and not economically viable considering the low concentration of specific phenolic compounds in OMWs and the cost and complexity of purification techniques (Dermeche *et al.* 2013).

Table 1. 2. Characteristic parameters (average results) of fresh OMW samples obtained from pressure olive mills and 3-phase centrifugation systems

Parameter	Pressure system	3-Phase centrifugation system
pH	5.27	5.23
Dry matter (g L^{-1})	129.7	61.1
Specific weight	1.049	1.020
Oil (g L^{-1})	2.26	5.78
Reducing sugars (g L^{-1})	35.8	15.9
Total phenols (g L^{-1})	6.2	2.7
o-Diphenols (g L^{-1})	4.8	2.0
Hydroxytyrosol (mg L^{-1})	353	127
Precipitate with alcohol (g L^{-1})	30.4	24.6
Ash (g L^{-1})	20	6.4
COD (g O_2 L^{-1})	146.0	85.7
Organic nitrogen (mg L^{-1})	544	404
Total phosphorus (mg L^{-1})	485	185
Sodium (mg L^{-1})	110	36
Potassium (mg L^{-1})	2470	950
Calcium (mg L^{-1})	162	69
Magnesium (mg L^{-1})	194	90
Iron (mg L^{-1})	32.9	14.0
Copper (mg L^{-1})	3.12	1.59
Zinc (mg L^{-1})	3.57	2.06
Manganese (mg L^{-1})	5.32	1.55
Nickel (mg L^{-1})	0.78	0.57
Cobalt (mg L^{-1})	0.43	0.18
Lead (mg L^{-1})	1.05	0.42

(Arvanitoyiannis and Kassaveti, 2008)

1.1.2. Polluting power of OMWs

By disposing OMWs into the land without control, foul smelling and possibly pathogenic substances are spread into the environment, while undesirable (and finally completely uncontrolled) fermentations of the organic fraction are performed. Moreover, transfer of the residue into the underground waters or the adjacent water areas (lakes, rivers, etc) is performed with obvious negative effects into the environment. The disposal of OMWs on soil affects its physical characteristics (porosity and aggregation), as well as its chemical (acidity, salinity, organic and inorganic chemical compounds, leaching) and biological (impact on the soil microbial communities, effects on plants/crops) properties. When OMW is disposed directly and without previous treatment (common practice to date) into nearby rivers and streams, a considerable impact on the receiving waters occurs. Specifically the most visible effect of this pollution is the discoloring of natural waters attributed to the oxidation and subsequent polymerization of tannins giving darkly colored polyphenols, which are difficult to be removed from the effluent (Lanciotti *et al.*, 2005; Niaounakis and Halvadakis, 2006; Paraskeva and Diamadopoulos, 2006; Amaral *et al.*, 2008). Moreover, the considerable OMWs content of reducing sugars (e.g. glucose) would result in an increased number of microorganisms that could use these easily assimilable compounds as substrates. Therefore, dissolved oxygen concentration into the water is consumed, reducing thus the share available for other living organisms leading into a serious imbalance of the whole ecosystem (Lanciotti *et al.*, 2005). The lipids in OMWs may form an impenetrable film on the surface of rivers, their banks and surrounding farm lands. This film blocks out sunlight and oxygen to microorganisms in the water, leading to reduced plant growth in the soils and river banks and in turn erosion (Niaounakis and Halvadakis, 2006). High phosphorous content could lead to a similar process as it encourages and accelerates the growth of algae and increases the chances to eutrophication destroying the whole natural waters ecological balance. The presence of large quantity of OMWs nutrients may provide a perfect medium for pathogens growth and water infection, having severe consequences to the local aquatic life and humans coming into contact with water. Finally, if OMWs are dumped in sewers (common disposal method for olive-millers) corrosion phenomena to the sewer pipes occur due to high concentration of organic acids (mainly volatile fatty acids) (Lanciotti *et al.*, 2005; Niaounakis and Halvadakis, 2006; Paraskeva and Diamadopoulos, 2006; Amaral *et al.*, 2008; Massadeh and Modallal, 2008).

Characteristic odors that olive-mills generate as gas emissions and can be detected around the olive oil producing facilities are attributed to volatile organic acids and other low-boiling point organic substances. When OMWs are stored in open lagoons and/or discharged on the land or into natural waters, fermentation phenomena take place. As a result, methane and other pungent gases

emanate from pond evaporation plants and pollute waters or soil leading to considerable pollution by odors (even in great distances) (Niaounakis and Halvadakis, 2006).

1.1.3. Treatment of OMWs

Both the variety of OMWs components and the tremendous seasonal volume production makes their treatment difficult and therefore their elimination and disposal one of the main and critical environmental problems related to the olive oil industry. The development of cost-effective OMWs treatment technologies remains a priority, since OMWs are in most cases discharged directly to the environment without any other treatment performed. Several physicochemical processes have been proposed for OMWs treatment, but these methods, in most cases, are limited to small-scale operations (Mantzavinos and Kalogerakis, 2005; Sarika *et al.*, 2005; Ginos *et al.*, 2006; Arvanitoyiannis *et al.*, 2007; Arvanitoyiannis and Kassaveti, 2008). On the other hand, recent developments indicate that OMW should be considered as fermentation medium to be valorized rather than a waste to be discharged, being a potential substrate for various fermentation processes (Crognale *et al.*, 2006). Therefore research should focus on both waste bioremediation and production of high-added value products, simultaneously, under cost-effective technologies. Below some of these processes are presented in brief (for details see: Μπαλατσούρας, 1999; Niaounakis and Halvadakis, 2006; Arvanitoyiannis and Kassaveti, 2008).

1.1.3.1. Physical processes

In this chapter treatments involving the separation of different phases (variety from solid-liquid to liquid-liquid) through mechanical means are included.

Dilution

A simple way to reduce OMWs organic load is dilution, using water being in abundance during the winter olive-mills operation period. The dilution water may come from nearby streams or brooks, wells or irrigation. Dilution itself decreases the concentration of OMWs and thus makes it easier to reach the required standards of the effluent. The OMWs polluting load and the resident surrounding population ratio can play an important role in the selection of the treatment process. When the load due to OMWs is low (compared to domestic effluents), OMWs can be disposed to an existing sewage treatment plant. Dilution is also carried by the continuous process of olive oil production (three-phase) (Niaounakis and Halvadakis, 2006).

Sedimentation/Settling

The most widely used traditional physical pretreatment method of OMWs is that of settling. Considering that much of the OMWs organic matter is in suspended form rather than in solution, the removal of the sludge or sediment results in a large reduction in BOD_5. The sedimentation is a natural process (though considered as slow and usually costly method) which results in two liquid fractions. A low COD supernatant and a high COD settled sludge which after being removed, dried and hardened can be used as fuel product (replacement of wood, coal etc.), as fertilizer and as insulator and protector for maintaining humidity in certain types of plantation (Niaounakis and Halvadakis, 2006).

Flotation

Flotation includes the removal of solid or liquid particles (such as oil droplets or suspended solids) from OMWs. By adding gas (i.e. air) separation is facilitated as rising gas bubbles either adhere to or are trapped in the particle structure of the suspended solid. Thus, its specific gravity is decreased relative to liquid phase and separation of the suspended particles is affected (Niaounakis and Halvadakis, 2006).

Centrifugation

Three segregated phases are formed (a surface layer containing oil, an aqueous layer with the soluble materials and a sediment layer concentrating suspended and colloidal matter) when OMW are subjected to centrifugation. Suspended solids may be fully separated when using this process, which in turn can improve COD removal and oil recovery (Niaounakis and Halvadakis, 2006).

Filtration and Membrane Technology

Filtration (occasionally used in small-scale olive mills) resembles settling and serves the same purpose, by removing suspended and colloidal solids. Even though COD removal may be high (almost higher than centrifugation), by using press filtration as treatment process, the physicochemical characteristics of OMWs (suspended solids, oils and fats) lead to a rapid clogging and the formation of an impenetrable cake of solids and oils, making this process practically unsuitable (Niaounakis and Halvadakis, 2006).

Membrane technology (differentiated into microfiltration, ultrafiltration, nanofiltration and reverse osmosis) is based on the separation of particle sizes that are in the same phase. Microfiltration leads to the separation of particles with diameter more than 2 μm (thus colloidal

constituents are completely removed). Ultrafiltration has a cut-off of 0.1 μm eliminating besides colloidal, suspended pollutants such as oils or phenolic compounds, although dissolved components are insufficiently removed. Another disadvantage of this method is the severe fouling of the membrane affecting its performance and rendering it as quite expensive. Nanofiltration separates different fluids or ions using respective, partially permeable membranes. Even though is a treatment not as fine as reverse osmosis, it presents lower energy performance. Reverse osmosis has a cut-off (depending on the membrane) from 20 to 1000 mol L^{-1} classifying this process in the molecular range. It has an efficiency more than 90% in removing organic matter, however, the waste amount resulting from this process as also the operating cost are rather high (Niaounakis and Halvadakis, 2006).

Thermal processes

The condensation of the OMWs by reducing their water content and eventually reducing the total amount of the waste (converting it into a stable and usable end product) prior to final disposal is the common clue of numerous thermal processes. Generally speaking, thermal processes are used to accomplish the detoxification of hazardous waste by decomposing organic compounds contained in it as also to accomplish the reduction of the volume of the waste and the conversion of the waste into solids by vaporizing its water and/or its other liquids. Such processes are divided into three main categories: physico-thermal processes (evaporation and distillation and drying of olive cake), irreversible thermo-chemical processes (combustion and pyrolysis) and a combination of physical and biological processes (lagooning). (Μπαλατσούρας, 1999; Niaounakis and Halvadakis, 2006; Arvanitoyiannis and Kassaveti, 2008).

Evaporation is the vaporization of a liquid from a solution leading to the production of a concentrated solution and a volatile stream consisting of water vapor and volatile substances. This process gives a large reduction in COD and BOD_5 but the concentrate that remains has to be treated before final disposal (e.g. by biological treatment). The high energy demand, the considerable air emission, the complex control process requiring specialized personnel, the post-treatment and disposal of the produced emissions are considered as serious process drawbacks. The evaporation of OMWs reduces its volume by 70-75% and brings down the polluting load to more than 90% in terms of COD (even though there is a disagreement among several technical reports) (Niaounakis and Halvadakis, 2006; Arvanitoyiannis and Kassaveti, 2008).

Combustion (rapid chemical reaction of raw material and oxygen to from carbon dioxide, water and heat) and pyrolysis (thermal decomposition of an organic material in the absence of oxygen) are the main irreversible thermo-chemical processes that eliminate any possibility of

further uses of OMWs. However they cause environmental problems arising from the emission of toxic substances in gas form and they require high energy consumption and very expensive infrastructures (Niaounakis and Halvadakis, 2006; Arvanitoyiannis and Kassaveti, 2008).

While lagooning (use of artificial pond or storage lakes), the energy of the sun is used to speed-up the process of OMW evaporation and drying. Moreover, OMW components are partially degraded by a natural biological route. Nevertheless, this occurs over very long time periods, in practice (from one milling season to the subsequent season). Most Mediterranean countries use lagooning as common practice. However, the need of large collecting basins, the threat of leakage through the soil and into the groundwater, the useless (either as fertilizer or for irrigation) end product and the serious negative environmental impacts on nearby areas that are caused by the ponds due to the foul odors, insect proliferation, leakages, infiltrations, and silting with sludge are considered as serious drawbacks of this practice (Μπαλατσούρας, 1999; Niaounakis and Halvadakis, 2006).

1.1.3.2. Physicochemical processes

The use of additional chemicals for the neutralization, flocculation, precipitation, adsorption, chemical oxidation and ion exchange of OMWs are included in physicochemical processes.

Neutralization

This technique can be used as a pretreatment procedure for the removal of the suspended or colloidal matter of OMWs by restoration of the hydrogen (H^+) or hydroxyl (OH^-) ion balance in solution so that the ionic strength of each are equal. This is performed either by reducing pH to the point of zero charge (pH~2-4) via the addition of acids (e.g. H_2SO_4, HCl, HNO_3) or by increasing it (pH=11) via the addition of bases (e.g. $CaCO_3$, $Ca(OH)_2$, NaOH). By increasing the hydrogen ion (H^+) concentration or by adding specifically absorbed ions (Ca^{2+}) the negative surface charge of the suspended hydrophilic colloids is reduced and this leads to their neutralization and destabilization (Niaounakis and Halvadakis, 2006).

Precipitation/Flocculation/Coagulation

Precipitation is the chemical conversion (by the addition of a precipitate-inducing agent) of soluble substances into insoluble particles. Coagulation and flocculation initiate a chemical reaction that promotes the formation, agglomeration or clumping of such particles and thus facilitate their removal from solution. They can be described as the destabilization of process of a stable colloidal

dispersion by the addition of a chemical known to effect destabilization. During storage, OMW undergoes natural self-purification due to a spontaneous flocculation/denaturation of the proteins. The long chain proteins act as flocculating agents helping the suspended material to interact. The individual particles combine together to form a flock, which becomes denser than the surrounding medium and then settles. Flocculation happens also upon heating, leading in the conclusion that other results will be obtained with fresh OMWs directly from the mills than with cold and old OMWs. Flocculation gives partial, not quantitative results (as the separated fraction is consisted by only a fraction of the initial content of OMWs) and the precipitated material has to be disposed of (Niaounakis and Halvadakis, 2006; Arvanitoyiannis and Kassaveti, 2008) and, thus, should be subjected to further treatment as it may cause serious pollution.

Adsorption

Another physicochemical process is adsorption which consists of the attachment of dissolved compounds from OMWs to the surface of a solid substance – adsorbent (such as activated carbon, bentonite etc.) and takes place both at the visible surface of the solid, but also in its pores. This process presents low space requirements, no water pollution, no odor emission and low raw material costs (for adsorbent) whereas it has limited purification efficiency, running plant costs and the need of qualified personnel to ensure trouble-free operation. The organic compounds which can be removed from OMWs when using this practice are coloring substances (mainly tannic acids), hardly or non-biodegradable pollutants and inhibiting compounds (Niaounakis and Halvadakis, 2006).

Chemical oxidation

This treatment includes the use of oxidizing agents (chosen from the group formed by oxygen, oxygen, chlorine, chlorinated derivatives, potassium permanganate or a mixture of them) for the remediation of OMWs. An interesting clue of this sector of processes is that with the use of ozonation (as pretreatment method), the total COD would be decreased (because the toxic compounds are present in minor concentration) and simultaneously the biomass potential to feed an anaerobic reactor would not be lost. This occurs because of the fact that ozone is rather selective towards double bonds. Theoretically, it would leave intact the proteins and sugars contained in OMWs, which are biodegradable anyway, and attack selectively the double bonds of unsaturated fatty acids and phenols.

In an attempt to optimize oxidation processes, advanced oxidation processes (AOPs; such as O_3/H_2O_2, photolysis of O_3, photolysis of H_2O_2, photocatalysis, fenton reaction) have been

employed. They have been characterized by the production of the highly oxidative hydroxyl radical at ambient temperature generated by a number of photochemical and non-photochemical pathways. Due to its strong oxidative nature, which is much greater than other traditional oxidants, the hydroxyl radical is able to completely transform organic carbon compounds to CO_2. It is indicated that oxidation rate limitations coly be lowered if conventional oxidants are replaced by combination of oxidants as well as combinations of oxidants with ultraviolet radiation (Mantzavinos and Kalogerakis, 2005; Niaounakis and Halvadakis, 2006).

1.1.3.3. Biological processes

Recent developments indicate that OMWs should be considered as a fermentation medium to be valorized rather than a waste to be discharged, being a potential microbial substrate for various fermentation processes (Crognale *et al.*, 2006). Therefore research should focus on both waste bioremediation and production of (high-) added value products, simultaneously, under cost-effective technologies.

The contents of the OMWs organic fraction can be divided into three main categories:

- Easily biodegradable (simple sugars, organic acids, amino acids)
- Relatively easy biodegradable polymers (proteins, hemicellulose, pectin)
- Persistent (phenolic compounds, tannins, lipid compounds)

Even though the persistent compounds participate in a small amount within the organic fraction compared to the other two fractions, they attach special properties to OMWs and they are practically responsible for treatment difficulties. Specifically, various phenols are toxic against plants in their germination and against aquatic organisms (Μπαλατσούρας, 1999).

The breakdown of phenolic compounds should be considered as the limiting step in OMWs treatment by biotechnological processes (Kissi *et al.*, 2001; Tsioulpas *et al.*, 2002; Aggelis *et al.*, 2003; Ahmadi *et al.*, 2006) as such compounds are not easily biodegradable (Aggelis *et al.*, 2003; Lanciotti *et al.*, 2005; Crognale *et al.*, 2006; Pasaskeva and Diamadopoulos, 2006; Di Serio *et al.*, 2008; Papanikolaou *et al.*, 2008a). The biological approach requires deep knowledge of the biochemical routes used by the microorganisms for the different compounds of OMWs so as to select the most appropriate species or “design” new strains that effectively degrade the wide variety of these substances. It should be stressed out that biological processes (especially anaerobic ones) have been found to be more economic and efficient than physical/chemical processes (Niaounakis and Halvadakis, 2006). OMW-based media enriched with other carbon sources have been used for the cultivation of molds, prokaryotic microorganisms, yeast and yeast-like species leading to the remediation of the waste as also to the production of added-value compounds such as yeast and

fungal biomass (Scioli and Volaro, 1997; Ben Sassi et al., 2008; Papanikolaou et al., 2008a), exopolysaccharide (Crognale et al., 2006), various enzymes (Zervakis *et al.*, 1996; Scioli and Volaro, 1997; Aggelis *et al.*, 2003; Crognale *et al.* 2006; D'Annibale *et al.*, 2006), citric acid (Papanikolaou *et al.*, 2008) and finally few for the production of bioethanol (Bambalov *et al.*, 1989; Zanichelli *et al.*, 2007; Massadeh and Modallal, 2008).The biological treatment of OMWs occurs under anaerobic or aerobic conditions. Aerobic process (usually used on lower concentration streams or as polishing step to further remove residual organic matter and nutrients) relies on microorganisms that thrive under conditions where plentiful of oxygen is available and a sufficient amount of food is present. In anaerobic biodegradation (digestion) (used for removing organic matter in higher concentration streams) no oxygen is present and a series of microbiological processes convert organic compounds to methane and carbon dioxide (Niaounakis and Halvadakis, 2006; Arvanitoyiannis and Kassaveti, 2008).

Anaerobic processes

Anaerobic biodegradation (digestion) consists of a series of microbiological processes that convert organic compounds to methane and carbon dioxide in absence of oxygen while several types of microorganisms have been reported to be implicated in aerobic processes, anaerobic processes are driven mostly by bacteria. The anaerobic process includes three major steps:

- Hydrolysis, during which anaerobic bacteria break down complex organic molecules of the waste such as proteins, cellulose, lignin, and lipids (reactions catalyzed by extracellular enzymes such as cellulases, proteases and lipases) into soluble monomer molecules (such as amino acids, simple sugars, glycerol and fatty acids) making them directly available for the next step group of bacteria)
- Acidogenesis, which includes fermentation executed by fermentative acidogenic bacteria (converting sugars, amino acids, and fatty acids into organic acids such as acetic, propionic, formic, lactic, butyric, or succinic, into alcohols and ketones such as ethanol, methanol, glycerol, acetone, and finally into acetate, carbon dioxide and hydrogen) and anaerobic oxidation carried out by acetogenic bacteria (converting fatty acids like long chain fatty acids as also alcohols into acetate, hydrogen, and carbon dioxide, which are used by the methanogenic bacteria)
- Methanogenesis, where acetate, hydrogen, and carbon dioxide are converted (by methanogenic bacteria composed of both gram-positive and gram-negative bacteria with a wide variety of shapes) into methane

The anaerobic process is the most widely investigated technique for reducing the polluting load of OMWs. It presents feasibility both to wastewaters treatment with a high organic load and to the techno-economic structure of the olive-mills. Moreover, it offers the advantages of low energy consumption, production of an energy-rich gas (methane) that may be amenable to further uses after some preparation and relatively small amount of sludge that must be subjected to subsequent treatment. Nevertheless, this same low rate of sludge accumulation is responsible for the high sensitivity of anaerobic systems to the recalcitrant components of the inflowing OMWs and for the increased discharge of microorganisms. This process also presents a high sensitivity to variability in operational parameters and toxicants (Niaounakis and Halvadakis, 2006).

Aerobic processes

The process that relies on microorganisms that thrive under aerobic conditions (plentiful availability of oxygen and sufficient amount of food is present) is called aerobic process. When applying aerobic processes both the type of microbial fauna utilized making the distinction between mono- and poly-culture and the technology utilized in order to achieve the desired goal should be specified. The currently available and studied aerobic practices applied on OMWs treatment are attached-growth (biofilm, fixed-film), trickling filter, packed-bed reactor, rotating (disk) biological contactor (RBC), suspended growth, activated sludge, sequencing batch reactor (SBR), aerated lagoons (stabilization ponds), controlled wetlands. Several investigations have been carried out concerning the uses of specific aerobic microorganisms [fungi, bacteria and (micro-)algae] capable of growing aerobically on OMWs in order to neutralize their heavy polluting effect (reduce the initial organic load and phenolic content) and either obtain high added value products or rendering them susceptible to further degradation treatment (Niaounakis and Halvadakis, 2006).

The structure of the aromatic compounds present in OMWs resembles to many of the components of lignin. The ability of higher fungi to break down phenolic compounds is based on the secretion of extra-cellular oxidases (ligninolytic enzymes) laccase, lignin peroxidase and manganese dependent (or independent) peroxidase (Fountoulakis *et al.*, 2002; Aggelis *et al.*, 2003; Crognale *et al.*, 2006; Lakhtar *et al.*, 2010). The secretion of these enzymes is strain-dependent and influenced by various culture conditions (Sayadi and Ellouz, 1992; Aggelis *et al.*, 2003; Tsioulpas *et al.*, 2002; Crognale *et al.*, 2006; Aloui *et al.*, 2007; Massadeh and Modallal, 2008). Only few microorganisms, mainly white rot basidiomycetes, are able to degrade lignin by means of oxidative reactions catalyzed by the aforementioned phenol oxidases and peroxidases. Both the low degree of specificity, which characterizes these enzymes, and the structural relationships of many aromatic

pollutants with the natural substrates of enzymes, have suggested the use of ligninolytic organisms and their enzymes for the treatment of these kinds of substrates (Niaounakis and Halvadakis, 2006).

The use of filamentous fungi for OMWs pretreatment has been shown to reduce toxicity and improve the biodegradability in aerobic degradation. In particular, the pretreatment of OMWs with higher fungi, which produce poly-aromatic hydrocarbon-degrading enzymes, has been used to detoxify and decolorize them. However, the application of such processes on a large scale compared to bacteria presents limitations due to the difficulty of achieving continuous culture because of the formation of filamentous pellets and mycelia. Another process drawback could be the variation of COD reduction and color removal values obtained after OMW biotreatment, even when using the same microorganism and operating conditions (Niaounakis and Halvadakis, 2006).

Non-genetically modified yeast strains (like *S. cerevisiae*), in general, do not contain in their genetic arsenal the potentiality of producing such types of enzymes (Sayadi and Ellouz, 1992), and, thus, the removal of phenol compounds and the decolorization of OMWs that are subjected to fermentation by these yeast species through the use of the above-mentioned enzymes should be excluded. On the other hand, Rizzo *et al.* (2006) suggested a potential exclusively physical mechanism involving the establishment of weak and reversible interactions, mainly between anthocyanins and yeast walls, by means of adsorption. Moreover, (potentially very weak) assimilation of several phenolic compounds by the yeast could, also, not be excluded (Chtourou *et al.*, 2004).

1.1.4. Utilization and applications of OMWs

A wide range of technological processes - mentioned above - are nowadays available for reducing the pollutant effects of OMW and transforming it into valuable products; the most suitable practices involve recycling rather sole detoxification of this waste. The OMW organic matter (15-18%) implies million tons of substance that may be used as raw material either to recover valuable natural constituents/by-products [such as uses as fertilizer/soil conditioner, herbicide/pesticide, animal feeding/human consumption (edible fungi), recovery of residual oil, recovery of organic compounds (like pectins, antioxidants, bioactive phenolic compounds, enzymes)] or as a substrate for the growth of microorganisms and the production of new (potentially high-added value) products [biosurfactants, biopolymers, activated carbon, enzymes, production/generation of bioenergy/biofuels (such as alcohols-bioethanol, biohydrogen, biomethane, biodiesel)] (Niaounakis and Halvadakis, 2006; Demerche *et al.*, 2013). Some fields for the end products of treated olive mill by-products and wastes are summarized in Fig. 1.1..

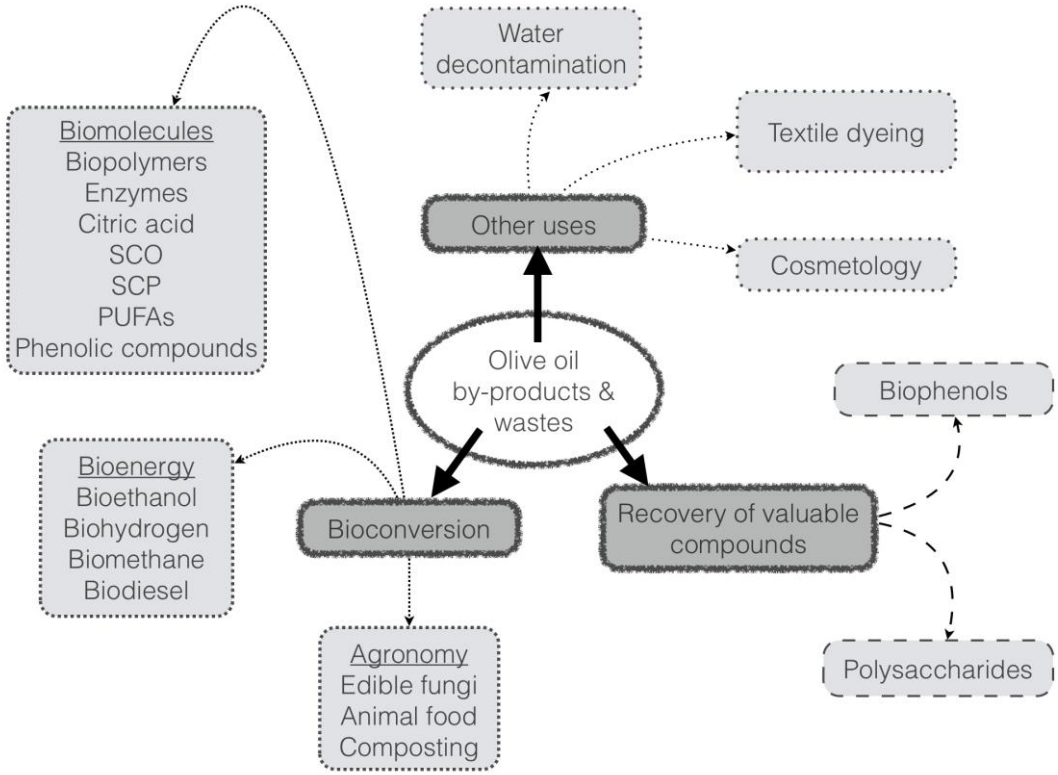


Figure 1. 1. Valorization opportunities for olive mill by-products and wastes (adapted by Demerche *et al.*, 2013)

Fertilizer/Soil conditioner

OMWs contain high organic load and substantial amounts of plant nutrients. They are considered also as a low cost source of water, with characteristics favoring their use as soil fertilizer or organic amendment to poor soils (such soils are usually noted in the same countries where OMWs are produced). Those effluents affect positively soil aggregation and structure stability and the hydrodynamic properties of sandy soils (thus OMWs may substitute expensive commercial soil conditioners). However there is a controversy amongst researchers about the use of OMWs as fertilizer. The antimicrobial and phytotoxic properties of OMW (associated with their high mineral content, low pH and the presence of phytotoxic compounds like phenols; something that may allow the use of OMWs also as pesticide/herbicide) indicate that it should not be directly applied to soils and crops. Consequently, their use after the removal of their phenolic components for soil fertilization could be considered valuable (Niaounakis and Halvadakis, 2006; Demerche *et al.*, 2013).

A potential bioremediation treatment of olive-mill wastes could be composting. By composting OMWs (fresh or sludge from pond-stored OMWs) blended with appropriate waste materials (namely carriers such as maize straw, cotton waste, olive leaves, cotton gin waste and

sewage sludge as bulking agents), a new material can be obtained with high level of humification and without phytotoxic effects used as organic fertilizer (compost) to improve soil fertility and plant production. A simultaneous process involving microbial degradation of the OMWs polluting load is also takes place (Cegarra *et al.*, 1996; Paredes *et al.*, 2000; 2002; 2005; Garcia-Gómez *et al.*, 2003; Niaounakis and Halvadakis, 2006).

Recovery of organic compounds

OMWs appear to be a suitable source of valuable organic compounds to be extracted. Such compounds are: pectins (natural hydrocolloids used as gelling agents, stabilizers and emulsifiers in the food industry) and antioxidants (mainly simple and complex water soluble phenolic compounds with potential antioxidant properties). Specific solvents and ultrafiltration/reverse osmosis techniques are used by current state-of-the-art, which require the application of sophisticated technologies leading to the necessity of complex chemical facilities availability (Niaounakis and Halvadakis, 2006; Demerche *et al.*, 2013).

Animal feeding/Human consumption

Negative opinions exist about the nutritional acceptability of OMWs as animal feeding due to the presence of anti-digestive agents which induce diarrhea to ruminants because of high concentration of potassium and phenolic compounds (though removal of these compounds from the waste-waters could be performed before animal feeding). A non-negligible amount of sugars and minerals contained in the OMWs make them an ideal substrate for yeast or other fungi leading to the production of a microbial mass of high digestibility that includes carbohydrates, lipids, minerals and vitamins. Finally edible (or other) fungi, especially *Pleurotus* or *Lentinula* species but also *Agaricus bisporus* and *Geotrichum candidum*, are able to grow using olive oil by-products (as also blends of OMWs with other wastes such as cheese whey) as a nutrient source (Zervakis *et al.*, 1996; Niaounakis and Halvadakis, 2006; Aouidi *et al.*, 2010; Lakhtar *et al.*, 2010; Demerche *et al.*, 2013).

Production/generation of energy

With the actual development of bioenergy, the cost of plant biomass and fermentable organic matter will increase significantly in the near future and might compete with other industrial areas. The recent success of first-generation biofuels has been seriously discussed with regards to its negative impact on plant production for food. The development of second- (biofuels from cellulosic and hemicellulosic materials or other waste streams) and third-generation (biofuels from microalgae) biorefineries is one of the proposed solutions for producing bioenergy and valorizing

non-edible biomass. OMW treatment technologies could aim at energy recovery as an interesting alternative for sustainable disposal of residues, able to reduce the environmental impact and to generate electric energy for sale or for the satisfaction of olive-mills energy needs (though, amongst other issues, high technological level, requiring remarkable capital investments and qualified personnel, characterizes such disposal systems). In this content, OWMs are interesting substrates which at the same time poses an environmental problem. As stated, OMWs contain sugars, phenolic compounds and potentially polyols and lipids, thus, these residues could serve as a source for bioethanol (recovery by distillation), biohydrogen, biodiesel precursors (e.g. triglycerides) and biomethane production. Studies have tended to focus on biomethane obtained through the anaerobic digestion of OMW substrates, however, work has also been performed in the areas of bioethanol generation and bioH_2 production by photofermentative processes and dark fermentation (Niaounakis and Halvadakis, 2006; Demerche *et al.*, 2013).

Anaerobic digestion can convert organic substrates to CH_4 and CO_2 (biogas) through the concerted action of a mixture of microbes (consortia) (see paragraph 1.1.3.3. - anaerobic digestion). Biogas can substitute natural gas and medium calorific gases. This process can be carried out in relatively inexpensive and simple reactor designs and operating procedures. Nevertheless the low efficiency of the process due to the waste toxicity is a serious drawback.

A large number of microbial species with significant taxonomic and physiological differences can produce biohydrogen through single or combined metabolic pathways. Several studies though emphasize that systems producing photobiological hydrogen using photosynthetic bacteria and OMWs will clearly require enhancements due to their dark color and inhibitory effects [either by using high dilution rates (requiring the addition of nitrogen supplementation and adjustments to OMWs' pH but proven impracticable at large scale as the volume of the effluent is increased) or by blending with other wastes such as cheese whey] (Niaounakis and Halvadakis, 2006; Aouidi *et al.*, 2009; 2010; Demerche *et al.*, 2013).

Microbial products

Various biological processes have been used for the treatment of OMWs (for more information see paragraph 1.1.3.3.). OMWs should be considered as a fermentation medium to valorize rather than a waste to discharge, being a potential substrate (for the cultivation of molds, prokaryotic microorganisms, yeast and yeast-like species) for fermentation processes (Crognale *et al.*, 2006). Such processes can lead to the simultaneous reduction of COD values and phenol compounds degradation and to the production of added-value compounds such as biomass, citric acid, ethanol, enzymes [mainly extracellular laccase and manganese peroxidase (MnP), lipases

(applicable in the dairy, pharmaceutical, detergent and other industries), pectinases and phenol oxidases, used in the olive oil extraction process to improve olive oil yield and quality, turbidity, oxidation induction time, contents of aromatic compounds and to reduce the toxicity of many aromatic compounds] (Zervakis *et al.*, 1996; De Felice *et al.*, 1997; Scioli and Vollaro, 1997; Aggelis *et al.*, 2003; Lanciotti *et al.*, 2005; Crognale *et al.*, 2006; D'Annibale *et al.*, 2006; Niaounakis and Halvadakis, 2006; Papanikolaou *et al.*, 2008a; Lopes *et al.*, 2009; Yousouf and Pirozzi, 2009; Yousouf *et al.*, 2010; Demerche *et al.*, 2013).

1.2. Molasses

Molasses are the viscous by-product of the sugar cane or sugar beet processing into sugar, containing sugars (44-60% *w/w*) and various minerals (Curtin, 1983; Chen and Chou, 1993). 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. The waters remaining after such bioprocesses, called molasses wastewaters (MWWs), are characterized by high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) values, strong odor and dark brown color (Satyawali and Balakrishnan, 2008). The high molecular weight polymer melanoidin is the dark brown pigment found in the molasses wastewaters (Kumar and Chandra, 2006; Plavšić *et al.*, 2006). The composition of this effluent and its release into the environment without appropriate previous treatment, may lead to eutrophication phenomena in the water. Its dark color hinders photosynthesis by blocking sunlight labeling molasses deleterious to aquatic life (FitzGibbon *et al.*, 1998).

MWWs are one of the main environmental threats for soil and water (Evershed *et al.*, 1997), as they resist to microbial degradation and to conventional biological treatment processes. Therefore, there is an adequate treatment necessity of such wastewater before the effluent is discarded to the environment (Kumar and Chandra, 2006). The decolorization of molasses and MWWs and degradation of melanoidins have been achieved by the use of chemical and physicochemical treatment processes (Kim *et al.*, 1985; Migo *et al.*, 1993; Yaylayan and Kaminsky, 1998; Chandra and Singh, 1999; Zhao *et al.*, 2000; Mutlu *et al.*, 2002; Pala *et al.*, 2005; Ryan *et al.*, 2008; Zeng *et al.*, 2009). Those treatment methods are considered to be quite expensive and unstable especially in large scale operations (Sirianuntapiboon *et al.*, 2004; Tondee *et al.*, 2008a; 2008b). Fungi, bacteria and yeasts have been cultivated on molasses either for melanoidin degradation and the reduction of color and of BOD and COD values (Ohmomo *et al.*, 1988; Kumar and Chandra, 2006) or for the production of added-values metabolites such as ethanol, gluconic acid, citric acid, fruto-oligosaccharides (FOS), pullulan, succinic acid, single cell oil (SCO),

erythromycin and bacteriocins (Roukas *et al.*, 1996; Nahvi *et al.*, 2002; Baptista *et al.*, 2006; Kopsahelis *et al.*, 2007; 2012; Cáceres-Farfán *et al.*, 2008; El-Enshasy *et al.*, 2008; Liu *et al.*, 2008; Sharma *et al.*, 2008; Zhu *et al.*, 2008; Chatzifragkou *et al.*, 2010; Metsoviti *et al.*, 2011). In a limited number of reports, the products of added-value compounds and the detoxification - decolorization of molasses were simultaneously studied (Chatzifragkou *et al.*, 2010; Metsoviti *et al.*, 2011).

1.2.1. Composition and polluting power of molasses

The composition of molasses is presented in Table 1.3. and variations concerning the chemical composition can be seen, that depend on the origin (either sugar cane or sugar beet) of these low-cost renewable materials. When molasses are discarded directly to the environment without previous treatment they create significant problems due to the extremely high COD ($80x10^3$-$100x10^3$ mg L^{-1}) and BOD ($40x10^3$-$50x10^3$ mg L^{-1}) values, due to low pH, strong odor and dark brown color (Satyawali and Balakrishnan, 2008). Besides the heavy organic load, molasses include vast amount of nutrients and minerals like nitrogen (1.660-4.200 mg L^{-1}), phosphorus (225-3.038 mg L^{-1}) and potassium (9.600-17.475 mg L^{-1}) that, equally, may lead to eutrophication phenomena. Moreover, its dark color hinders photosynthesis by blocking sunlight labeling molasses deleterious to aquatic life (FitzGibbon *et al.*, 1998). Baruah *et al.* (1993) state that studies on water quality have shown that rivers intoxicated with molasses wastewaters present high BOD values (~1600-21000 mg L^{-1}) even in range of 8 km from the disposal point.

Table 1. 3. Composition of molasses

Component / Characteristic	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 and Chou, 1993; Godbole, 2002; Teclu *et al.*, 2009)

1.2.2. Melanoidins; properties and formation

Melanoidins are dark brown to black pigments, produced by natural condensation between sugars and amino-acids through non-enzymatic browning reaction (known as Maillard reaction) (Plavšić *et al.*, 1998). Natural melanoidins can be easily found in several types of foods and beverages (Painter, 1998) as also in various agricultural products in large quantities (Kumar and Chandra, 2006). Melanoidin structure is not yet fully understood as it is believed that it may not possess a specific one as both their elemental structure and chemical composition are high dependable on the nature and the molecular concentration of the reactants as also dependable on the reaction conditions like pH, temperature, time of heating etc. (Ikan *et al.*, 1990; Yaylayan and Kaminsky, 1998). Melanoidns when present, they attach to food antioxidant, antiallergic and antimicrobial properties and enhance human health as they act as chemical factors of metals and free radicals neutralization. On the other hand melanoidins may be also harmful by provoking mutations and/or carcinogenesis (Silván *et al.*, 2006).

As mentioned above, melanoidins are produced by condensation between sugars and amino-acids through Maillard, one of the most important reactions that occur during thermal process of sugar cane or sugar beet for the production of crystal sugar (Painter, 1998). The mechanism of melanoidins synthesis, proposed by Hodge (1953), through the amino-carbonylic reaction between amino-acids and sugars is the most receivable. Namiki and Hayashi (1983), proposed a new browning reactions pathway which includes the creation of a Schiff base at the early stages of amino-carbonylic reaction before the Amadori rearrangement, followed by the formation of C_2 (glyoxal diakylamine) products and of free radicals. Following, Hayase *et al.* (1984) reported the formation of C_3 sugar molecules at the first stages of browning reactions which were identified as methylglyoxal diakylamine. Fay and Brevard (2005) have studied the first stages of Maillard reaction reporting that its first stable intermediate products are called Amadori components which are N-substituted 1-amino-deoxy-ketones representing an important class of Maillard reaction products.

1.2.3. Treatment of molasses and MWWs

The simultaneous decolorization of molasses and the abduction of their content in melanoidins together with the use of this residue as microorganism's substrates for the production of various biotechnological products is a basic aim of research. Low international price of molasses as also their high content in assimilable sugars render this residue as a suitable substrate for the production of numerous biotechnological products.

Even though literature presents various chemical (Chandra and Singh, 1999), and physicochemical processes, such as ozonolysis (Kim *et al.*, 1985) and adsorption in activated carbon for the decolorization of molasses, MWWs and melanoidins which may lead in satisfying results (for more information see paragraph 1.2.3.1.), such treatment processes are not financially feasible in large scale, in contrast with biological treatment with use of fungi (such as *Coriolus*, *Aspergillus*, *Phanerochaete*) and bacteria (such as *Bacillus*, *Alkaligenes*, *Lactobacillus*) (Aoshima *et al.*, 1985; Ohmomo *et al.*, 1985; 1988; Kumar and Chandra, 2006). It should be stressed out though, that biological treatment is highly dependable on various conditions such as pH, temperature, concentration of nutrients and oxygen, inoculum volume etc.. Moreover, the biological degradation system includes mainly oxidases and peroxidases such as glucose oxidase, dependent manganese peroxidases (MnP) and independent manganese peroxidases (MIP) (Aoshima *et al.*, 1985; Ohmomo *et al.*, 1985). Boer *et al.* (2006), have proved that both MnP and MIP possess the ability to decolorize molasses in presence of H_2O_2 as also the decolorization ability of oxidases and peroxidases reaches a maximum performance at certain pH value, certain temperature and that it depends on substrate specificity.

1.2.3.1. Physicochemical processes

Pyrolysis is one of the molasses treatment processes for the degradation of melanoidins. Yaylayan and Kaminsky (1998) applied pyrolytic degradation to melanoidins coming from the first stages of Maillard reaction resulting in the production of various compounds, mainly acetic acid. Hayase *et al.* (1984), studied the decolorization (64% in pH=7.0 and 97% in pH=10.0) and degradation products (mainly 2-methyl-2 4-pentanediol, N N-dimethylacetylamide, phenol, acetate, 2-furancarboxylic acid, furandixarboxylic acid and 5-(hydroxymethyl)-2-furancarboxyllic acid) of the melanoidins by H_2O_2. The degradation of melanoidins (derived from glucose and glycine) by applying ozonolysis at -1˚C, resulting in decolorization 84% (after 10 minutes) and 97% (after 90 minutes), was studied by Kim *et al.* (1985). There was also a reduction of the average molecular weight of melanoidins from 7000 to 3000 (after 40 minutes). An interesting treatment method for the colloids and natural organic matter (NOM) abduction from wastes is flocculation following coagulation (Zhao *et al.*, 2000). Melanoidins present similar chemical structure with NOM, thus, the same process has been applied. The basic elements used for flocculation are minerals of aluminum and ferrum. Liang *et al.* (2009), used ferric chloride for biological treatment of molasses reporting 86% COD reduction and 96% decolorization (the respective values when used sulphur aluminum). When flocculation occurs with the use of various mineral salts, it should be noticed that the procedure is highly affected by the solution pH, the type and dose of flocculant and the mixing

conditions (Liang *et al.*, 2009). Migo *et al.* (1993), used polyferric sulphate and Ryan *et al.* (2008) used electro-flocculation for the decolorization of molasses. The use of membrane demarcation systems (Mutlua *et al.*, 2002), Fenton reagent (Pala *et al.*, 2005) and ozone (for melanoidins oxidation with SnO_2 as catalyst; Zeng *et al.*, 2009) have been reported in literature for melanodins abduction.

1.2.3.2. Biological processes

There are various reports in literature suggesting the use of bacteria (Kumar *et al.*, 2006), yeasts (Tondee *et al.*, 2008a) and fungal strains (Miranda *et al.*, 1996) employed for decolorization of molasses, MWWs and melanoidins. Biological treatment is more environmental friendly (and financially more feasible) compared to physicochemical processes, but nevertheless (due to differential on the needs of microorganisms) highly dependable on certain growth conditions (Chandra *et al.*, 2008). For instance, the fungi *Cunninghamella echinulata* and *Mortierella isabellina* were cultivated on molasses, and growth was accompanied by non-negligible substrate decolorization, reaching up to ~75% for *C. echinulata* (400 h of culture) and ~20% for *M. isabellina* (200 h after inoculation) (Chatzifragkou *et al.*, 2010). Moreover, waste molasses (with initial total sugars, TS_0, concentrations 20 g L^{-1} and 30 g L^{-1}) were used as growth medium for *Leuconostoc mesenteroides* so as to produce bacteriocin. Decolorization up to ~27% of this residue was performed together with appreciable bacteriocin production (Metsoviti *et al.*, 2011). Ohmomo *et al.* (1985), used the fungus *Coriolus versicolour* Ps4a for the decolorization of melanoidins, with a decolorization rate achieved up to ~80%. Following, Ohmomo *et al.* (1988) used *Aspergillus oryzae* strain Y-2-32 which absorbed in its mycelia low molecular weight melanoidins. *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 presented the ability to quickly degrade the high molecular weight fraction. Tondee *et al.* (2008a) cultivated *Issatchenkia orientalis* strain No. SF9-246 (isolated from rotten banana) in a malt extract-glucose-peptone broth containing melanoidins, and a decolorization rate of 60.2% was obtained within 7 days.

The capability of bacteria of the genus *Pseudomonas*, *Enterobacter*, *Stenotrophomonas*, *Aeromonas*, *Acinetobacter* and *Klebsiella* to reduce COD of anaerobically treates molasses spent wash was tested by Ghosh *et al.* (2004) resulting in various final decolorization values. Following, Ghosh *et al.* (2009) cultivated *Pseudomonas putinda* on the same substrate achieving 24% decolorization whereas when used immobilized (in calcium alginate beads) cells they managed to two-fold increase the decolorization yield. Sirianuntapiboon *et al.* (2004) cultivated strain no.

BP103 of acetogenic bacteria on molasses wastewaters, achieving a 76.4% decolorization yield. Tondee and Sirianuntapiboon (2008b) cultivated *Lactobacillus plantarum* strain No. PV71-1861 (isolated from pickle samples) under anaerobic and facultative (static) conditions showing a high potential for use in decolorization of molasses wastewater (maximum yield 68.12% within 7 days). Kumar and Chandra (2006) used *Bacillus thuringiensis* MTCC 4714, *Bacillus brevis* MTCC 4716 and *Bacillus* sp. MTCC 6506 on substrates containing synthetic melanoidins. The use of individual cultures did not present significant affection on melanoidins whereas mixed cultures resulted in maximum decolorization of 50%. Kalavathi *et al.* (2001) achieved degradation of the melanoidins (decolorization up to 96%) in distillery effluents by the marine cyanobacterium *Oscillatoria boryana* BDU 92181. Cyanobacteria *Lyngbya* sp. and *Synechocystis* sp. manage to decolorize distillery effluents by 81% and 26% respectively (Patel *et al.*, 2001).

1.2.4. Biotechnological applications of molasses

As mentioned above, molasses as they possess a high sugar content they become highly attractive (together with their low international price) as substrate for the production of various biotechnological compounds. Literature reports the use of various microorganisms grown on molasses for the production of (high-) added value products such as ethanol, citric acid, gluconic acid, fruto-oligosaccharides (FOS), pullulan, succinic acid, single cell oil, erythromycin, bacteriosins etc. (Roukas, 1996; El-Enshasy *et al.*, 2008; Liu *et al.*, 2008; Sharma *et al.*, 2008; Chatzifragkou *et al.*, 2010).

1.2.4.1 Ethanol production

Molasses and molasses waste-waters have been used as substrates in various configurations (immobilized yeast strains, fed batch, repeated fed batch and continuous bioreactor trials, mixed yeasts, non-aseptic conditions etc.) for the biotechnological production of ethanol (Roukas, 1996; Pinal *et al.*, 1997; Nahvi *et al.*, 2002; Baptista *et al.*, 2006; Kopsahelis *et al.*, 2007; 2012; Cáceres-Farfán *et al.*, 2008).

1.2.4.2 Other products

Citric acid

Citrate, the intermediate metabolite of TCA cycle, may be produced by biotechnological means when molasses are used as substrate, with the use of strains of the fungus *Aspergillus niger*. It could be produced either by submerged fermentation (SmF) or liquid surface fermentation (LSF)

(Milson and Meers, 1985). The production of citric acid by *A. niger* is highly affected by molasses metal content (such as ferrum, zinc, copper, manganese). According to Majolli and Aguirre (1998) the concentration of such heavy metals should be significantly reduced before mycelial growth. Pera and Callieri (1997) reported that the porduction of citric acid when molasses are used as substrate is highly affected by the presence of ferrum ions in concentrations above 0.2 ppm. Adham (2002), attempted to improve citric acid fermentation (Cit_{max}~73 g L^{-1} against ~30 g L^{-1}) by *A. niger* grown in beet-molasses medium by adding natural oils [at concentrations of 2% and 4% (*v/v*)] containing high unsaturated fatty acids. Ikram-ul *et al.* (2004), produced citric acid by selected mutants of *A. niger* [improved by chemical mutagenesis using N-methyl, N-nitro-N-nitroso-guanidine (MNNG)] when grown on cane molasses. The best mutant selected, produced ~96 g L^{-1} citric acid 168 h after fermentation of potassium ferrocyanide and H_2SO_4 pre-treated blackstrap molasses in Vogel's medium. The production of citric acid from cane molasses by *A. niger* in a pilot study using surface or submerged fermentation, studied by Hamissa and Radwan (1977) and Qazi *et al.* (1990) resulted in a maximum concentration of citric acid of 60.8 and 67.0 g L^{-1}, respectively. Likewise, cell recycling of *A. niger* in surface fermentation of cane molasses was performed reducing significantly fermentation time compared to the normal single cycle batch submerged or surface fermentation process. About 80% of the sugar was converted to citric acid in 5 days of batch fermentation, and three batches were carried out with the same fungal mat without any significant loss of productivity (Garg and Sharma, 1991). The production of citric acid from beet molasses by *A. niger* was improved with the addition of phytate (plant constituent that can be found in the seeds of cereals and legumes) to the medium. The effect of phytate addition was found to be dependent on concentration and the stage of fermentation at which it was added. When added at the beginning of incubation, the optimal concentration of phytate in the medium for citric acid production was 10.0 g L^{-1} and resulted in a ~3.1-fold increase in citric acid accumulation. Addition of 16.0 g L^{-1} phytate to the medium, after 3 days of incubation, resulted in the maximum citric acid concentration ~2.4-fold higher than the control experiment (Wang, 1998). A novel method of citric acid production from beet molasses in which an anion exchange resin packed column was connected to the bioreactor for separation of citric acid from fermentation broth was developed by Wang *et al.* (2000). In comparison with a conventional batch, the new fermentation technique increased citric acid productivity and sugar conversion from 0.34 g L^{-1} h^{-1} and 82.2% to more than 0.5 g L^{-1} h^{-1} and 94.8%, respectively.

Gluconic acid

Gluconic acid (pentahydroxycaproic acid) is a mild organic acid derived from glucose by a simple oxidation reaction facilitated by the enzyme glucose oxidase (from fungi) and glucose dehydrogenase (from bacteria such as *Gluconobacter*). Gluconic acid is produced from glucose through a dehydrogenation reaction catalysed by glucose oxidase. Oxidation of the aldehyde group on the C-1 of b-D-glucose to a carboxyl group results in the production of glucono-d-lactone ($C_6H_{10}O_6$) and hydrogen peroxide. Glucono-d-lactone is further hydrolysed to gluconic acid either spontaneously or by lactone hydrolysing enzyme, while hydrogen peroxide is decomposed to water and oxygen by peroxidase (Ramachandran *et al.*, 2006).

Gluconic acid and its derivatives (principally sodium gluconate) can be applied on pharmaceutical, detergent, food and leather industry. It is produced by genetically modified strains of *A. niger* when undergoes solid-state fermentation using molasses as substrate. The components for the medium solidification should be carefully chosen as they offer the necessary nutrients for microorganism's growth but also they can be suspending factors. Sharma *et al.* (2008) employed tea waste as solid support (to molasses). The fungus growth was enhanced by the tea components and the maximum yield of gluconic acid production was 80.5 %.

Fructooligosaccharides (FOS)

FOS are classified as prebiotics. Prebiotics are non-digestible food ingredients that stimulate the growth and/or activity of bacteria (indigenous bifidobacteria) in the digestive system in ways claimed to be beneficial to health. FOS were initially produced in industrial scale using pure sucrose as substrate with the use of enzymes of *Aspergillus* (Hidaka *et al.*, 1988; Hirayama *et al.*, 1989) or *Aureobasidium* (Yun *et al.*, 1992). Shin *et al.* (2004), cultivated *Aureobasidium pullulans* on molasses (initial sucrose concentration 360 g L^{-1}) substrate achieving total FOS concentration 166 g L^{-1}, 24 hours after inoculation with 46% product yield (glucose and fructose suspended the production of FOS).

Succinic acid

Liu *et al.* (2008) cultivated *Actinobacillus succinogenes* for the production of succinic acid which is a precursor of numerous products [such as chemicals, pharmaceuticals, food additives, solvents and biodegradable plastic; see: Willke and Vorlop (2004)]. They have reported the production of ~44 g L^{-1} of succinic acid 60 hours after inoculation with acetic acid and formic acid being the by-products of the process. A process in which previously treated molasses for the

abduction of heavy metals were used as microbial substrate, led to a maximum succinic acid concentration ~51 g L^{-1} (consuming 95% of initial sugar concentration) (Liu *et al.*, 2008).

Single cell oil

The yeast *Trichosporon fermentans* was cultivated on molasses under nitrogen limited conditions for the production of single cell oil (SCO) that could potentially be used as precursor for biodiesel production. SCO consisted of fatty acids having composition similar to vegetable fatty acids (involving mainly palmitic acid, stearic acid, oleic acid and linoleic acid) (Zhu *et al.*, 2008). The factors affecting such fermentations are various, mostly related both to substrate composition (such as C/N ratio) and to culture conditions (such as the incubation temperature, the oxygen presence into the medium and the pH) (Suutari and Laakso, 1993; Suutari *et al.*, 1993; Koike *et al.*, 2001; Fakas *et al.*, 2006 and 2009; Papanikolaou *et al.*, 2007; Chatzifragkou *et al.*, 2010; Papanikolaou and Aggelis, 2011a; 2011b). Finally, in recent developments, the Zygomycete fungi *Cunninghamella echinulata* and *Mortierella isabellina* were cultivated on sugar-based media including molasses and it has been demonstrated that the assimilation rate of the sugars employed as substrates played a crucial role upon lipid accumulation process. Both fungi presented satisfactory dry cell weight production on media composed of molasses, while equally remarkable quantities of SCO and the medically and nutritionally important γ-linolenic acid (GLA) were synthesized (Chatzifragkou *et al.*, 2010).

Pullulan

Roukas and Liakopoulou-Kyriakides (1999), produced the water-soluble polysaccharide named pullulan from beet molasses by *Aureobasidium pullulans* in a stirred tank fermentor. Pullulan is consisted by molecules of maltotriose united by α 1,6 glucosidic bonds. This compound presents the ability to form films with tolerance on oils being not permeable from oxygen. Aeration was an important factor for the production of pullulan (maximum concentration 23 g L^{-1} under 1 vmm aeration versus 0.5 vvm and 0.0 vvm giving maximum pullulan concentration 14 g $L^{-1}$and 12 g L^{-1} respectively)

Erythromycin

El-Enshasy *et al.* (2008) cultivated *Saccharopolyspora erythraea* in molasses based medium under submerged fermentation for the production of erythromycin. Erythromycin is a secondary metabolite the production of which depends on substrate composition and on culture conditions.

Through cultivation medium optimization (by also adding n-propanol and ammonium sulphate) they managed to produce 800 mg L^{-1} erythromycin.

Bacteriocins

Bacteriocins, synthesized ribosomically by lactic acid bacteria, are peptides that may act as biopreservatives as they exert antimicrobial activity against a range of microorganisms. They constitute a naturally produced eco-friendly tool amendable for the control of the growth of various undesirable microorganisms as they are capable of inhibiting an important range of (principally) Gram-positive bacteria (including food spoilage and food-borne pathogen microorganisms). Metsoviti *et al.* (2011) used waste molasses (with TS_0 concentrations 20 g L^{-1} and 30 g L^{-1}) as growth medium for *Leuconostoc mesenteroides* so as to produce bacteriocin while simultaneous decolorization of the residue up to ~27% was observed.

Animal feed

Molasses can be added as silage to animal feed. Xandé *et al.* (2010) studied the effects of the level of sugarcane molasses on growth and carcass performance of growing pigs reared under a ground sugarcane stalks feeding system finding that the addition of molasses had no particular effect on their carcass. Finally, Bórquez *et al.* (2009) studied the feeding value of ensiling fresh cattle manure with molasses in lambs noting insignificant differences but for a slight enhancement of raw meat quality.

1.3. Bioethanol production

1.3.1. General approaches

Due to the continuous and rapid increase of the world's population and the predominance of industrialization, current need of energy generation, especially deriving from various renewable or non-renewable resources is of significant importance. The decrease of petroleum stock, which up to date remains the main energy source, that constitutes practically a sole non-renewable energy source, as well as its continuously increasing price, have led to the worldwide rise both of the need and the of interest in alternative energy sources. Therefore, utilization of various renewable biofuels as energy sources in engines and heating systems has become of remarkable and continuously growing importance (Bozell, 2004; Shaine-Tyson *et al.*, 2004, Sarkar *et al.*, 2012). The types of renewable biofuels that are currently available in the market are: biodiesel (green diesel) (defined as methyl-, ethyl- or butyl-esters deriving from trans-esterification of vegetable oils, animal fats, waste/cooked oils and microbial lipids), bioethanol [with both types of these fuels already being used for various types of diesel engines and heating systems (Bozell, 2004; Shaine-Tyson *et al.*, 2004)], biogas (defined as a biofuel with a high energy value, basically consisting of methane, produced by anaerobic digestion of agricultural organic waste or manure in rural areas) and biohydrogen (hydrogen produced biologically by mainly algae and bacteria, characterized also as the fuel of the future) (Ginkel and Sung, 2001; Reith *et al.*, 2003; Kapdan and Kargi, 2006; Meher Kotay *et al.*, 2008; Martins das Neves *et al.*, 2009).

Ethanol as fuel is considered as one of the most important renewable energy sources due to its economic and environmental benefits (Cardona Alzate and Sánchez Toro, 2006). Production of ethanol in a large commercial scale by locally available resources can decrease dependence on foreign oil without remarkably high capital investments, while this process can reduce trade benefits and create new employment opportunities (Cardona Alzate and Sánchez Toro, 2006). With its clean burning characteristics (Prasad *et al.*, 2006) ethanol as a fuel can play a significant role in the reduction of the greenhouse gas emissions (Cardona Alzate and Sánchez Toro, 2006; McMillan, 1997; Marchetti *et al.*, 2005) given that it reduces polluting gases, mainly carbon dioxide. Moreover, by using crude agro-industrial residues as raw alcoholic fermentation starting materials, not only alternative substrates are provided but also their disposal problem can be solved. Therefore, the main purpose of research is currently focused upon the commercially high ethanol production by fermentation technology means, using cheap raw materials as carbon source (e.g. agro-industrial wastes or co-products, lingo-cellulose-type materials etc.). Thus, the discovery of new naturally occurring or the "construction" of "new" "over-producing" strains (by the means of

classical mutagenesis or genetic engineering approaches) as well as the optimization of ethanol production in various fermentation configurations (batch, fed-batch, continuous, recycling-continuous operations, or cell immobilization) in order to achieve higher yields, final product concentrations and higher volumetric productivities is of high importance in our days (Rehm and Reed, 1996; Cardona Alzate and Sánchez Toro, 2006). One of the advantages of ethanol as fuel (unlike fossil fuel) is that the raw materials used to produce it are natural and renewable. Moreover, ethanol as a biodegradable and relatively highly soluble in water material, has low toxicity, therefore the consequences of large fuel spilling threaten far less the environment than those associated with crude oil or gasoline (McMillan, 1997).

Fermentation processes of any material that contains carbohydrates can produce ethanol. These can be categorized into four main types of raw materials: sugar (sugar beets, molasses, fruits, sweet sorghum), starch (potato, wheat, oat, rice), lignocellulosic material (agricultural residues, grasses, forestry wastes, sawdust) and urban – industrial wastes (Sun and Cheng, 2002; Prasad *et al.*, 2006; Koutinas *et al*., 2014a). Lignocellulosic biomass and starch may represent cheaper material than simple sugar, however, the energy (therefore the funds) needed for converting the starch or the lignocellulosic biomass into simple fermentable sugars is the major disadvantage concerning the utilization of these substrates (Lin and Tanaka, 2005).

1.3.2. Global ethanol production

Ethanol is an essential chemical which is used as a raw material for a vast range of applications including chemicals, fuel (bioethanol), beverages, pharmaceuticals and cosmetics (Najafpour, 2006). The majority (90-95%) of the percentage of ethanol that is produced globally derives from biological fermentation technology (bioethanol), whereas the rest is produced using ethene (coming from cracking of crude oil and/or natural gas) using steam and phosphoric acid as catalyst (synthetic ethanol). The reaction of synthetic ethanol production also produces toxic by-products, thus is not used for human consumption. Bioethanol as fermentation product stream is processed with subsequent enrichment by distillation/rectification and dehydration.

Global ethanol production peaked in 2010. The United States of America is the world's largest producer of ethanol, having produced about 50 billion liters in 2012 alone. The majority of the world's ethanol is produced by U.S. and Brazil together reaching values from 62% (Kim and Dale, 2004; Balat *et al.*, 2008) to 87% (http://www.afdc.energy.gov/; Fig. 1.2.). The vast majority of U.S. ethanol is produced from corn, while Brazil primarily uses sugar (http://www.afdc.energy.gov/). According to Lin and Tanaka (2006) Brazil has more than 300

ethanol production plants and supplies 3 million cars with pure ethanol. In the global ethanol production, China, India, Eastern Europe, Western Europe and finally Canada are following.

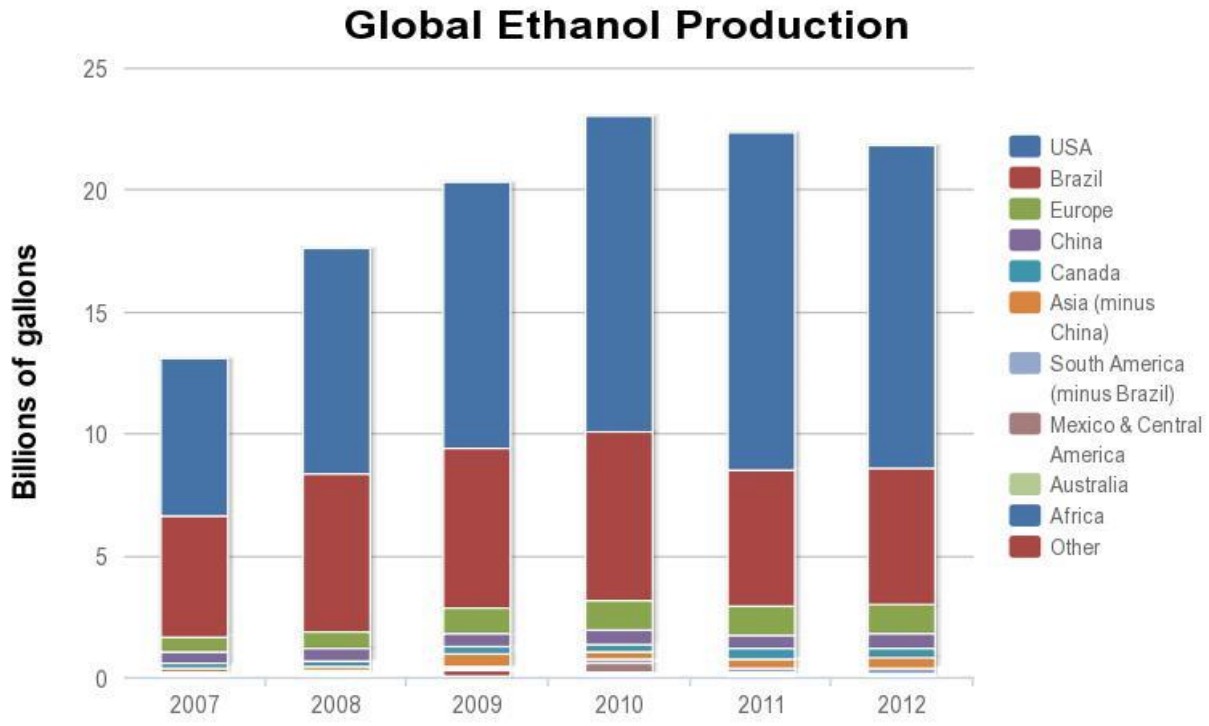


Figure 1. 2. Global ethanol production by country or region (2007-2012) (http://www.afdc.energy.gov/data/tab/all/data_set/10331)

1.3.3. Ethanol as fuel

The majority of the environmental problems are created due to the use of conventional energy sources. The constant enlargement of such problems as also the reduction of fossil energy resources have led to the worldwide rise of the need for alternative renewable energy sources. Therefore, utilization of various renewable biofuels as energy sources in engines and heating systems has become of remarkable and continuously growing importance. Besides the traditional use of ethanol as an essential chemical raw material in a vast range of applications including chemicals, beverages, pharmaceuticals and cosmetics, the latest trend is its use as fuel.

Ethanol fuel can be used as blend with gasoline in percentages from 5% to 85% (Kim and Dale, 2006; Balal *et al.*, 2008; Festel, 2008). In accordance with the EU quality standard EN 228, bioethanol can be used as a 5% blend with petrol without requiring conventional engine modification (Festel, 2008). The most popular blends are known as E85 (85% bioethanol, 15% gasoline), E20 (20% bioethanol, 80% gasoline) and E10 (10% bioethanol, 90% gasoline; called also gasohol in USA) (Kim and Dale, 2006; Balal *et al.*, 2008; Festel, 2008). There is also a mixture

used in Brazil, called gasohol and contains 24% bioethanol blended with 76% gasoline (Balal *et al.*, 2008). Blends having higher concentrations of bioethanol can be used in flexible-fuel vehicles (FFVs) that can operate on mixtures up to 85% bioethanol (E85) (Balal *et al.*, 2008; Festel, 2008). Bioethanol can be also converted into etherized bioethanol (ethyl tertiary butyr ether; ETBE), which can be used as a 15% blend with gasoline (Festel, 2008). Finally, ethanol may be used in the transesterification of vegetable oils for the production of Fatty Acid Ethyl Esters (FAEEs; on of the commonly used forms of biodiesel) (Marchetti *et al.*, 2007).

1.3.4. Raw alcoholic fermentation materials

Besides its chemical synthesis, ethanol is mainly produced by biotechnological means through microbial fermentations. Every raw material that contains sugars or can be transformed into sugars can become a fermentation substrate for the production of ethanol. Various lignocellulosic or starchy materials are considered to be the most suitable raw materials for the industrial production of bioethanol, as they are a natural renewable carbon sources, found in abundance in nature. The basic ingredients of such raw materials are glucose and xylose. On the other hand, it must be pointed out though that principal microbial ethanol producers (*Saccharomyces cerevisiae* and *Zymomonas mobilis*) cannot break down xylose, thus, this point needs to be solved by discovery of xylose-consuming ethanol producers or by the means of genetic engineering (creation of mutan strains expressing the enzymes involved in the catabolism of xylose). Moreover, as far as complex substrates are concerned, in most of the cases a sugar recovery is needed through enzymatic or acid hydrolysis, while, as previously indicated, crude - waste agro-industrial residues could be envisaged as raw alcoholic fermentation materials. Thus, not only alternative substrates are provided but also their disposal problem can be solved. The suitability of the raw material is estimated by its availability, its production volume, the pre-treatment cost (in the case of complex materials used as substrates) and finally by the ethanol production yield on total sugars consumed. In general, the substrates already used in the literature are presented below:

Directly fermentable feedstock: Simple sugars (industrial - raw glucose low-added value product used in confectionary industries, sugar beets, molasses, fruits, fruit juices, sweet sorghum, cane juice, whey permeate, waste-waters containing significant quantities of simple sugars) can readily be utilized as the sole substrate by microbial strains capable to produce ethanol. In this case the process cost is decreased given that the pre-treatment of substrate in many cases is not needed at all (Lin and Tanaka, 2005).

The raw material that is used widely for ethanol fermentation is that of molasses, derived from the sugar industry (Lin and Tanaka, 2005; Prasad *et al.*, 2006). Various types of molasses exist, and these low-added value residues contain about 45-50 % (w/w) of fermentable sugars and 50-55% organic and inorganic compounds (Prasad *et al.*, 2006). In order to carry out the fermentation process, the medium is firstly diluted with water (for reducing the sugar concentration so as to avoid toxicity and, therefore, the abrupt end of the bioprocess due to inhibition exerted by the substrate). After dilution with water, pH adjustment, sterilization and inoculation (with yeasts or bacteria) are needed. Concerning OMWs, there are only a few reports in the literature suggesting the use of such effluents as fermentation process water and substrate directly for the production of ethanol, while either the dilution of this residue or the necessity for removing the phenolic (toxic) fraction by an efficient pre-treatment of the waste before alcoholic fermentation, was considered of primordial importance (Bambalov *et al.*, 1989; Zanichelli *et al.*, 2007; Massadeh and Modallal, 2008). Other media containing simple sugars that have been in the ethanol fermentation were raw glucose, whey-permeate, akalona hydrolysates, contaminated with fungicides grape musts etc. (Roukas, 1994; Lin and Tanaka, 2005; Prasad *et al.*, 2006; Wang *et al.*, 2007; Arifeen *et al.*, 2009; Sarris *et al.*, 2009). It is noted that the fermentation cost of sterilization is significantly high and there would have been a significant benefit in the process if fermentations that do not include sterilization step could have been carried out (Αγγελής, 2007).

Starch: Starch, an abundant and important substrate, is the polymer of glucose and it first needs to be converted into glucose (saccharification), which will be used by microorganisms to produce ethanol. It is composed by the polymers amylose and amylopectin in different proportion based on its source. Glucose linked mainly in linear chains by α-1,4 bonds gives the amylose polymer whereas amylopectin is a highly branch polymer of glucose also including at the branch point α-1,6 bonds.

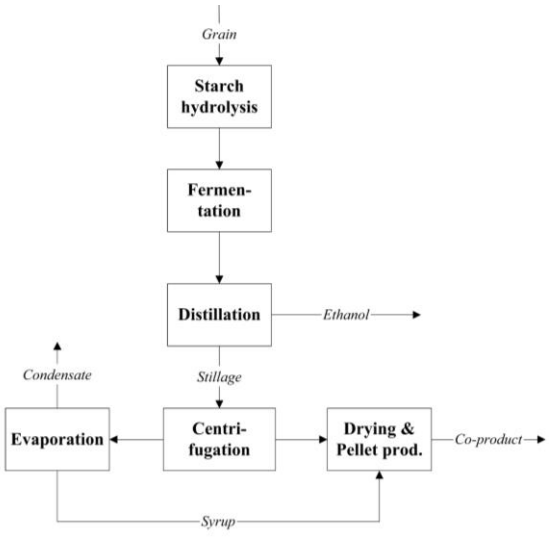


Figure 1. 3. Flow chart of ethanol production from starch-containing materials (Galbe *et al.*, 2007)

Starch has to be broken down by the combination of two enzymes. It is first hydrolyzed by the enzyme α-amylase, an endo-amylase that attacks α-1,4 bonds having as a result the reduction of starch

molecular size. The starch is then cooked at high temperature (140 - 180 C) and in its liquefied form, the exo-amylase enzyme glucoamylase hydrolyzes amylose and amylopectin chains to produce glucose.

Microorganisms in general prefer saccharified starch but this needs a high amount of energy to be consumed, thus, research is focused upon the production and study of enzymes (therefore microorganisms, mainly fungi) that are able of degrading raw (non saccharified) starch (Prasad *et al.*, 2006). It should be also noted that various approaches in genetic engineering level include insertion of genes encoding for the synthesis of enzymes capable of breaking down starch (e. g. α-amylase, isoamylase, amyloglucosidase) in the genome of microorganisms carrying out alcoholic fermentation process (e. g. *Zymomonas mobilis*, *Saccharomyces* spp.) so that to obtain simultaneously and by one and the same microorganism the secretion of hydrolytic enzymes and the production of ethanol. Industrial ethanol production could take place using starchy materials as corn, wheat, potatoes, cassava root etc.. Ethanol production from corn might not be practical because of the competition with the agricultural land needed for food production. Schematically, the production of ethanol by starchy materials is depicted in Fig. 1.3.

Lignocellulosic biomass

Lignocellulosic biomass includes wood (wood chips, forestry wastes), agricultural crop residues, bagasse, grass, straw, ground nut shell, sawdust, cotton, rapeseed, mustard, mulberry, sunflower stalks etc.. It is an alternative energy source as it is renewable and available throughout the world (Sun and Cheng, 2002). Therefore it represents an enormous and zero (or even negative) cost raw material compared with directly fermentable feedstock (sugars) and a competitive biomass resource as compared with the starchy materials (mainly corn). Therefore, lignocellulosic biomass is a promising resource for various fermentation technologies including bioethanol production. Lignocellulose is a more complex substrate than starch. It is a mixture of lignin (non fermentable phenylopropene) and the carbohydrate polymers cellulose (glucose polymer) and hemicellulose (sugar heteropolymer). The carbohydrate polymers have tight hydrogen bonds with lignin, which represents a physical barrier to be removed from them so as to be available for further transformations. The three steps of the biological process for converting the lignocellulosic biomass to ethanol are as follows: delignification which sets free the cellulose and hemicellulose from lignin, depolymerization which produces free sugars and fermentation of the hexoses and pentoses blends for the production of ethanol, having as most difficult and, therefore, limiting step the delignification process (Lin and Tanaka, 2005; Prasad *et al.*, 2006).

In order for lignocellulosic biomass to be utilized, it needs to be pretreated so as to increase surface area and porosity of the material, to increase the bulk density and decrease the crystallinity of the cellulose to make it accessible for hydrolysis (Prasad *et al.*, 2006; Sun and Cheng, 2002). Pretreatment is the most important processing challenge in the production of ethanol, being one of the most costly steps and is of crucial importance because it influences the whole ethanol production process. There are various techniques that can be used for this scope, some of which are going to be briefly mentioned in the following paragraphs. The lignocellulosic biomass pretreatment can be divided into four main categories: physical, physicochemical, chemical and biological (Sun and Cheng, 2002; Sarkar *et al.*, 2012).

Pyrolysis is a physical endothermic process which includes the material treatment at temperatures higher than 300 °C leading to the rapid decomposition of cellulose towards gaseous products and residual char (Prasad *et al.*, 2006; Sun and Cheng, 2002). Following, a mild acid hydrolysis has as a result the conversion of the pyrolysis residues (cellulose) into reducing sugars.

Mechanical size reduction (usually wet or dry milling, vibratory ball milling, compression milling) is another physical pretreatment. It is a process that reduces cellulose crystallinity and improves the efficiency of downstream process (Sarkar *et al.*, 2012).

Steam explosion (or autohydrolysis) is a physicochemical pretreatment that is commonly used over lignocellulosic biomass and makes it more accessible to cellulase attack (McMillan, 1997; Sun and Cheng, 2002; Sarkar *et al.*, 2012). This process includes the treatment of chipped biomass with high- pressure saturated steam and the following pressure reduction, which leads to the explosive decompression of the materials (Prasad *et al.*, 2006). As a result, lignin is transformed and hemicellulose is lead to degradation. This is a fact that increases the potential cellulose hydrolysis. By adding H_2SO_4, SO_2 or CO_2 the production of inhibitory compounds decreases and improvement of enzymatic hydrolysis occurs. Steam explosion requires lower energy compared to conventional mechanical methods but it also includes the incomplete disruption of the lignin-carbohydrate matrix and might produce inhibitory of the process compounds to the microorganisms, therefore, treated biomass might need to be washed with water (Sun and Cheng, 2002).

Ammonia fiber explosion (AFEX) is also a physicochemical (alkaline thermal) pretreatment of lignocellulosic biomass. Lignocellulose resources are exposed to liquid ammonia under pressure at high temperature for certain period of time and rapid pressure release follows. The above process improves the saccharification rates of various crops. An advantage for this method could be mentioned the fact that it does not produce inhibitors for the rest of the process, and, hence, washing with water is not necessary (Prasad *et al.*, 2006; Sun and Cheng, 2002). For reducing the cost and for protecting the environment, ammonia should be recycled at the end of the pretreatment.

Carbon dioxide (CO_2) explosion is the third physicochemical pretreatment similar to the other two (however more cost effective than AFEX, not causing formation of inhibitors and with higher conversion yields compared to steam explosion) which is based on the formation of carbonic acid leading to the increase of the hydrolysis rate.

Ozonolysis, acid hydrolysis, alkaline hydrolysis, oxidative delignification and organosolv process can be categorized as chemical pretreatment processes of lignocellulosic biomass. Such methods are easy in operation and demonstrate good conversion yields in short period of time (Sarkar *et al.*, 2012). Precisely, ozonolysis is mainly focused upon degradation of the lignin and hemicellulose whereas it can hardly affect cellulose. The application of mild operating conditions (e. g. room temperature and low pressure reaction conditions) constitutes great advantage of this method. Moreover, ozonolysis removes effectively the lignin without producing toxic inhibitors for the rest of the process. In acid hydrolysis, concentrated, powerful, corrosive and hazardous acids such as H_2SO_4 and HCl are used. They demand corrosion resistant reactors as also their recovery for financial reasons. A variation of the method is dilute acid hydrolysis (like cellulose hydrolysis) that has less severe process conditions and improves the reaction rates (Prasad *et al.*, 2006). It has been demonstrated that this method achieves high conversion yields of xylan into xylose. This fact affects the process financially, since xylan constitutes almost the one third of the total carbohydrate in various lignocellulosic biomass (Sun and Cheng, 2002). pH should be neutralized before the sequence of the process. On the other hand, a major disadvantage of this process refers to the fact that this pretreatment results in the production of several growth inhibitors of microorganisms such as acetic acid, furfural and 5 hydroxymethylfurfural (Sarkar et al, 2012). Alkaline hydrolysis digests the lignin matrix, producing cellulose and hemicellulose amenable to be subjected to enzymatic degradation (Sarkar *et al.*, 2012). This mechanism is based on the use of bases for the saponification of intermolecular ester bonds cross-linking xylan, hemicellulose and various components such as lignin and hemicellulose (Sun and Cheng, 2002). An increase of the internal surface area and, at the same time, a decrease in the crystallinity and the degree of polymerization as also the separation disruption of the lignin structure and separation of structural linkages between carbohydrates and lignin can be caused by dilute NaOH treatment (Sun and Cheng, 2002). The use of alkaline chemicals for improving the cellulose digestibility is supposed to be effective but not of financial interest (recovery and recycling of bases) for the production of fuel. In the oxidative delignification the pretreatment is done with the aid of hydrogen peroxide (H_2O_2), which catalyses the lignin biodegradation. Finally, the ogranosolv (or organic solvent) chemical process breaks down the internal lignin and hemicellulose bonds with the aid of a mixture consisted of an organic or aqueous

solvent with inorganic acid catalysts (Sun and Cheng, 2002). For reducing the process cost, the solvents used should be removed (for avoiding also inhibitory actions) and recycled.

Ending, the fourth main pretreatment category is biological pretreatment, where fungi are used to degrade hemicellulose and lignin that are contained in several waste and residue materials. Certain genera of fungi have been reported capable of breaking down cellulose (brown rot fungi). Others attack both cellulose and lignin (white and soft rot fungi) (Sun and Cheng, 2002; Prasad *et al.*, 2006). The essential enzymes secreted and involved in the biodegradation lignin compounds during cultivation of various molds lignocellulosic materials are laccase, lignin-peroxidase, manganese-independent peroxidase and manganese-dependent peroxidase, the secretion of which is strain-dependent and is influenced by various culture conditions (Sayadi and Ellouz, 1992; 1995; Tsioulpas *et al.*, 2002; Aggelis *et al.*, 2002; Wesenberg *et al.*, 2003; Ayed *et al.*, 2005). The mild environmental conditions as also the low energy requirement are the advantages of this process. On the other hand, serious disadvantages of the biological process, constituting in fact major limiting steps, refer to the very low rate of hydrolysis and the low yields as well as the fact that a limited number of naturally occurring microorganisms is capable of breaking down these compounds (Sun and Cheng, 2002; Prasad *et al.*, 2006).

Simultaneous saccharification and fermentation (SSF)

In simultaneous saccharification and fermentation (SSF) process, enzymatic hydrolysis and alcoholic fermentation are combined. Glucose is fermented to ethanol as soon as it appears in the solution thus the concentration of the substrate glucose is kept low. Hydrolysis products (glucose and short cellulose chains) inhibit strongly cellulase, the responsible enzyme for the enzymatic hydrolysis of pretreated cellulose. SSF achieves the removal of the end-product inhibition, eliminates the need for separate reactors (one for saccharification and another for fermentation) and reduces the fermentation time (Αγγελής, 2007). It reduces the contamination risk of external microflora because of high process temperature, anaerobic conditions and ethanol presence in the reaction medium. It requires lower amounts of enzyme. On the other hand the different optimum temperature needed for the process of hydrolysis and that of fermentation as also the ethanol itself (as an inhibitor in the fermenting action of microorganisms and cellulose activity) introduce the method's disadvantages. More method disadvantages are the low rates of cellulose hydrolysis and the fact that most of the microorganisms used in the procedure cannot utilize xylose, the hemicellulose hydrolysis product (Lin and Tanaka, 2005; Αγγελής, 2007). Hence this procedure requires research to increase the rate of hydrolysis something that will reduce the ethanol production cost.

1.3.5. Biochemistry of alcoholic fermentation

Fungi, including yeasts draw their necessary energy from organic nutrients degradation. The free energy (the rest is dissipated in the form of heat) produced by degradation reactions of a substrate (catabolism) that is transferred to the chain of synthesis reaction (anabolism). Part of this free energy may be used for active transport, movement or biosynthesis. The free energy transporter is the rich in energy molecule adenosine triphosphate (ATP). The hydrolysis of ATP into adenosine diphosphate (ADP) results in the liberation of a large quantity of free energy (7.3 kcal/mol). Microbial growth as well as other anabolic activities are directly related to the quantity of ATP furnished by catabolic pathways, thus, ATP is considered to be "the universal money of free energy" (Ribéreau-Gayon *et al.*, 2006).

The processes which produce ATP are substrate-level phosphorylation and oxidative phosphorylation. Substrate-level phosphorylation takes place during glycolysis and can be either aerobic or anaerobic. During oxidation by electron loss, an ester - phosphoric bond is formed. This energy-rich bond between the oxidized carbon of the substrate and a molecule of inorganic phosphate, is then transferred to the ADP by transphosphorylation, thus forming ATP. Oxidative phosphorylation is an aerobic process and occurs in the mitochondria. The production of ATP is linked to the transport of electrons to an oxygen molecule by the cytochromic respiratory chain. This oxygen molecule is the final acceptor of the electrons (Ribéreau-Gayon *et al.*, 2006).

Alcoholic fermentation biochemistry includes sugar degradation pathways (glycolysis, alcoholic fermentation, glyceropyruvic fermentation, respiration) and regulation between fermentation and respiration (Pasteur effect and Crabtree effect). Matters of alcoholic fermentation biochemistry have been studied in depth by many. In this chapter, the basic steps referred to the present study will be presented briefly.

1.3.5.1. Glycolysis / Embdem-Meyenhorf-Parnas (EMP) pathway

Glycolysis or Embdem-Meyenhorf-Parnas (EMP) pathway, a series of reactions that take place completely in the cytosol, is the process of intracellular transformation of glucose (and fructose) into pyruvate together with the formation of ATP.

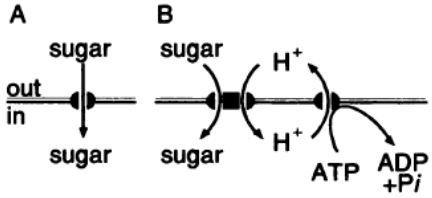


Figure 1. 4. Schematic representation of two mechanisms of sugar transport in yeasts
Weusthuis *et al.* (1994)

Glycolysis involves a sequence of 11 chemical reactions for breaking down hexoses and releasing energy in the chemical form of ATP (Fig. 1.5.). Initially, hexoses are transported inside the cell by facilitated diffusion without the need of energy consumption (not an active transport system, as the inner sugar concentration is lower than the external sugar

concentration; Fig. 1.4. A). When internal and external solution concentrations are equal and therefore the driving force for sugar uptake becomes zero, this process does not allow the uptake of sugars against a concentration gradient. For instance, during growth at very low extracellular sugar concentrations, intracellular accumulation of sugars may be necessary to allow the cytoplasmic sugar kinases and disaccharide hydrolases to function optimally. This can be accomplished by coupling the uptake of a sugar molecule to the uptake of one or more protons via proton symport systems. Thus, the proton motive force over the plasma membrane can be used to drive intracellular accumulation of sugar. This proton motive force is generated mainly by the plasma membrane H^+-ATPase complex, which couples the hydrolysis of ATP to ADP plus Pi to the outward translocation of protons (Fig. 1.4. B).

The first stage of glycolysis is the conversion of glucose into fructose 1,6-biphosphate, requiring 2 ATP molecules, which comprises three steps. An initial phosphorylation of glucose towards the formation of glucose 6-phosphate, catalyzed by a family of enzymes called hexokinases (PI hexokinase is not active until stationary phase, as it is repressed partially by glucose; PII hexokinase is essential and predominantly active during the log phase in a high sugar concentration medium). This reaction consumes ATP, but it keeps the intracellular hexose concentration low and thus favors the continuous transport of sugars into the cell through the plasma membrane transporters. Afterwards, the isomerization of glucose 6-phosphate into fructose-6-phosphate by phosphoglucose isomerase takes place, followed by the phosphorylation of fructose-6-phosphate by the action of phosphofructokinase, forming fructose-1,6-biphosphate and consuming one ATP molecule.

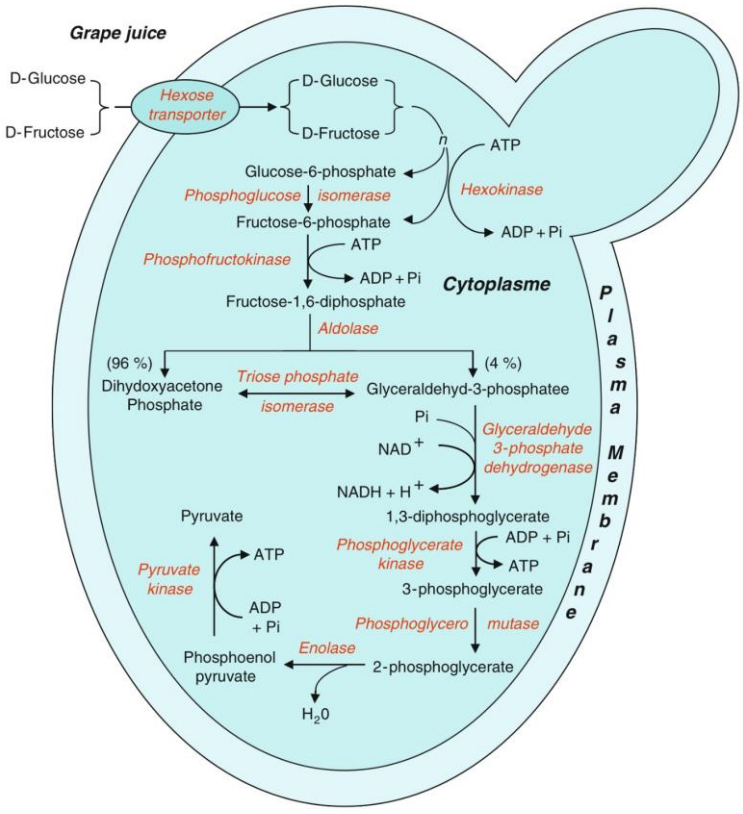


Figure 1. 5. Biochemical mechanism of glycolysis (Zamora, 2009)

At the second stage of glycolysis, two triose phosphate isomers named glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate are formed when fructose 1,6-biphosphate is

cleaved by the catalytic action of aldolase. This reaction produces a much greater proportion of dihydroxyacetone phosphate (96%), which is rapidly transformed into G3P by triose phosphate isomerase (Αγγελής, 2007).

The third step of glycolysis includes the transformation of G3P into 3-phosphoglycerate, recovering part of the energy from G3P. Initially G3P is converted by 1,3-biphosphoglycerate (1,3-BGP) catalyzed by glyceraldehyde 3-phosphate dehydrogenase. This reaction involves the oxidation (coupled with a substrate-level phosphorylation) of the molecule that is linked to reducing NAD^+ (the cofactor of dehydrogenation) to NADH in order to redress the redox balance. An energy rich bond between the oxidized carbon group and inorganic phosphate is formed by the substrate-level phosphorylation. The NAD^+ accepts two electrons and a hydrogen atom lost by the oxidized substrate. Phosphoglycerate kinase catalyzes the transfer of the phosphoryl group of the acylphosphate from 1,3-BPG to ADP. Finally, 3-phosphoglycerate and ATP are formed. This reaction, releases all the energy contained in the previously formed energy-rich bond, which the cell uses to phosphorylate one molecule of ADP into ATP. The last step of glycolysis transforms 3-phosphoglycerate into pyruvate which is the final product of glucolysis. Phosphoglycero-mutase catalyzes the conversion of 3-phospho-glycerate into 2-phosphoglycerate which is dehydrated by the enzyme enolase, forming phosphoenolpyruvate. This compound has a high phosphoryl group transfer potential (energy-rich bond). The enzyme pyruvate kinase catalyzes the phosphorylation of ADP, forming pyruvic acid and ATP (Ratledge, 1991; Μπαλατσούρας, 1993; Αγγελής, 2007). In this manner, from one molecule of glucose, glycolysis creates two molecules of pyruvic acid, four of ATP and one of NADH. Since two molecules of ATP are immediately used to activate a new hexose molecule, the net energy gain of glycolysis for the cell is therefore two ATP molecules per molecule of hexose metabolized. Pyruvate produced by glycolysis can be used by yeasts for several metabolic pathways. However, the microorganism must regenerate NAD^+ from the NADH to reestablish the oxydoreduction potential of the cell. This can be done by fermentation or respiration. This stage marks the end of the common trunk of glycolysis. Alcoholic fermentation, glyceropyruvic fermentation or respiration follow, depending on various conditions (Weusthuis *et al.*, 1994; Ribéreau-Gayon *et al.*, 2006; Zamora, 2009).

It should be stressed out that the microorganism *Zymomonas mobilis* catabolizes the sugar substrate (for the production of ethanol) through the Entner-Dudoroff glycolysis pathway whereas, yeast strains in general break down sugar substrates through the EMP glycolysis pathway (for more information see paragraph 1.3.5.6.).

1.3.5.2. Alcoholic fermentation

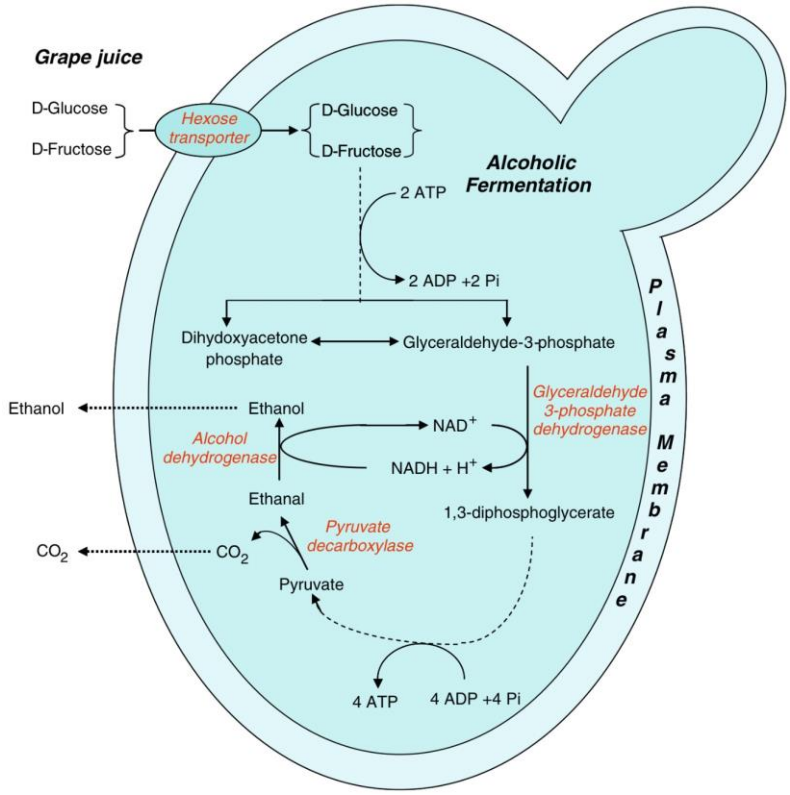


Figure 1. 6. Biochemical mechanism of alcoholic fermentation (Zamora, 2009)

The reducing power of NADH produced through glycolysis, must be transferred to an electron acceptor to regenerate NAD^+ consumed by glycolysis. In the case of *Saccharomyces cerevisiae* and other yeasts species, this process is called alcoholic fermentation and takes place within the cytoplasm (Fig. 1.6.). In the alcoholic fermentation, acetaldehyde (ethanal; the decarboxylation product of pyruvate acid) serves as the terminal electron acceptor (Zamora, 2009). With respect to glycolysis, alcoholic fermentation contains two additional enzymatic reactions. Pyruvate is initially decarboxylated into acetaldehyde by pyruvate decarboxylase. The cofactors are thiamine pyrophosphate (TPP) and magnesium. TPP and pyruvate form an intermediary compound, by the ionization of the carbon atom located between the nitrogen and the sulfur of the TPP thiazole cycle. A carbanion is formed and readily combined with the pyruvate carbonyl group. Following, acetaldehyde is reduced into ethanol recycling NADH to NAD^+. This reaction is catalyzed by the alcohol dehydrogenase using zinc as cofactor. Both final products of alcoholic fermentation, ethanol and carbon dioxide, are transported outside the cell by simple diffusion (Ribéreau-Gayon *et al.*, 2006; Αγγελής, 2007; Zamora, 2009).

The microorganism, besides having the production of ethanol as the most important pathway to regenerate NAD^+, it can also use alternative metabolic pathways for this purpose. The main pathway used in *Saccharomyces cerevisiae* strains is called glyceropyruvic fermentation and generates glycerol as its final product.

1.3.5.3. Glyceropyruvic fermentation

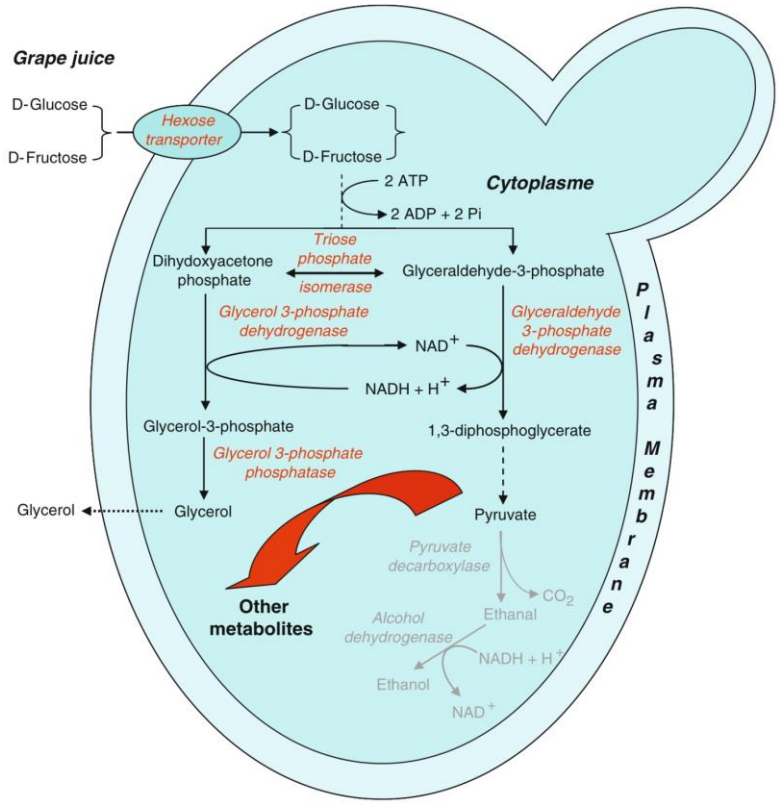


Figure 1. 7. Glyceropyruvic fermentation (Zamora, 2009)

As mentioned above, besides production of ethanol as the most important pathway to regenerate NAD^+, glyceropyruvic fermentation is the alternative pathway for this purpose (Fig. 1.7.) generating glycerol as its final product (Ribéreau-Gayon *et al.*, 2006; Αγγελής, 2007). Initially, glyceropyruvic fermentation was described through the effect of sulphites which resulted in an increased glycerol production. Specifically, sulphite combines with acetaldehyde which then prevents NAD^+ from regenerating via alcohol dehydrogenase. Under these conditions, the yeasts need to oxide NADH through an alternative pathway in order to compensate for the NAD^+ deficit, and the only way to do so, is by producing glycerol. Dihydroxyacetone phosphate (the main product of aldolase reaction) is oxidized to glycerol-3-phosphate (by the enzyme glycerol-3-phosphate dehydrogenase) and a molecule of NADH is simultaneously oxidized to NAD^+. Following, the enzyme glycerol-3-phosphate phosphatase catalyzes the production of glycerol by dephosphorylating glycerol-3-phosphate. The production of glycerol consumes ATP but it is necessary to compensating for the redox imbalance in the cell.

Glyceropyruvic fermentation besides being affected by the presence of sulphites, it can also be active in other situations. At the first stages of fermenation, when yeasts perform a very active growth rate accompanied by the biosynthesis of proteins, lipids and nucleotides, glyceropyruvic fermentation ocurrs preferentially. Most of the aforementioned biomolecules are synthesised using pyruvate as substrate. Each time a molecule of pyruvate is used anabolically, a NAD^+ deficit is created which must be recovered through the glyceropyruvic pathway. For this reason, glycerol is mainly produced during the first steps of alcoholic fermentation, when yeasts are growing and they need a large proportion of pyruvate to increase their biomass. Furthermore, yeasts produce glycerol as a protector against high osmotic pressures (Ribéreau-Gayon *et al.*, 2006; Zamora, 2009).

1.3.5.4. Respiration

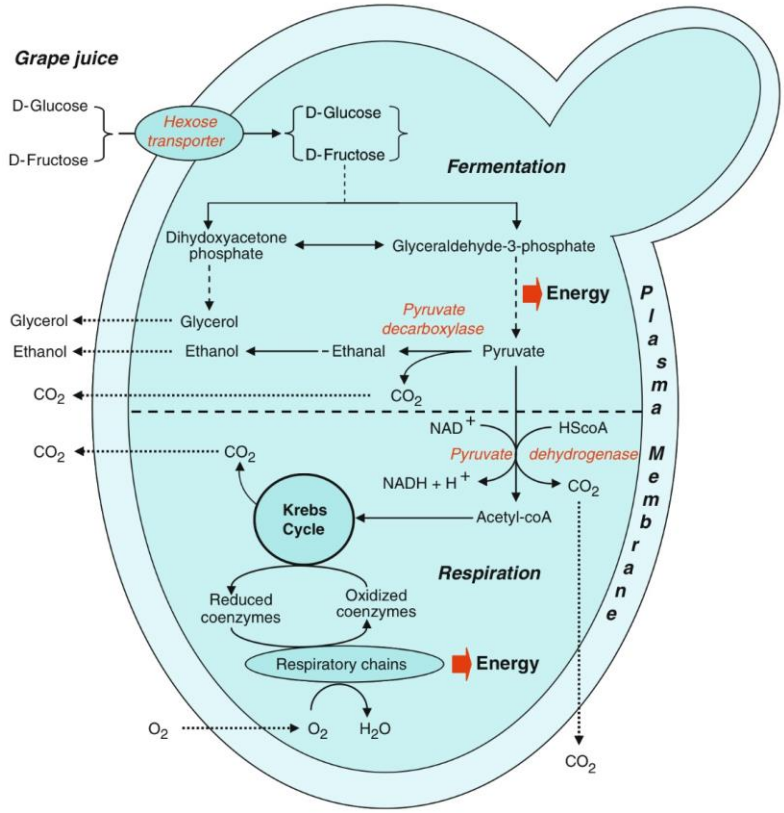


Figure 1. 8. Fermentation and respiration (Zamora, 2009)

Yeasts are facultative anaerobic microorganisms because they possess the genetic equipment for metabolizing sugars aerobically or anaerobically. Therefore, sugars can be consumed also by respiration (Zamora, 2009) (Fig. 1.8.). Respiration, just like fermentation, begins with glycolysis generating pyruvate as a final product. When sugar is used by the respiratory pathway, pyruvic acid undergoes an oxidative decarboxylation, catalyzed by pyruvate dehydrogenase in the interior of mitochondria. Thiamine pyrophosphate (TPP), lipoamide and flavin–adenine dinucleotide (FAD) serve as catalytic cofactors (Αγγελής, 2007). This reaction reduces NAD^+ to NADH and must incorporate acetyl coenzyme A (acetyl-CoA) (the acetyl unit issued from pyruvate is activated in the form of acetyl-CoA). Acetyl-CoA can then be incorporated to the citric acid cycle (also called the tricarboxylic acids cycle and Krebs cycle), completely oxidized into carbon dioxide and producing molecules of reduced coenzymes (NADH and FADH). These reactions also occur in the mitochondria (Αγγελής, 2007). The reduced coenzymes produced by the citric acid cycle and also by glycolysis, are later reoxidized in the respiratory chains, reducing molecular oxygen to water. The respiration of 1 mole of glucose yields an overall energy gain of 36-38 moles of ATP (Ratledge, 1991; Αγγελής, 2007). The respiration of the same amount of sugar produces 18 to 19 times more biologically usable energy available to yeasts than fermentation meaning that is more beneficial in terms of energy. However, it needs oxygen as a substrate and it is inhibited by high sugar concentration. In industry, respiration is used for biomass production. The transformation of pyruvate into acetaldehyde or acetyl-CoA is therefore a key point for regulating yeast metabolism (Ribéreau-Gayon *et al.*, 2006; Zamora, 2009).

1.3.5.5. Regulation between respiration and fermentation: Pasteur effect and Crabtree effect

According to Pasteur effect, alcoholic fermentation is inhibited by respiration. For low concentrations of glucose on culture media (where glucose does not suppress oxidative phosphorylation), yeasts utilize sugars through either respiration or fermentation. The presence of oxygen, though induces the respiratory chain and the production of biomass but simultaneously decreases the kinetics of sugar fermentation and simultaneous ethanol production. These conditions are used for the industrial production of selected dry yeast (Αγγελής, 2007). On the other hand, low concentrations of oxygen result in the catabolite repression of the first enzymes of the Krebs cycle with simultaneous increase of enzymes-keys of the EMP glycolysis (e.g. phospho-fructokinase), resulting in the direction of the cellular metabolism towards ethanol production. This is the so-called "Pasteur effect", while yeast species are generally categorized as either Pasteur positive or negative (Ratledge, 1991; Αγγελής, 2007). In fact, two are the enzymes that compete to catalyze either the respiration or fermentation of pyruvate, namely pyruvate decarboxylase and pyruvate dehydrogenase. This competition explains the respiratory inhibition of fermentation. The pyruvate decarboxylase is involved in the fermentative pathway. It has a lower affinity towards pyruvate than pyruvate dehydrogenase. Respiration needs very high amounts of ADP and inorganic phosphate, which migrate to the mitochondria, as subtract for oxidative phosphorylation. Therefore, when respiration takes place, the cytoplasm lacks ADP and inorganic phosphate. This in turn can limit the phosphorylation and thus slows the transmembrane sugar transport.

Once the microorganism starts to consume sugars, large quantities of carbon dioxide are produced, the oxygen is displaced and semi anaerobic conditions that favor fermentation are created. However, even in the presence of oxygen, if sugar concentration is higher than c. 9 g L^{-1} respiration is impossible and the microorganism only metabolizes sugars by the fermentative pathway. This phenomenon is known as the Crabtree effect, Pasteur contrary effect and as catabolic repression by glucose. In a high sugar concentration the mitochondria degenerate, the proportion of cellular sterols and fatty acids decreases and both the enzymes of citric acid cycle and the constituents of respiratory chains are repressed. Therefore, *S. cerevisiae* can only use respiration when the sugar concentration is really low and when oxygen is present in the medium (Boulton *et al.*, 1996; Ribéreau-Gayon *et al.*, 2006; Zamora *et al.*, 2009).

1.3.5.6. Ethanol production by the bacterial strain Zymomonas mobilis

Besides eukaryotic microorganisms such as *Saccharomyces cerevisiae, Saccharomyces uvarum* (*carlbergensis*), *Saccharomyces rouxii, Kluyveromyces fragilis, Kluyveromyces lactis, Candida tropicalis, Candida oleophila, Hanseniaspora* spp. and *Mucor rouxianus* that have been

reported capable of producing alcohol in various culture conditions and configurations, ethanol can be produced by various prokaryotic microorganisms belonging to the species *Zymomonas mobilis*, *Clostridium acetobutylicum*, *Clostridium thermosaccharolyticum*, *Clostridium sporogenes*, *Thermoanaerobacter ethanolicus* and *Bacillus stearothermophilus* (Αγγελής, 2007).

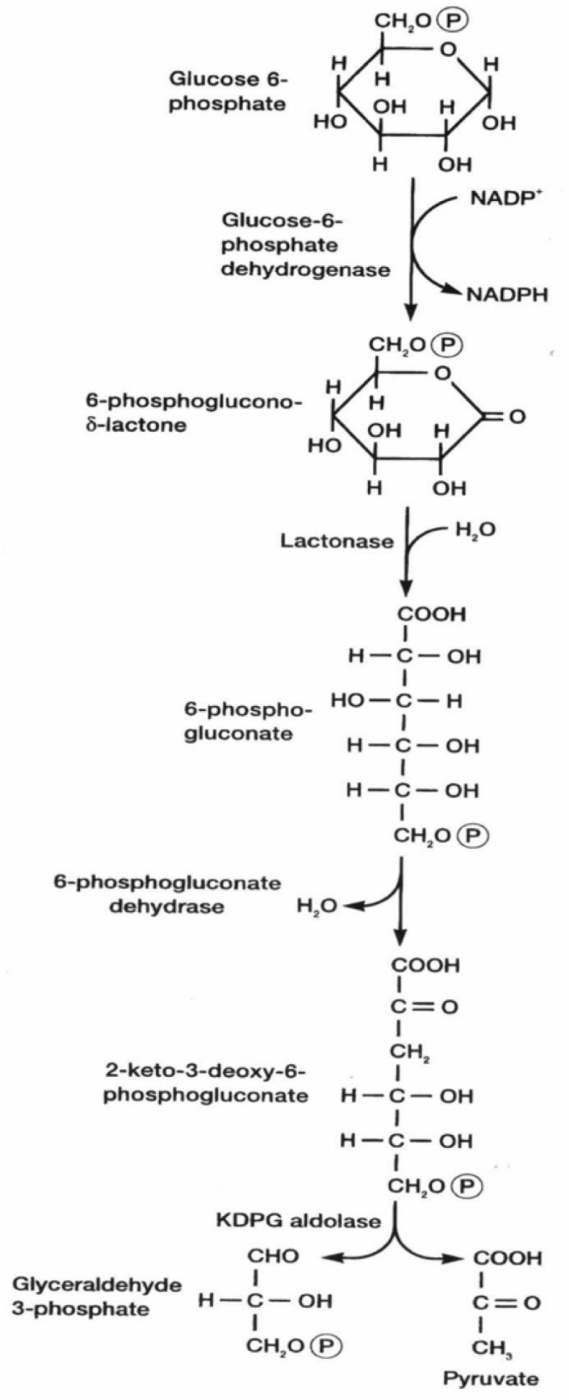


Figure 1. 9. Entner – Dudoroff pathway carried out by strains of *Zymomonas mobilis* (Rehm and Reed, 1996)

Given that in an industrial point of view bioethanol has been in general produced by strains belonging to the species *Zymomonas mobilis* and *Saccharomyces* spp. (usual species that are mentioned in the literature to produce large amounts of ethanol are *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* - *uvarum*), the present part will focus upon the biosynthesis of ethanol by *Zymomonas mobilis*. Like the yeast *Saccharomyces* spp., the bacterium *Zymomonas mobilis* is incapable of metabolizing pentoses (e. g. xylose or urban, industrial, or other agro-industrial wastes containing xylose), hence, genetically engineered microorganisms of the above species capable of breaking down xylose are needed in order to proceed with this fermentation (Sun and Cheng, 2002; Prasad *et al.*, 2006). *Zymomonas mobilis* produces ethanol under anaerobic conditions whereas *Saccharomyces* spp. produces ethanol under both aerobic and anaerobic conditions. The reaction efficiency in both instances is the same, giving generation of 2 moles of ethanol and 2 moles of carbon dioxide from 1 mole of catabolized sugar (Rehm and Reed, 1996). Fundamental biochemical differences exist between the fermentation carried out by *Zymomonas mobilis* compared with the one conducted by the yeast strains. The microorganism *Zymomonas mobilis* catabolizes the sugar substrate through the Entner-Dudoroff glycolysis pathway (Fig. 1.9.) whereas, yeast strains – as mentioned above – in general break down sugar substrates

through the EMP glycolysis pathway (Fig. 1.5.) (Rehm and Reed, 1996; Αγγελής, 2007; Koutinas *et al.*, 2014a).

From the above analysis (see also Figs. 1.5. and 1.8.) it can be deduced that in the fermentation carried out by *Zymomonas mobilis* strains, for 1 mol of catabolized hexose there is a generation of 1 mole of ATP produced, whilst in the conversion carried out by yeast strains, 2 moles of ATP are produced per mole of hexose catabolized. The above fact has as a consequence, hence, production of less biomass when *Zymomonas mobilis* strains are used compared with the fermentation carried out by yeasts. In contrast, as it has been already reported (and can easily been observed by Figs. 1.5. and 1.8.) no alterations in the final spectrum of end-fermentation products is observed, given that in both instances the stoichiometric reaction is the same, giving generation of 2 moles of ethanol and 2 moles of carbon dioxide from 1 mole of catabolized sugar, the maximum theoretical yield, hence, in both cases (utilization of both *Zymomonas mobilis* or yeast strains) is ~0.51 g of ethanol produced per 1 g of sugar consumed (Rehm and Reed, 1996; Lin and Tanaka, 2005; Αγγελής, 2007; Koutinas *et al*., 2014a).

Various other biochemical differences are observed between the fermentation carried out by *Zymomonas mobilis* or yeast strains, given that in the former case the metabolism of *Zymomonas mobilis* can also lead to the production of fermentation by-products such as mannitol, sorvitol, dihydroxy-acetone and, principally, the exo-polysaccharide levane, whilst *Saccharomyces* spp. strains produce (besides ethanol) mainly glycerol (Αγγελής, 2007). It is also noted that pyruvic acid decarboxylase of *Zymomonas mobilis* strains is not common (it does not require as activation co-factor pyrophosphate thiamine - TPP) while in bacterial membranes the presence of various opanoids (specific triterpenoids), the biosynthesis of which is not at all related with some oxygenation of the culture medium, even in the early fermentation steps, is common. Additionally, uncommon cellular fatty acids (e.g. $^{\Delta 11}$C18:1) are detected in the membranes of *Zymomonas mobilis* strains (Αγγελής, 2007). In contrast, the necessity of slight oxygenation even at the early growth stage is obligatory for the case of *Saccharomyces* spp. strains, in order for the yeasts to synthesize their cellular unsaturated fatty acids [e.g. $^{\Delta 9}$C16:1, $^{\Delta 9}$C18:1, $^{\Delta 9,12}$C18:2 - it is known that reactions of dehydrogenation occur only under aerobiosic conditions (Ratledge, 1994; Papanikolaou and Aggelis, 2010)], while unusual cellular fatty acids are not common (Ratledge, 1994). Yeast strains also present in non-negligible quantities ergosterol in their total cellular lipids, given that this component provides cellular stability and tolerance against ethanol (Αγγελής, 2007). Both *Zymomonas mobilis* and *Saccharomyces* spp. strains can tolerate up to 120 - 140 g L^{-1} ethanol (Lin and Tanaka, 2005; Αγγελής, 2007; Koutinas *et al.*, 2014a).

It should be also mentioned that the yeast strains capable of producing ethanol by fermentation, present a so called diauxic growth, if oxygen is found into the growth medium. Given that they metabolize available sugar by fermentation, biomass is created and after depletion of sugar from the growth medium ethanol is oxidized for the production of new biomass (Αγγελής, 2007; Piškur *et al.*, 2006). Two different maximum specific growth rates (μ_{max}) are observed: the first one corresponds to glucose fermentation and the second one to ethanol oxidation (Αγγελής, 2007). Also, as previously stressed, in the conversion carried out by the yeast strains, the production of ethanol is critically affected by the initial glucose concentration in the growth medium, given that high sugar concentrations causes catabolic repression of the first enzymes of the citric acid cycle (e. g. iso-citrate dehydrogenases), resulting, thus, to direction of cellular metabolism towards the fermentation of ethanol instead of glucose oxidation (Crabtree effect - see: Ratledge 1991).

1.4. Secondary metabolites by *Yarrowia lipolytica*: Citric acid and Single Cell Oil (SCO)

1.4.1. Citric acid production

Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid), is an intermediate organic compound (six-carbon tricarboxylic acid) in the tricarboxylic acid (TCA) cycle when carbohydrates are oxidized into carbon dioxide. It is found naturally in citrus fruits (such as lemon, orange), pineapples, plums and pears, in the seeds of different vegetables, in animal bone, muscle, and blood and crystallized as calcium citrate (Najafpour, 2006; Roukas, 2006). Citric acid is a commodity chemical produced and consumed throughout the world with over 1.6 million tones production in 2007 (Berovic and Legisa, 2007), with China accounting for 35-40% of its worldwide production (Soccol *et al.*, 2006). It is used mainly in the food and beverage industry, primarily as an acidulate. It is an important chemical used as pharmaceutical as also in other industrial uses such as the manufacture of ink and dyes, in cosmetics, toiletries and in detergents and cleaning products (Najafpour, 2006; Soccol *et al.*, 2006). Citric acid applications are summarized in Table 1.4..

Although citric acid can be chemically synthesized, there is no chemical method that is superior to microbial fermentation (Roukas, 2006). Mayilvahanan *et al.* (2006), mention that this compounds is exclusively manufactured by fermentation. Citric acid is the main organic acid produced in tonnage today by fermentation (Soccol *et al.*, 2006; Berovic and Legisa, 2007). Literature presents various strains of fungi (mainly *A. niger*) and yeasts (mainly *Yarrowia lipolytica*) for the biotechnological production of citric acid (Papanikolaou et al., 2002b; 2008a; 2008b; 2009; 2013; Najafpour, 2006; Roukas, 2006; Papanikolaou and Aggelis, 2009). Yeast

strains when compared to *A. niger* strains present some advantages including rapid growth and short fermentation time leading to high productivity rates, higher tolerance in contamination, capability of metabolizing high initial sugar concentrations, broader spectrum of substrates amenable to be converted into citric acid, insensitivity to substrate variations thus can be used for developing a continuous process and greater tolerance for metal ions allowing the use of less refined substrates like wastes (Roukas, 2006).

Table 1. 4. Citric acid applications

Applications	Industry	Functions
Beverages	Wines and ciders	Prevents browning in some white wine, prevents turbidity of wines and ciders, pH adjustment
	Soft drinks and syrups	Provides tartness, stimulates natural fruit flavor. As acidulant in carbonated and sucrose based beverages
Food	Jellies, jams and preservatives	pH adjustment, acts as acidulant, provides the desired degree of tartness, tang and flavor, increases the effectiveness of antimicrobial preservatives
	Dairy products	Emulsifier in ice creams and processed cheese, acidifying agent and antioxidant in many cheese products
	Candies	Acts as acidulant, provides tartness, minimizes sucrose inversion, produces dark color in hard candies, prevents crystallization of sucrose
	Frozen fruit	Protects ascorbic acid by inactivating trace metals, lowers pH to inactivate oxidative enzymes
	Fats and oils	Synergist for other antioxidants, as sequestrant, stabilizing action
	Animal feed	Feed complementation
Agriculture		Micronutrient evaluation in fertilizers, enhances P availability in plants
Pharmaceutics	Pharmaceuticals	Effervescent in powders and tablets in combination with bicarbonates, anticoagulant, provides rapid dissolution of active ingredients, acidulant in mildly astringent formulation
	Cosmetics and toiletries	Buffering agent, pH adjustment, antioxidant as a metallic–ion chelator
Other	Industrial applications	Buffer agent, sequestring metal ions, neutralizes bases, used in nontoxic, noncorrosive and biodegradable processes that meet current ecological and safety standards
	Metal cleaning	Removes metal oxides from the surface of ferrous and nonferrous metals, for operational cleaning of iron and copper oxides. In electroplating, copper plating, metal cleaning, leather tanning, printing inks, bottle washing compounds, floor cement, textiles, photographic reagents, concrete, plaster, refractories and moulds, adhesives, paper, polymers, tobacco, waste treatment, chemical conditioner on teeth surface, ion complexation in ceramic manufacture.

(Soccol *et al.*, 2006)

1.4.2. Raw materials of citric acid fermentation

There are various raw materials used as substrates for citric acid production through fermentation which will be mentioned in general. Those can be categorized in fruit extracts (date syrup, carob pods, figs), cereal constituents (mainly starch), oils (coconut oil, olive oil, palm oil, rapeseed oil, soybean oil), hydrocarbons (n-paraffins, n-alkanes, α-olefins with main use of *Y. lipolytica* strains) and finally agroindustrial wastes and by-products [molasses, cheese whey and whey permeate, olive mill waste-waters, apple pomace, grape pomace, kiwifruit peel, pineapple waste, brewery wastes (spent grain liquor), sugar cane bagasse, coffee husk, kraft black liquor, cassava bagasse, wheat bran, citrus waste, carrot waste] (Soccol *et al.*, 2006; Roukas, 2006; Papanikolaou and Aggelis, 2009; Demerche *et al.*,2013).

Likewise ethanol production, mentioned above, in order to obtain a result of high financial interest, the raw material used as substrate for the biotechnological production of citric acid should be cheap. Moreover, optimization of citric acid production in fermentation configurations should take place to result in higher yields, final product concentrations and volumetric productivity. Commercially sufficient maximum citric acid concentrations would be that of 80-130 g L^{-1} with citric acid yield on substrate (g formed g^{-1} substrate consumed – $Y_{Cit/S}$) more than 0.5 g g^{-1} for sugar-based substrates and 1.0 g g^{-1} for hydrophobic substrates (Steinbüchel, 1996).

Another important low cost renewable feedstock (produced in huge quantities by several industries such as bio-diesel, fat saponification and alcoholic beverage production units) for the production of citric acid, is glycerol. Papanikolaou *et al.* (2002b) studied the biochemical response of *Y. lipolytica* LGAM S(7)1 (ACA-DC 50109) during growth on raw glycerol. Citric acid up to 35 g L^{-1} (yield 0.42-0.44 g citric acid g^{-1} glycerol consumed) was reported when growth was performed under nitrogen-limited conditions and initial glycerol concentration media ranging from 80 to 120 g L^{-1}. Even higher final citric acid quantities (c. 63 g L^{-1}, yield of total citric acid produced per glycerol consumed c. 0.52 g g^{-1}) were reported at higher glycerol concentration media (Papanikolaou *et al.*, 2008b). The aforemetnioned strain was alsocultivated in nitrogen-limited glycerol-based repeated batch bioreactor cultures, resulting in citric acid production up to ~34 g L^{-1} (yield 0.52±0.05 g citric acid g^{-1} glycerol consumed) (Makri *et al.*, 2010).

The acetate mutant *Y. lipolytica* strain 1.31 was cultivated on biodiesel derived raw glycerol with initial concentration of 200 g L^{-1} resulting in citric acid production of ~125 g L^{-1} (yield 0.62 g citric acid g^{-1} glycerol consumed) (Rymowicz *et al.*, 2006). When glycerol generated by the biodiesel industry used as the carbon source (initial concentration 150 g L^{-1}) for the growth of acetate-negative mutant of *Y. lipolytica* strain Wratislavia K1 in fed-batch fermentations, maximum citric acid production was 110 g L^{-1} (Rymowicz *et al.*, 2008). Crude glycerol, discharged from

biodiesel production, used as substrate for the production of natural *Y. lipolytica* strains under nitrogen-limited submerged shake-flask experiments. Maximum total citric acid 50.1 g L^{-1} was produced with yield on glycerol consumed 0.44 g g^{-1} (André *et al.*, 2009). Rywińska *et al.* (2009) used pure and crude glycerol from biodiesel production as substrates for citric acid production by acetate-negative mutants of *Y. lipolytica* in fed-batch fermentation. *Y. lipolytica* strain Wratislavia AWG7 when cultivated on a medium containing 200 g L^{-1} of glycerol, production reached a maximum of citric acid production of 139 g L^{-1}, 120 h after inoculation (up to 0.69 g of citric acid per gram of glycerol consumed).

1.4.3. SCO production

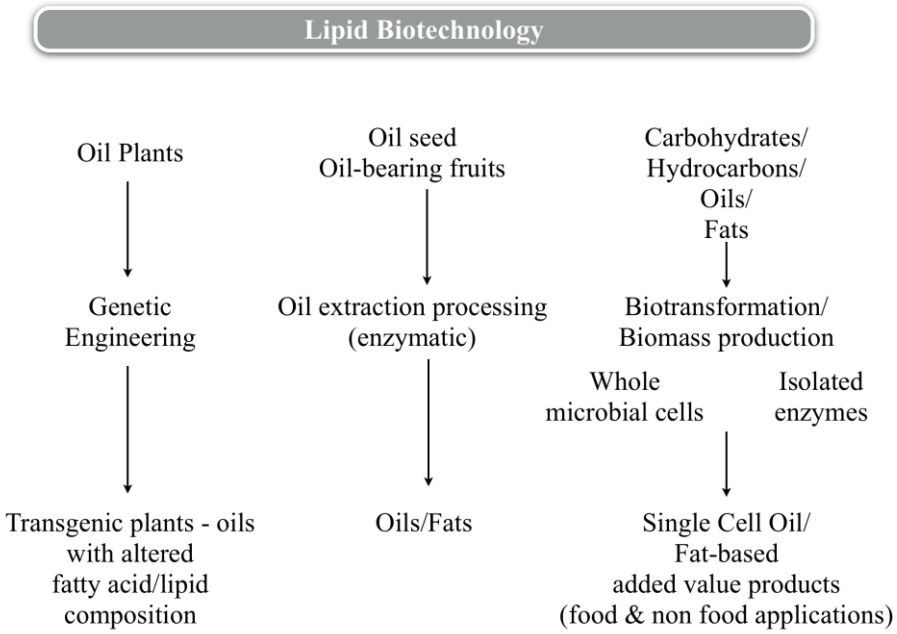


Figure 1. 10. Major areas of lipid biotechnology
(adapted by Mukherjee, 2006)

The major areas of lipid biotechnology are depicted in Fig. 1.10.. Single cell oil (SCO) is the edible oil extracted from microorganisms, resembling animal or plant oil, that could be safely used in foods or in other products where fats and oils (coming from more conventional sources) are normally used. Microorganisms that can accumulate lipid of about 20% (*w/w*) of the dry cell biomass are termed as "oleaginous" (Wynn and Ratledge, 2006). Some oleaginous microorganisms (yeasts, molds, microalgae) (Mukherjee, 2006; Wynn and Ratledge, 2006) are able to produce lipid (rich in unsaturated fatty acids) up to 70% (*w/w*) of their dry weight (Wynn and Ratledge, 2006). In contrast, in other (more rare) cases dependent on the culture conditions, cellular fatty acids can be remarkably saturated, presenting thus, composition similarities with saturated high value exotic fats

like cocoa-butter, illipe butter, shea butter etc. (Papanikolaou *et al.*, 2001; 2002a; 2003; Papanikolaou and Aggelis 2003; 2010; 2011a; 2011b).

Microbial lipids could contribute to cover the global growing demand of fats and oils. In general, it is difficult to calculate the exact costs of SCO production by fermentation technology. The variables that need to be taken into consideration, include cost of the substrate and the process itself, cost of the waste fermentation broth disposal, the cost of the separation of the SCO produced, the process developing research cost as also the final marketing, sales and distribution of the oil (the aforementioned factors may, in some cases, present equal or even higher costs than the whole process of SCO fermentation). Only recently, techno-economical studies have been performed illustrating the approximate cost of SCOs production (Koutinas *et al.*, 2014b). Therefore, SCO production is of higher industrial and financial importance if the substrate used is of low value such as crude fats or fatty acid wastewaters or other agro-industrial byproducts and wastes. SCO can be used as raw material of oleochemical industries and in the production of second and third generation biofuels. The modification in the composition of already existing lipids (of hydrophobic substrates used) lead to the biotechnological production of "new" lipids such as storage lipids mainly composed by triglycerides (TAGs) rich in Polyunsaturated Fatty Acids (PUFAs), including Essential Fatty Acids (EFAs) used as specific dietetic products (dietary supplements – nutraceuticals) (Ratledge, 1994; Papanikolaou and Aggelis, 2010; 2011b). Moreover the production of storage lipids resembling exotic fats in structure and physical properties (e.g. cocoa butter) may occur (Papanikolaou and Aggelis, 2003a; 2003b; 2011a; 2011b; Wynn and Ratledge, 2006).

1.4.4. Substrates of SCO production

Oleaginous microorganisms present a potential of growing on various substrates which are used as carbon and energy source. Such substrates are divided into hydrophilic and hydrophobic ones. Lipid biosynthesis from sugars and related substrates is called "*de novo*" lipid accumulation and lipid accumulation from hydrophobic substrates is called "*ex novo*" lipid accumulation. *De novo* accumulation of cellular lipids is a secondary anabolic activity, conducted after essential nutrient (usually nitrogen) depletion in the medium. Due to this exhaustion, the carbon flow is directed towards the accumulation of intracellular citric acid that is used as acetyl-CoA donor in the cytoplasm. Acetyl-CoA generates cellular fatty acids and subsequently triacylglycerols. On the contrary, *ex novo* lipid accumulation is a growth associated process (thus biomass and lipid accumulation occur simultaneously), independent from nitrogen exhaustion in the medium (see also paragraphs 1.4.5. and 1.6.2.).

Hydrophilic substrates could be sugar-based media such as (analytical grade or industrially derived) simple sugars [(e.g. glucose and fructose (also as products of starch, rice straw and inulin hydrolysis)], sucrose, lactose, whey, glucose-enriched wastes (e.g. glucose-enriched tomato waste hydrolysate, glucose-enriched sewage sludge), molasses, xylose-based media (with xylose deriving from chemical hydrolysis of various lignocellulosic materials), more complicated (compared with glucose) sugar-based substrates such as polysaccharides (e.g. starch and pectin-containing materials; presenting though notable differences in terms of both lipid and fatty acid composition of the SCO produced) as also glycerol, glycerol-enriched tomato waste hydrolysate, ethanol, propanol, citric acid, acetic acid, propionic acid and other low molecular weight organic acids. Hydrophobic substrates could be vegetable oils (e.g. olive oil, corn oil, sunflower oil, hydrolyzed rapeseed oil etc.), fatty esters (methyl-, ethyl-, butyl-, or vinyl-esters of fatty acids), soap-stocks, pure or industrially derived free-fatty acids, mixtures of free fatty acids and n-alkanes. Moreover, several agro-industrial by-products and wastes of low value such as industrial fats composed of free-fatty acids of animal (e.g. stearin, a fully saturated derivative of tallow) or vegetable origin, thermally processed oils, crude fish oils, OMWs could be offered as substrates for the biotechnological production of SCOs (Chen and Chang, 1996; Aggelis and Sourdis, 1997; Papanikolaou and Aggelis, 2002; 2010; 2011a; 2011b; Fickers *et al.*, 2005; Mukherjee, 2006).

1.4.5. Biochemistry of secondary metabolites production: Citric Acid and SCO

Citric acid is synthesized as part of the tricarboxylic acid (TCA) cycle within the mitochondrion of eukaryotic cells. In oleaginous microorganisms, citric acid accumulation is correlated with the activity of isocitrate dehydrogenase (ICDH) as a component of the TCA cycle. ICDH is dependent on the presence of AMP concentration which is regulated by the activity of AMP deaminase. AMP deaminase activity is up-regulated at the onset of nitrogen limitation in the growth medium (possibly due to the need of obtaining additional ammonium ions from intracellular materials).

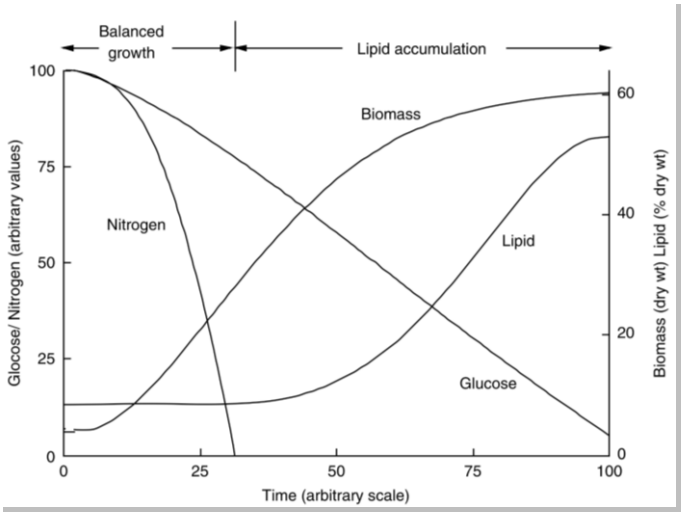


Figure 1. 11. The course of lipid accumulation by a typical oleaginous microorganism (Wynn and Ratledge, 2006)

When nitrogen is exhausted from the culture medium, oleaginous cells show an increased activity of AMP deaminase followed by the decrease of AMP cellular

content (including its content in the mitochondrion). The reduced content of AMP in the mitochondrion stops the activity of ICDH (strictly dependent on AMP concerning oleaginous cells) resulting in isocitrate not having the ability to be metabolized. It is accumulated and equilibrated with citric acid (via aconitase). Therefore citric acid is accumulated in the mitochondrion and its export to cytosol (in exchange for malate) is generated by an efficient efflux system (existing in the mitochondrial membrane). The generalized kinetics of SCO production in oleaginous microorganisms is seen in Fig. 1.11., while the intermediate metabolism is seen in Fig. 1. 12..

Following, if ATP:citrate lyase (ACL; an enzymatic complex that is considered to be the

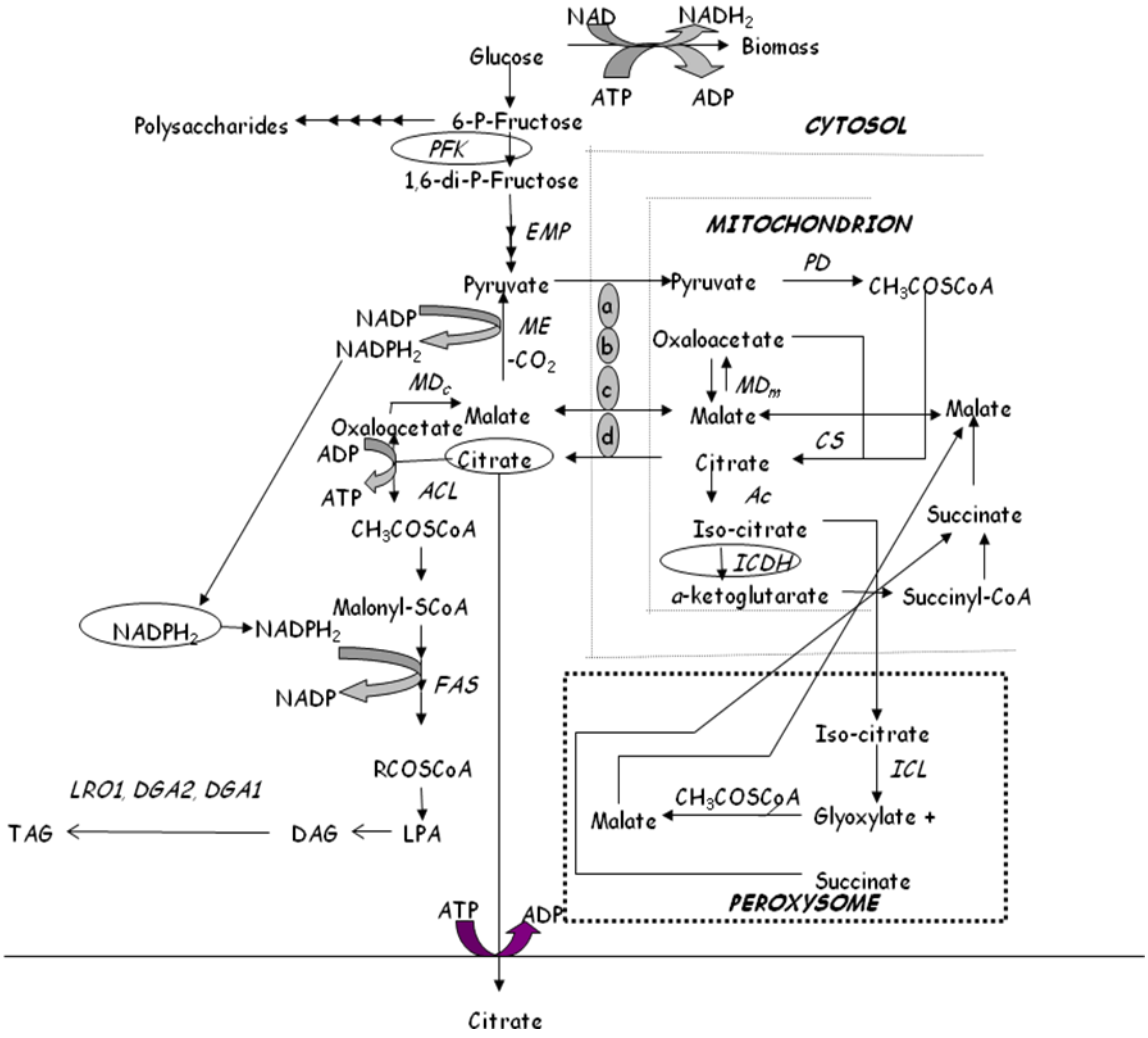


Figure 1. 12. Pathways involved in the breakdown of glucose by microbial strains capable of producing SCO, polysaccharides and/or citric acid in nitrogen-limited conditions
LPA: Lysophosphatidic acid; DAG: Diacylglycerols; TAG: Triacylglycerols; TRSP: citric acid transporting system; a, b, c: systems transporting pyruvic acid from cytosol to mitochondrion and inversely; d: system transporting citric and malic acid from cytosol to mitochondrion and inversely; ACL: ATP-citrate lyase; FAS: fatty acid synthetase; ICDH: iso-citrate dehydrogenase; MD_c: malate dehydrogenase (cytoplasmic); MD_m: malate dehydrogenase (mitochondrial); ME: $NADPH^+$-malic enzyme; PD: pyruvate dehydrogenase; CS: citrate synthase; ICL: iso-citrate lyase; PFK: Phospho-fructokinase; DGA1: Diacylglycerol acyltransferase; DGA2: Phospholipid diacylglycerol acyltransferase; LRO1: Lysophosphatidic acid acyl transferase; EMP: Embden-Mayerhoff-Parnas pathway. Pathways described by Ratledge and Wynn, (2002); Papanikolaou and Aggelis, (2009); (2011a); Koutinas and Papanikolaou (2011); Koutinas *et al.* (2014a).

most important factor to account for the oleaginicity of the various microorganisms, being absent in the majority of non-oleaginous microbial cells) is not present or its activity is suspended, then the accumulated citric acid is eiter moved outside the cell or will provoke the inhibition of the 6-phosphoro-fructokinase, having as result intracellular accumulation of polysaccharides based on 6-phosporo-glucose. If ACL is activated, citric acid present in the cytosol is cleaved producing acetyl-CoA and oxaloacetate (converted to malate via malate dehydrogenase and used as the counterion in the citrate efflux system mentioned before). Acetyl-CoA is used [with the activation of fatty acid synthetase (FAS) and acetyl-CoA carboxylase (ACC)] for free fatty acid (occurring in the cytosol) and, ultimately triacyglycerides (TAGs) biosynthesis leading finally to microbial oil accumulation. On the other hand, it should be stressed out that biochemistry of citric acid and lipid accumulation are highly correlated (mainly in the first steps) and in order to achieve their accumulation, media with an excess of carbon substrate and a limiting amount of nitrogen should be used (Papanikolaou *et al.*, 2002a; 2002b; Ratledge, 2004; Wynn and Ratledge, 2006; Roukas, 2006; Papanikolaou and Aggelis, 2009; 2011a; 2011b; Makri *et al.*, 2010). If ICDH is present and active, then citric acid is converted into α-ketoglutaric acid followed by the rest reactions of TCA cycle which may lead to the accumulation of other intermediate organic acids of the cycle (e.g. succinate, fumarate, malate), if nutrient limitation besides carbon exists. Otherwise, TCA cycle is covered and microbial biomass is generated. The consecutive steps of *de novo* lipid biosynthesis is depicted in Fig. 1. 12.

Oleaginous microorganisms accumulate lipid following - in most cases - a similar pattern (Fig. 1.13.).

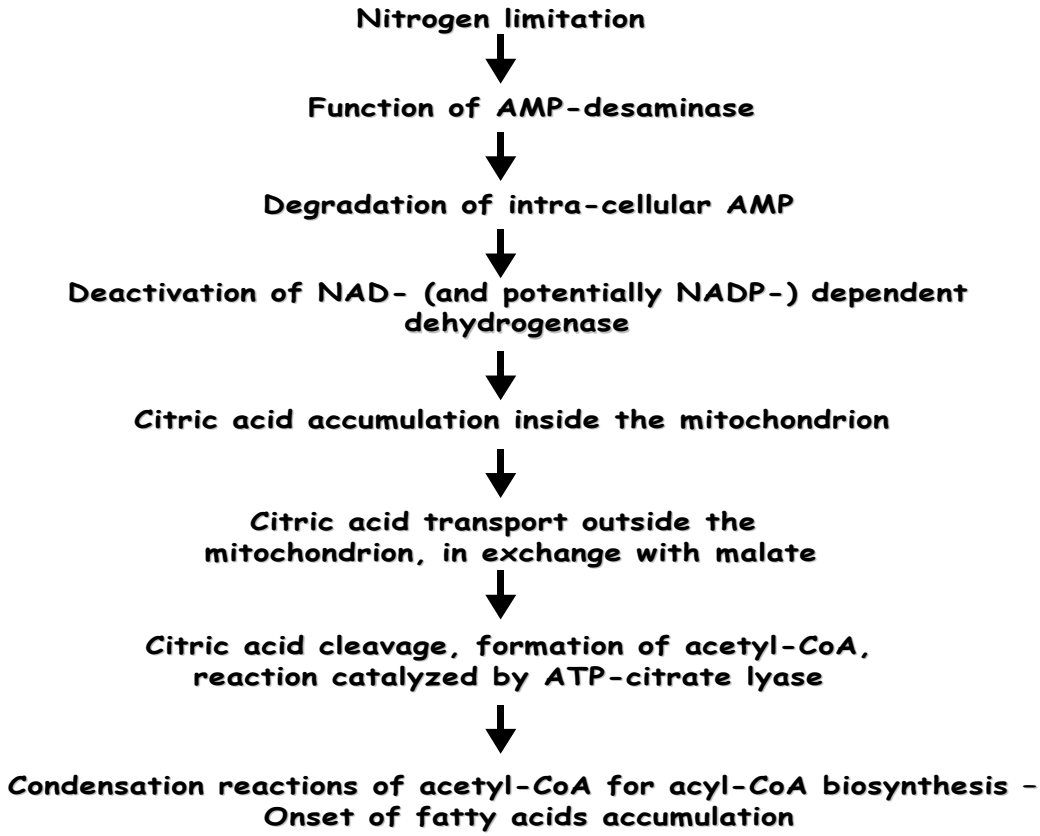


Figure 1. 13. Sequential steps of *de novo* lipid biosynthesis in oleaginous microorganisms growing under nitrogen-limited conditions (Papanikolaou and Aggelis, 2011a)

As mentioned above, culture media with high C/N ratio should be used. After the initial phase of balanced growth, when nitrogen source is exhausted (and all other nutrients are found in excess), the microorganism continues to assimilate the carbon source. It is when cells stop from dividing but continue to increase their size (as lacking nitrogen is needed for protein and nucleic acid synthesis which are both essential for new cells creation). As carbon source continues to be assimilated, oleaginous cells convert it into oils and fats until they reach a personal limit of obesity (Fig. 1.14.) with some cells continuing until they are physically unable to accumulate any more. In the final hours of fermentation, when the cell lipid content is reaching its maximum the residual carbon source may be exhausted too (Fig. 1.11.). It is when the cells sense starvation and investigate a survival mechanism where the store reserved of lipid is used for their own benefit. Thus, cells may be stimulated to consume the lipids that had just previously accumulated (the same moment that lipases are formatted), indicating that oil accumulation occurs as a means of the cells storing excess carbon as a reserve storage material (Aggelis *et al.*, 1995a; 1995b; Papanikolaou *et al.*, 2001; 2004a; Wynn and Ratledge, 2006; Papanikolaou and Aggelis, 2011a; 2011b). Lipid breakdown, usually followed by creation of new cell material (mostly polysaccharides), is

performed mainly through the glyoxylic acid by-pass (Papanikolaou *et al.*, 2002a; Vamvakaki *et al.*, 2010; Papanikolaou and Aggelis, 2011a). As indicated, when nitrogen is exhausted from the culture medium, oleaginous cells show an increased activity of AMP deaminase followed by the decrease of AMP cellular content (including its content in the mitochondrion). The reduced content of AMP in the mitochondrion stops the activity of ICDH resulting in isocitrate not having the ability to be metabolized. It is accumulated and equilibrated with citric acid (via aconitase). Therefore citric acid is accumulated in the mitochondrion and its export to cytosol (in exchange for malate) is generated by an efficient efflux system (existing in the mitochondrial membrane) (see Fig. 1.15.).

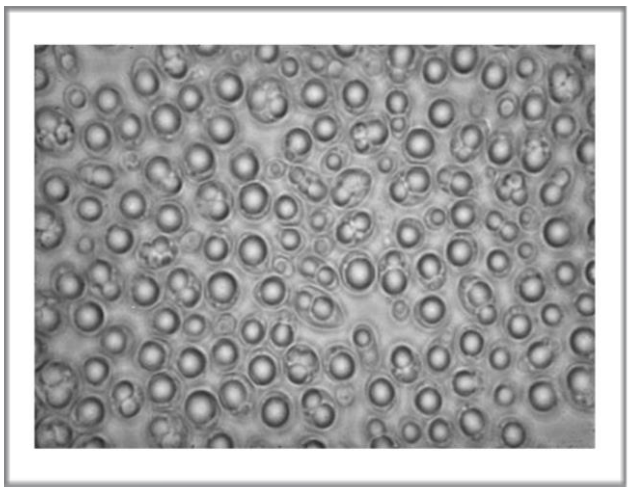


Figure 1. 14. Accumulation of lipid in the form of discrete intracellular droplets by cells of oleaginous yeast, *Cryptococcus curvatus* (Formerly *Candida curvata* D) Wynn and Ratledge (2006)

Following, citric acid present in the cytosol is cleaved by ACL producing acetyl-CoA and oxaloacetate (converted to malate via malate dehydrogenase and used as the counterion in the citrate efflux system mentioned before). Malic enzyme activity is present in most oleaginous microorganisms where it forms an integrated metabolon complex that combines with ACL and fatty acid synthase (FAS) to ensure a direct channelling of acetyl-CoA into fatty acids Acetyl-CoA is used [with the activation of FAS and acetyl-CoA carboxylase (ACC)] for free fatty acid biosynthesis (occurring in the cytosol), which are finally esterified with glycerol into triacyglycerols (TAGs) and incorporated via the endoplasmatic recticulum into fatty acid droplets (Ratledge, 2004).

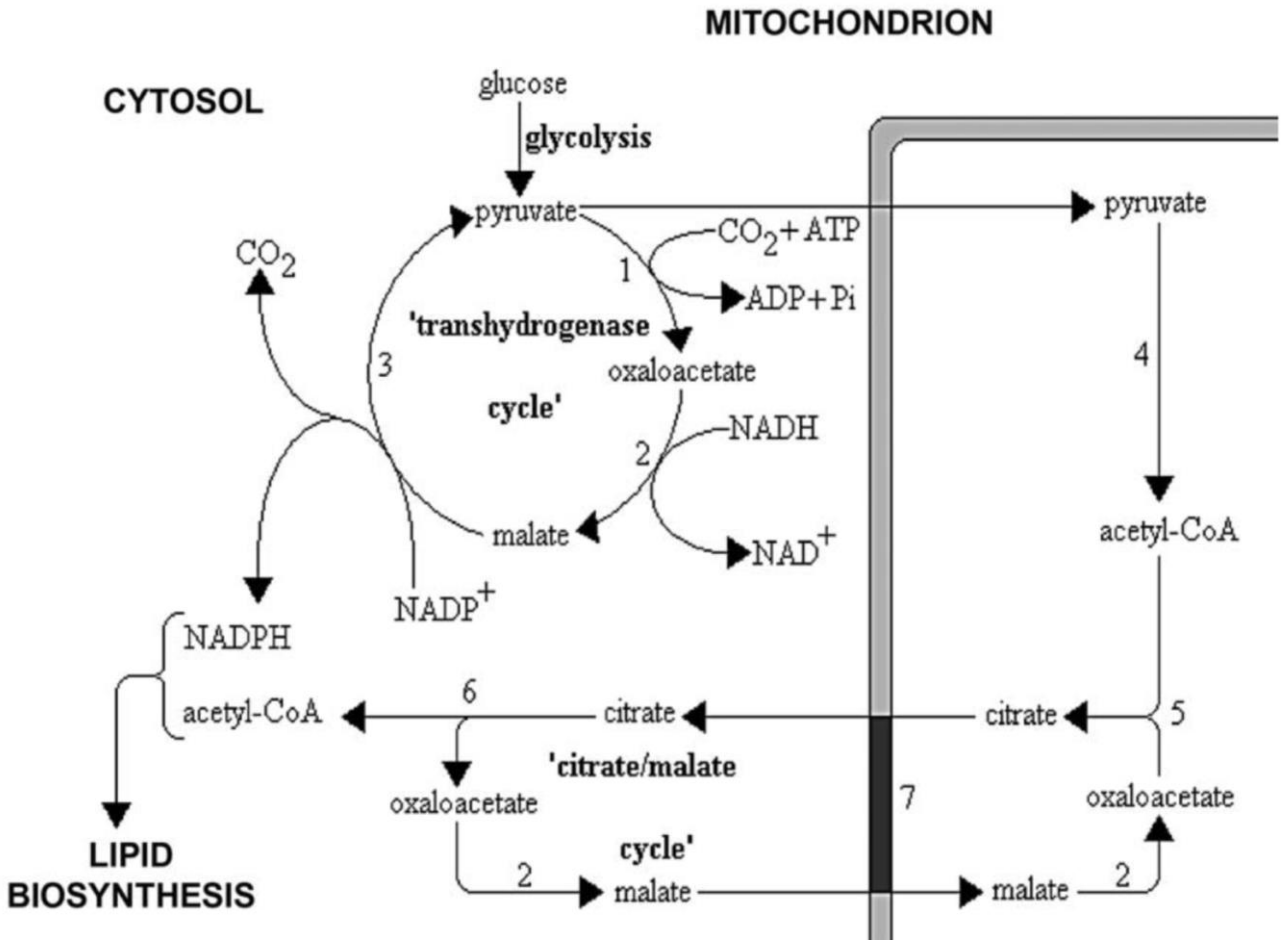


Figure 1. 15. Citrate/malate cycle and TCA cycle providing sufficient precursors of acetyl-CoA and NADPH for lipogenesis in oleaginous microorganisms.

Enzymes: 1, pyruvate decarboxylase; 2, malate dehydrogenase; 3, malic enzyme; 4, pyruvate dehydrogenase; 5, citrate synthase; 6, ATP:citrate lyase; 7, citrate/malate translocase (Ratledge, 2004).

Except from the concentration carbon and nitrogen source (high C/N ratio) of the growth medium used, there are several factors that affect lipid accumulation from an oleaginous microorganism. Such as the pH of the medium, the temperature (as such fermentations are mainly exothermic), the dilution rate (for the case of continuous cultures the total lipid yield on biomass decreases as the dilution rate increases), the dissolved oxygen and various substances like NaCl, EDTA etc. (Fidler *et al.*, 1999; Wynn and Ratledge, 2006). Concerning the affection of C/N ratio on the accumulation of lipids, it should be mentioned that in some microorganisms the content of their biomass in lipids increases in correlation with C/N ratio increment, up to an optimum value (Moreton, 1988; Fig. 1. 16.). In other microorganisms, the lipid percentage in dry matter raises proportionally to the C/N ratio increment (Papanikolaou *et al.*, 2004b; 2010).

Based on the previous analysis it is stressed out that when sugar-based (or similarly metabolized) substrates (hydrophilic) are used, lipid production occurs only when nitrogen is

limited (lipid synthesis as a secondary anabolic activity). On the contrary, when hydrophobic growth media (e.g. n-alkanes, free-fatty acids, TAGs or the like) are used, lipid accumulation is not correlated with nitrogen limitation, thus biomass and lipid accumulation occur simultaneously (Aggelis and Sourdis, 1997; Papanikolaou *et al.*, 2001; Papanikolaou and Aggelis, 2003a; 2003b; 2010; 2011a) (see also paragraph 1.6.2.).

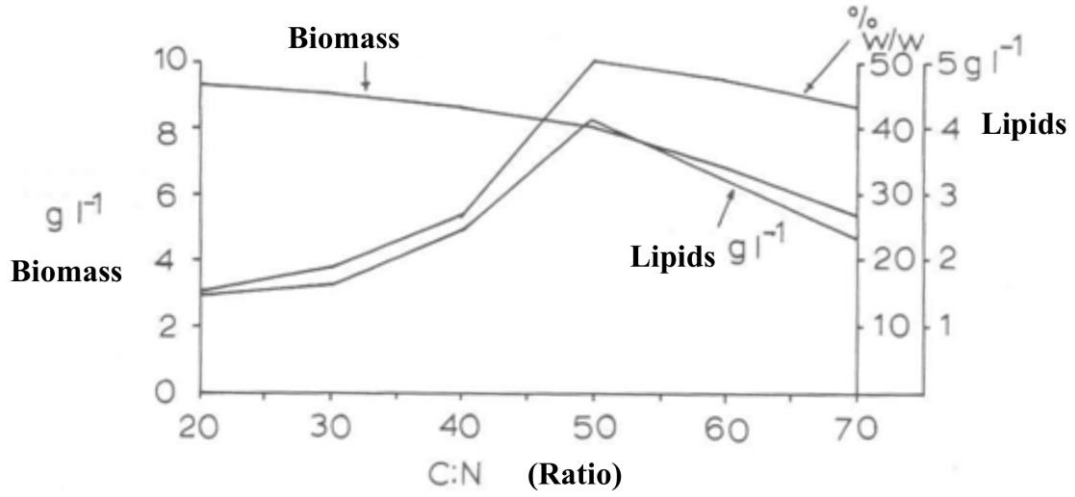


Figure 1. 16. Affection of the change of C/N ratio during the growth of *Rhodosporidium toruloides* IFO 0559 on substrate with stable glucose concentration and changing NH_4Cl concentration
(Moreton, 1988)

1.5. Genus *Saccharomyces*

Table 1. 5. Systematic classification of yeast *Saccharomyces cerevisiae*	
Kingdom	Fungi
Phylum	Ascomycota
Class	Hemiascomycetes
Order	Saccharomycetales
Family	Saccharomycetaceae
Genus	*Saccharomyces*
Species	*Saccharomyces cerevisiae*

1.5.1. Morphology of *Saccharomyces cerevisiae*

Yeasts are the most simple of the eucaryotes. The yeast cell contains cellular envelopes, a cytoplasm with various organelles and a nucleus surrounded by a membrane and enclosing the chromosomes (Fig. 1.17.). The yeast cell has two cellular envelopes: the cell wall and the membrane having a space between them called periplasmic space (a fluid gel wherein proteins move about). The first function of the cell wall (having as principal constituents β-glucans and manoproteins) is to protect the cell against bursting due to the internal osmotic pressure (determined by the composition of the cell's environment) (Μπαλατσούρας, 1993). In addition to its protective role, the cell wall is a dynamic and multifunctional organelle, evolving during the life of the cell (with its composition and functions), in response to environmental factors (Ribéreau-Gayon *et al.*, 2006).

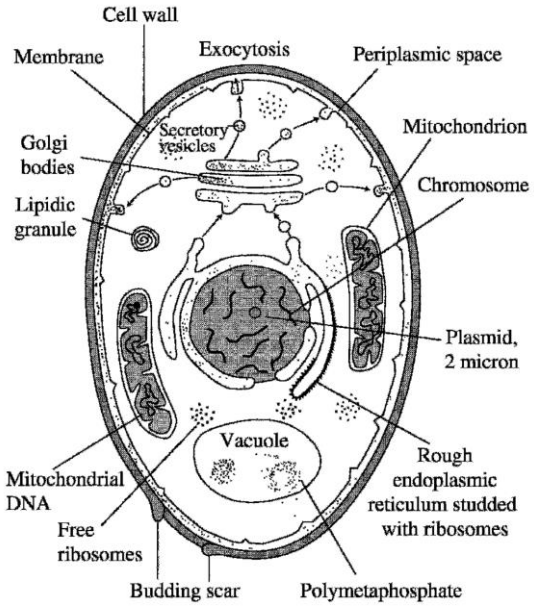


Figure 1. 17. A yeast cell (Ribéreau-Gayon *et al.*, 2006)

The cytoplasm and the membrane make up the protoplasm. The plasma membrane is a highly selective barrier, essential for the life of the yeast as it controls the exchanges between the living cell and its external environment. It is principally made of lipids (40%; essentially phoshpolipids and sterols) acting as amphiphilic molecules (possessing a polar or hydrophilic part made of a phosphorylated

alcohol and a non-polar or hydrophobic part comprising two parallel fatty acid chains) and proteins (50%). The membrane composition in fatty acids and its proportion in sterol controls fluidity.

In the genus *Saccharomyces*, between the plasma membrane and the nuclear membrane, the cytoplasm contains a basic cytoplasmic substance named cytosol. The subcellular organelles such as Golgi apparatus, endoplasmic reticulum and mitochondria are isolated from the cytosol by membranes. The endoplasmic reticulum (ER) is a double membrane system partitioning the cytoplasm. ER ensures the addressing of proteins synthesized by its attached ribosomes. The Golgi apparatus as an extension of ER, consists of a stack of membrane sacs and associated vesicles. Transfer vesicles transport the proteins issued from the ER to the sacs of Golgi apparatus. The dual function of Golgi apparatus is the glycosylation of protein and their direction via specialized vesicles either into a vacuole or into the plasma membrane. The spherically or rod-shaped organelles surrounded by two membranes called mitochondria, are distributed in the periphery of the cytoplasm. They are true respiratory organelles for the yeasts (in aerobiosis, the *S. cerevisiae* cell contains about 50 mitochondria where in anaerobiosis, these organelles degenerate and the enzymes of electron transport chain are not synthesized) (Boulton *et al.*, 1996; Ribéreau-Gayon *et al.*, 2006). The enzymes that catalyze the reactions of TCA cycle and the reactions of fatty acids degradation are located into the mitochondria. Yeast cellular envelopes play essential role as they contribute to a successful alcoholic fermentation and accumulate certain constituents. Thus a profound knowledge of these organelles is needed in order to take advantage of the properties of the microorganism (Boulton *et al.*, 1996; Ribéreau-Gayon *et al.*, 2006).

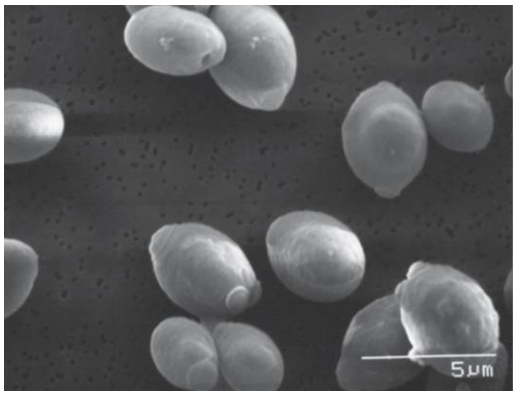


Figure 1. 18. *Saccharomyces cerevisiae* showing characteristic bud scars left upon separation of daughter cells (Fugelsang and Edwards, 2007)

Concerning reproduction, *S. cerevisiae* can multiply either asexually by vegetative multiplication (by a process called budding where the mother cell separates from the daughter cell; Fig. 1.18.) or sexually by forming ascospores (Μπαλατσούρας, 1993; Αγγελής, 2007). Most yeasts undergo vegetative multiplication but sporiferous diploid cells when present at hostile nutritive environment (e.g. depletion of fermentable sugars, low nitrogen concentration, high aeration) stop multiply. Some transform into a sac called asci containing haploid ascospores issued from meiotic division of the nucleus (Boulton *et al.*, 1996; Ribéreau-Gayon *et al.*, 2006).

1.5.2. Growth cycle of *Saccharomyces cerevisiae*

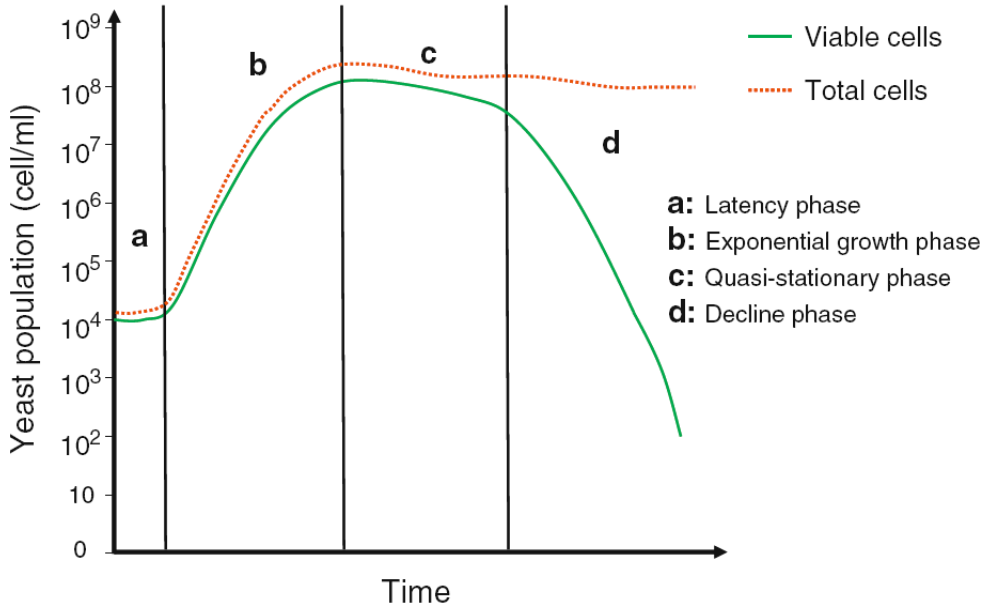


Figure 1. 19. Yeasts growth cycle

(Zamora, 2009)

The yeasts start to metabolize the carbon source and other nutrients of the medium to obtain energy and increase their population as shown in Fig. 1.19. (classic yeast growth cycle under standard conditions). The growth cycle has four principal phases. During the first hours (latency phase) the population does not increase as the cell needs to adapt to the new environmental conditions. Once the adaptation is over, cells begin to grow. An exponential growth phase (lasting from 2 to 6 days) increases the population up to 10^7-10^8 cells mL^{-1} (this growth phase is divided into accelerated, exponential and decelerating growth phase). As some nutrients become deficient, a quasi-stationary phase (lasting from 2 to 10 days) follows where the yeast stops growing and the population remains nearby stable. Finally the decline (death) phase begins and progressively reduces the viable population to 10^5 cells mL^{-1}.

It should also be remarked that during this cycle, growth is limited to four or five generations and the duration of these different phases is not equal (e.g. the death phase is three to four times longer than the growth phase) and that fermentation kinetics are obviously directly linked to the growth cycle (Ribéreau-Gayon *et al.*, 2006; Fugelsang and Edwards, 2007; Hornsey, 2007; Jackson, 2008; Zamora, 2009).

1.5.3. Environmental factors affecting the growth of *Saccharomyces cerevisiae*

The growth of yeasts (and therefore that of *S. cerevisiae*) is highly dependent on various environmental factors, such as carbon and energy sources, assimilable nitrogen, presence of oxygen, carbon dioxide and pressure, vitamins, inorganic elements, pH.

Carbon source

Various carbon sources are essential for the biosynthesis of basic cellular elements such as proteins, lipids etc.. Sugars are the main carbon source for yeasts. Through sugars, the yeast metabolism is activated and the necessary energy for its survival is created. The major carbon and energy sources for fermentation are glucose and fructose. Sucrose can be also fermented. It is enzymatically split into its component monosaccharides, glucose and fructose with the use of one of several invertases. Sucrose hydrolysis usually occurs external to the cell membrane by an invertase located between the cell wall and plasma membrane (periplasm) (Μπαλατσούρας, 1993; Ratledge, 1991). Sugars are moved across the plasma membrane into the cell with the help of proteinic transporters by facilitated diffusion. Besides the affection of the activation of sugar transport mechanisms, sugar concentration also regulates the expression of enzymes in the TCA cycle. Finally, it should be mentioned that over a wide range of sugar concentrations, ethanol production is directly related to sugar content (Jackson, 2008). Related to the concentration of glucose, *S. cerevisiae* (and other ethanol-producing yeasts) displays the Crabtree effect (for more information see paragraph 1.3.5.5.) where alcoholic fermentation occurs in the presence of oxygen when glucose concentration exceeds a certain threshold value (Verduyn *et al.*, 1990).

Nitrogen

Next to sugars, nitrogenous compounds are quantitatively the most important yeast nutrients. Nitrogen is essential for the biosynthesis of high molecular compounds, such as proteins. The most assimilable form of nitrogen for yeasts is that of ammonium ions. Nevertheless, most of the free amino acids (notably proline and arginine) can be used as nitrogen sources, because of the fact that *S. cerevisiae* presents proteolytic ability. At least 150 mg L^{-1} assimilable nitrogen is generally considered necessary for succesful fermentation without interruption. Optimum levels suggested are in the range of 400-500 mg L^{-1}. Higher concentrations promote cell multiplication and reduce the conversion of sugar to alcohol. On the other hand, inadequate nitrogen levels slow fermentation. This may result from the irreversible inactivation of sugar transport by ammonia starvation (Ribéreau-Gayon *et al.*, 2006; Jackson, 2008). The half-life of the main glucose transport system is approximately 12h, with complete inactivation occurring within approximately 50 h. This

results from the pause of protein synthesis and enzyme degradation. The lack of ammonia can also refute the allosteric activation of crucial glycolytic enzymes, such as phosphofructokinase and pyruvic kinase. This, in turn, further inhibits the uptake of glucose (Boulton *et al.*, 1996; Jackson, 2008).

Oxygen and aeration

As mentioned above, yeasts can gain the essential energy used for their growth either through respiration or fermentation (Pasteur effect - see: Ratledge 1991; Αγγελής 2007). The major factor regulating this course is the presence of oxygen into the medium (even though, regardless the amount of oxygen, *S. cerevisiae* will ferment anyway, when sugars are above a certain level; see paragraph 1.3.5.5.). Nevertheless, trace amounts of oxygen (or some oxygenation at the first fermentation steps) can favor fermentation by permitting the biosynthesis of sterols, long-chain unsaturated fatty acids and the vitamin nicotinic acid. The production and proper functioning of the yeast cell membrane require sterols (ergosterol and lanosterol), as well as unsaturated C16 and C18 fatty acids (Ribéreau-Gayon *et al.*, 2006; Jackson, 2008; Zamora, 2007).

Carbon dioxide and pressure

During fermentation, large volumes of carbon dioxide are generated, with a rough estimation that its escape removes about 20% of the heat generated through the conversion realized. Various volatile compounds are carried off with carbon dioxide. Ethanol loss is estimated at about 1-1.5% of the one that is biologically produced (Jackson, 2008). The generation of carbon dioxide produces strong convection currents within the fermentation apparatus that help equilibrate the nutrient and temperature status throughout the medium. At pressures above 700 kPa (~7 atm), yeast growth ceases, although pressure-related effects have been reported at pressures as low as 30 kPa above ambient. Moreover, low pH and high alcohol content increase yeast sensitivity to CO_2 pressure. Nevertheless, the fermentative ability of yeasts may not be inhibited completely until about 3000 kPa (Jackson, 2008). The yeast's metabolism may be affected by carbon dioxide accumulation as this compound may influence the balance between carboxylation and decarboxylation reactions. Some of the consequences of high pressure on cell growth and metabolism may arise from a decrease in water viscosity leading to a disruption of the intramolecular hydrogen bonding, which is vital to protein structure and function. In addition, critical changes in membrane composition could disrupt cell membrane permeability. On the other hand, the presence of CO_2 may be a part of biosynthesis enhancement of purines, pyrimidines and fatty acids (Ratledge, 1991; Jackson, 2008).

Vitamins

Vitamins play a crucial role in the regulation of yeast metabolism as coenzymes and enzyme's precursors and although they are not metabolized as energy sources, a concentration decrease is noted during fermentation. Even if yeast requirements typically are satisfied by either vitamin biosynthesis or assimilation from the medium, certain conditions can, however, significantly reduce their concentration or availability. For instance, fatty acids produced during fermentation can inhibit the uptake of thiamine. Amongst vitamins, mainly biotin may stimulate the growth of *Saccharomyces* cells (as its main metabolic role is fixed upon all carboxylation and decarboxylation reactions) (Jackson, 2008).

Inorganic elements

Inorganic elements (such as magnesium, manganese, copper, zinc, iron, potassium) often are essential components in the active (catalytic) sites of enzymes. They also play active roles in regulating cellular metabolism and in maintaining cytoplasmic pH and ionic balance. For instance, magnesium is involved in the catalytic action of several key glycolytic enzymes, the activation of fermentation enzymes and the stabilization of membrane structure. Organic compounds (like amino-acids) reduce the effective concentration of inorganic elements, while ions can antagonize each other's uptake (Boulton *et al.*, 1996; Jackson, 2008).

Lipids

Lipids are the basic constituents of cell membranes (phospholipids and sterols), while in several cases including *Saccharomyces cerevisiae* they function as energy storage materials. In several cases, lipophilic compounds can act as pigments (carotenoids) and can bind with proteins (lipoproteins) and carbohydrates (glycolipids). *Saccharomyces cerevisiae* yeast synthesize de novo its own cellular lipid from acetyl-CoA deriving from glucose catabolism with different mechanisms than the ones implicated in the oleaginous yeasts (i.e. there is absence of ATP-CL) when grown aerobically (Ratledge, 1994), but it is unable to produce long-chain unsaturated fatty acids and sterols under anaerobic conditions. *Saccharomyces cerevisiae* typically possesses sufficient reserves of these vital compounds to initiate fermentation and complete several cell divisions (typically four to five when a yeast inoculum is used), while lipids in this yeast species can be typically degraded through the β-oxidation process (Athenstaedt *et al.*, 1999). As in the case of the yeast *Yarrowia lipolytica*, the limiting step of the β-oxidation process is that of the synthesis of acyl-CoA oxidases, with three families of these enzymes existing in *S. cerevisiae* encoded by the respective genes (*POX*1, *POX*2 and *POX*3) (Athenstaedt *et al.*, 1999). In *Y. lipolytica*, 6 genes are implicated with

the biosynthesis of the family of acyl-CoA oxidases, namely *POX*1, *POX*2, *POX*3, *POX*4, *POX*5 and *POX*6 (Fickers *et al*., 2005; Beopoulos *et al*., 2009). When lipids are present in the fermentation medium, the ethanol tolerance of yeast cells is enhanced (Jackson, 2008).

The aforementioned lipid particles are consisted of 95% lipid and 5% protein. The lipid fraction of the particles consists of triacylglycerols (45-50%) and steryl esters (45-50%) together with minor amounts of phospholipids and free fatty acids as also about 2% diacylglycerol (Christiansen, 1978). Triacylglycerols and steryl esters of lipid particles were suggested to function as storage for components needed for membrane formation. Steryl esters of the yeast *Saccharomyces cerevisiae* are metabolically interconvertible with free sterols depending on the growth stage of the culture. During active growth (under sterol depletion), steryl esters can be hydrolysed and the free sterols can be recycled for membrane formation (Leber *et al.*, 1994). Under conditions of fatty acid deficiency, fatty acyl moieties of triacylglycerols and steryl esters can be incorporated into phospholipids (Athenstaedt *et al.*, 1999).

1.5.4. Environmental factors creating stress

A factor is considered to be stressful when it presents negative impact on the performance and functionality of the cells. These factors could be divided into physical (such as high temperature, osmosis, high osmotic pressure, lack of water, radiation), chemical (including the presence of various chemical compounds such as ethanol and other metabolites, lacking of nutrients, pH) and biological (cell aging, competition with other microorganisms, genotyping changes). Ethanol presence in the fermentation medium, osmosis and temperature are the most important and most studied factors concerning the physiology of *S. cerevisiae*.

Ethanol

All alcohols are toxic to varying degrees. *S. cerevisiae* shows considerable insensitivity to ethanol toxicity, thus much effort has been spent attempting to understand the nature of this tolerance, and the reason of its breaking down at high concentrations when oxygen is found into the medium. The ethanol tolerance of various microorganisms may be attributed to membrane (the main target of ethanol) changes (including higher concentration of unsaturated fatty acids) that decrease permeability and minimize the loss of nutrients and cofactors from the cell, notably magnesium and calcium. Although most strains of *S. cerevisiae* can ferment up to 13-15% ethanol, there is wide variation in this ability (Jackson, 2008).

Alcohol as toxic substance for cells when present at the medium, acts as a chemical stressful factor. Although buildup (at concentrations ranging between 8-18%) eventually inhibits

fermentation, this compound is considered to begin in the disruption of the yeast metabolism (suppression of sugar uptake) at much lower concentrations (2%). At higher concentrations, the transportation of various ammonium ions and amino-acid permeases is disrupted and in concentration ~11% the fermentation is fully suppressed (Glazer and Nikaido, 1994). This property becomes increasingly marked as the fermentation temperature rises and when lack in various nutrients (mainly magnesium) occurs into the medium (Walker, 1998; Jackson, 2008). Although higher (fusel) alcohols are inhibitorier than ethanol, their much lower concentration substantially limits their toxic influence. The main negative effect of ethanol is that of the disruption of the semi fluid nature of the cell membrane as ethanol is transferred in it destroying proteinic and lipid bonds (leading to continuous higher membrane permeability). It is believed that this results from alcohol's effect on lowering water activity. This destroys the ability of the cell to control cytoplasmic function, leading to nutrient loss and disruption of the electrochemical gradient across the membrane. The latter is vital for nutrient transport. Lowered water activity also disrupts hydrogen bonding, essential to enzyme function. High osmotic potential enhances ethanol toxicity (Jackson, 2008).

Temperature

Temperature is one of the most influential factors affecting fermentation as it directly and indirectly influences yeast metabolism. It is one of the features over which the biotechnologist could have the greatest control. At the upper and lower limits, temperature can cause cell death. However, inhibitory effects are experienced well within these extremes. The optimum growth temperature for most yeasts is between 20 and 30 ºC**.** As far as the maximum growth temperature (T_{max}) values are concerned, for *S. cerevisiae* strains a variance of a range between 35 and 43 ºC is presented. Even though T_{max} remains relatively stable amongst strains of the same species, the disruptive influences of high temperatures are increased by growth-limiting or growth-inhibiting factors (such as exhaustion of carbon source, accumulation of ethanol into the medium, presence of low-aliphatic chain fatty acids into the medium etc.). In contrast, low temperatures tend to diminish the toxic effects of ethanol. This may partially be a consequence of the higher proportion of unsaturated fatty acid residues in the plasma membrane. This property may help to explain the higher maximum viable cell count at the end of fermentations conducted at cooler temperatures (Walker, 1998; Jackson, 2008).

The growth rate of yeast cells is strongly influenced by the fermentation temperature, particularly during the exponential growth phase. For instance, cell division in *S. cerevisiae* was found to occur every 12 h at 10 °C, every 5 h at 20 ºC, and every 3 h at 30 °C (Jackson, 2008). Cells

face a rapid decrease in viability at the end of fermentation, at temperatures above 20 °C whereas at (excessively high) temperatures between 40 and 50 ºC there is a disruption of enzyme and membrane function and the cells are driven into autolysis. At lower temperatures, cell growth is retarded, but viability is enhanced, though cool temperatures prolong the fermentation lag phase, dramatically slow the fermentation rate and thus lead in its premature termination. Moreover, other important (but not directly related to the effect of temperature) influences arise such as the temperature affection on the rate of ethanol loss during fermentation (Glazer and Nikaido, 1994).

During alcoholic fermentation, much of the chemical energy produced into the medium is released as heat. In some cases, this is sufficient to increase fermentation temperature at about 30 °C. Part of the heat is lost with escaping carbon dioxide and water vapor. On the other hand, the rise in temperature can easily reach levels critical to yeast survival, if temperature-control measures are not implemented, and in the case of the non-aseptic alcoholic fermentation performed in traditional wine-making facilities, this could result in the failure of the alcoholic fermentation due to significant proliferation of lactic acid bacteria and the subsequent mannito-lactic instead of the alcoholic fermentation that would occur (Μπαλατσούρας, 1993). If heat transfer through the fermentor wall is sufficiently rapid, cooling the fermentor surface with water or by passing a coolant through an insulating jacket can be effective (Glazer and Nikaido, 1994; Walker, 1998; Jackson, 2008).

Osmotic pressure

The osmotic pressure can be increased either after loss of intracellular liquid or when the cells are present in high concentration media (Walker, 1998). At high values of osmotic pressure a vast decrease of the intracellular volume occurs due to water effluence resulting in a potential delay of the onset of fermentation. The resulting partial plasmolysis of yeast cells may be one of the causes of a lag period prior to active fermentation. In addition, cell viability may be reduced, cell division retarded, and sensitivity to alcohol toxicity enhanced (Meikle *et al.*, 1988; Marechal and Gervais, 1994).

Strains of *Saccharomyces cerevisiae* differ greatly in their sensitivity to sugar concentration. The nature of the remarkable tolerance of various yeasts (mainly wine yeasts) for the plasmolytic action of sugar is unclear. Nevertheless, it appears to be related to increased synthesis (or reduced permeability of the cell membrane) to glycerol. These responses to increased environmental osmolarity permit glycerol to equilibrate the osmotic potential of the cytoplasm to that of the surrounding medium. In that case, the expression of certain genes giving the signal for biosynthesis

of enzymes taking part at glyceropyruvic fermentation is induced (Brewster *et al*., 1993; Schüller *et al.*, 1994; Hirayama *et al*., 1995; Walker, 1998; Jackson, 2008).

1.5.5. Applications of *Saccharomyces cerevisiae*

The yeast *S. cerevisiae* has found numerous applications in the production of food and alcoholic beverages since ancient times. The production of enzymes and recombinant proteins and the development of drug screening assays are also commercial applications of such yeast cells. Moreover, the completed sequence of its genome, the understanding of its cellular physiology and the ease with which it can be manipulated has revolutionized the use of yeast as model system for the investigation of eukaryotic cell processes at a whole-genome level. The similarity of primary sequence and functional homology of yeast proteins and their corresponding proteins found in higher eukaryotic organisms accelerated the description of the function of numerous mammalian gene products (it should be noted that proteins from higher organisms may be expressed to a remarkable degree in yeast and confer activities comparable to their yeast counterparts) (Pausch *et al.*, 2005).

Specifically, ales (type of beer) are produced by *S. cerevisiae* "top yeasts" (rising to the surface of fermentation). The Japanese alcoholic beverage saké is prepared via two-stage feremantion of rice where a second fermentation is carried out by *S. cerevisiae* (following a first stage carried out by *Aspergillus oryzae*, in which starch hydrolysis is perform by the secreted amylases of the utilized fungus). Wine fermentation (as one of the earliest industrialized food production examples) is undergone by *S. cerevisiae*. Moreover, bread is produced through the use of various bakers' yeast strains of *S. cerevisiae* which aid to the development of dough structure and texture (through evolution of carbon dioxide during fermentation) and offer a distinctive and desirable flavor and to some extent improve its nutritive value. *S. cerevisiae* presents the ability to be genetically transformed with exogenous DNA by treatment with alkali cations or by electroporation (Pausch *et al.*, 2005). Due to these properties yeast cells may be employed as a genetically tractable eukaryotic model for dissection of a wide variety of cellular processes. Even though *S. cerevisiae* cells are limited as an expression system, they have been used by industry as sources of biochemical products and of enzymes capable of catalyzing specific chemical reactions (Rehm and Reed, 1996). Recent advances in the genetics and molecular biology of yeast have made available modified yeast strains able to produce new biologically active proteins and enzymes. Finally, compounds identified through the use of yeast-based drug discovery technologies can be used as pharmaceuticals or in agricultural biotechnology (Pausch *et al.*, 2005).

1.6. The yeast *Yarrowia lipolytica*

Table 1. 6. Systematic classification of yeast *Yarrowia lipolytica*

Kingdom	Fungi
Phylum	Ascomycota
Class	Saccharomycetes
Order	Saccharomycetales
Family	Hemiascomycetes
Genus	*Yarrowia*
Species	*Yarrowia lipolytica*

Yarrowia belongs to the family Hemiascomycetes and was formerly known as *Candida*, *Endomycopsis* or *Saccharomycopsis lipolytica*. A new genus was identified by David Yarrow of Delft Microbiology Laboratory and in acknowledgement Van der Walt and Von Arx (1980) proposed the generic name *Yarrowia*. The part of the species name "*lipolytica*" originates from the ability of this yeast to hydrolyze lipids. Most *Yarrowia* strains do not present the ability to grow above 32 °C and the species is strictly aerobic. It is considered as non-pathogenic and has been classified as Generally Regarded As Safe (GRAS) by the American Food and Drug Administration (FDA) for citric acid production (Fickers *et al.*, 2005).

In nature, *Y. lipolytica* strains are isolated from substrates rich in fat and proteins (Sinigaglia *et al.*, 1994), such as dairy products (cheeses like Camembert, Livarot, Rokpol as well as several types of yogurts) (Guerzoni et al, 1993; Barth and Gaillardin, 1996; Roostita and Fleet, 1996; Suzzi *et al.*, 2001; Vasdinyei and Deák, 2003), sausages (Gardini *et al.*, 2001), soil, sewage and oil-polluted media (rich in lipid content) or marine and hypersaline environments (Barth and Gaillardin, 1996; Kim *et al.*, 1999; Schmitz *et al.*, 2000; Zinjarde and Pant, 2002). Its ability to degrade proteins and lipids can be clearly visualized by the production of extracellular lipolytic and proteolytic activities (Roostita and Fleet, 1996). In peptone- or protein-rich media, the alkaline extracellular protease (AEP) may represent up to 1-2 g L^{-1}. In lipid-rich media, *Y. lipolytica* secretes extracellular lipase.

There is a large volume of studies using *Yarrowia* as a model organism due to its peculiar characteristics and phenotypes. In middle 1960s, it was grown (as first application) on n-alkane substrates for the production of single cell protein (SCP) (Fickers *et al.*, 2005). Following, due to its high-level protein secretory ability, studies were initiated on this sector, both in protein secretion

and in developing efficient tools for heterologous protein production (Fickers *et al.*, 2005). Later, its ability to efficiently utilize hydrophobic substrates led to the analysis of hydrophobic substrates utilization, peroxisome biogenesis, *ex novo* lipid production through fat fermentation and lactone production. Moreover, its ability to form true hyphae allowed *Y. lipolytica* to be used as a model to identify the genes involved in the yeast-to-hypha transition. Finally, *Yarrowia* was shown to be a very good model for the study of the mitochondrial complex I (Aggelis *et al.*, 1997; Casaregola *et al.*, 1997; Papanikolaou *et al.*, 2001; Papanikolaou and Aggelis, 2010; 2011a; 2011b; Nicaud, 2012).

1.6.1. Dimorphism of *Yarrowia lipolytica*

Y. lipolytica is able to undergo a true yeast-hypha transition. It grows both as in the yeast form with a polar budding pattern (Fig. 1.20.) as also in hyphal pattern (short mycelial cells) (Fig. 1.21.) depending on the yeast genetic background and on the culture conditions (e.g. yeast form when grown on YNB and hyphal growth by replacing glucose with N- acetylglucosamine or by adding serum to the culture medium). Wild type strains of *Y. lipolytica* exhibit diverse colony morphologies which can range from heavily convoluted and matt to smooth and glistening. Some of the aforementioned growth conditions that lead to this diversity are carbon source, nitrogen source, citrate and pH. For instance, Ruiz-Herrera and Sentandreu (2002) studied the different effectors of dimorphism in *Y. lipolytica* and concluded that mycelium formation was maximal at pH near neutrality and decreased as pH was lowered to become almost null at pH 3.

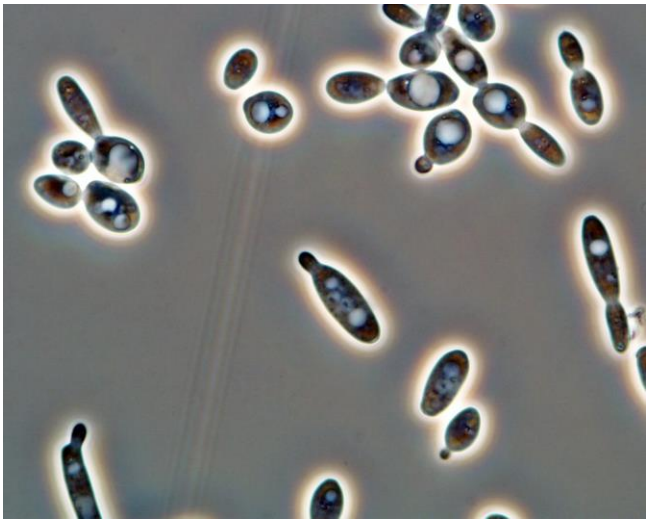


Figure 1. 20. Cells of *Yarrowia lipolytica* growing on glycerol
http://www.ufz.de/index.php?de=17937

Dimorphic fungus *Yarrowia lipolytica* is grown as in budding cells, pseudohyphae and septate branching hyphae. The proportion of cellular structure and hyphae existence is dependent on the strain used but mainly on the culture conditions. Various studies have been studied the differences in the physiology of the microorganism during the transition from budding cells to hyphae, concluding in higher concentration of amino-sugars and lower concentration of proteins at hyphae walls. Moreover correlation between certain genes and hyphae construction was observed. Thus, mutations at genes SEC14 and GPR1 as also STE11 gene deletion contribute to the

appearance of dimorphism phenomenon and increase the chance of moving from cell structure to hyphae. Likewise, the carbon source has been considered of significant importance as a *Y. lipolytica* strain cultured in nitrogen-limited cultures with glucose or industrial glycerol used as individual substrates or co-substrates developed only into its single-cell form while almost no mycelia were observed (Papanikolaou *et al.* 2002b) whereas, during cultivation on saturated free-fatty acids used as substrates (Papanikolaou *et al.* 2001), growth with mycelium morphology was equally enhanced (Papanikolaou *et al.*, 2007).

Dimorphism is of crucial importance because of the fact that it is correlated with the various fungi pathogenesis. *Y. lipolytica* is considered to be a (non-conventional) yeast model as the transition observed at dimorphism phenomenon could be easily take place *in vitro* while it can be easily studied using both classical genetic analysis and alternative molecular biology techniques. It should be also stressed out that *Y. lipolytica* when at dimorphism, presents great similarity to fungus *Candida albicans*, the most important pathogenic fungus (Kreger-van Riz and Veenhuis, 1973; Van der Walt and Von Arx, 1980; Rodriguez and Dominguez, 1984; Vega and Dominguez, 1986; Barnett *et al.*, 1990; Rodriguez *et al.*, 1990; Guevara-Olvera *et al.*, 1993; VandenBossche *et al.*, 1993; Lopez *et al.*, 1994; Barth and Gaillardin, 1996 and 1997; lLiu, 2001 and 2002; Ruiz-Herrera and Sentandreu 2002; Szabo and Štofaníková, 2002; Dhillon *et al.*, 2003; Dominguez *et al.*, 2003; Nicaud, 2012).

Figure 1. 21. Typical mycelium morphology of *Yarrowia lipolytica* cultivated on stearin used as the sole carbon source. Magnification x100 (Papanikolaou *et al.*, 2007)

1.6.2. Physiology of *Yarrowia lipolytica*

1.6.2.1. Hydrophobic substrates (HS) assimilation

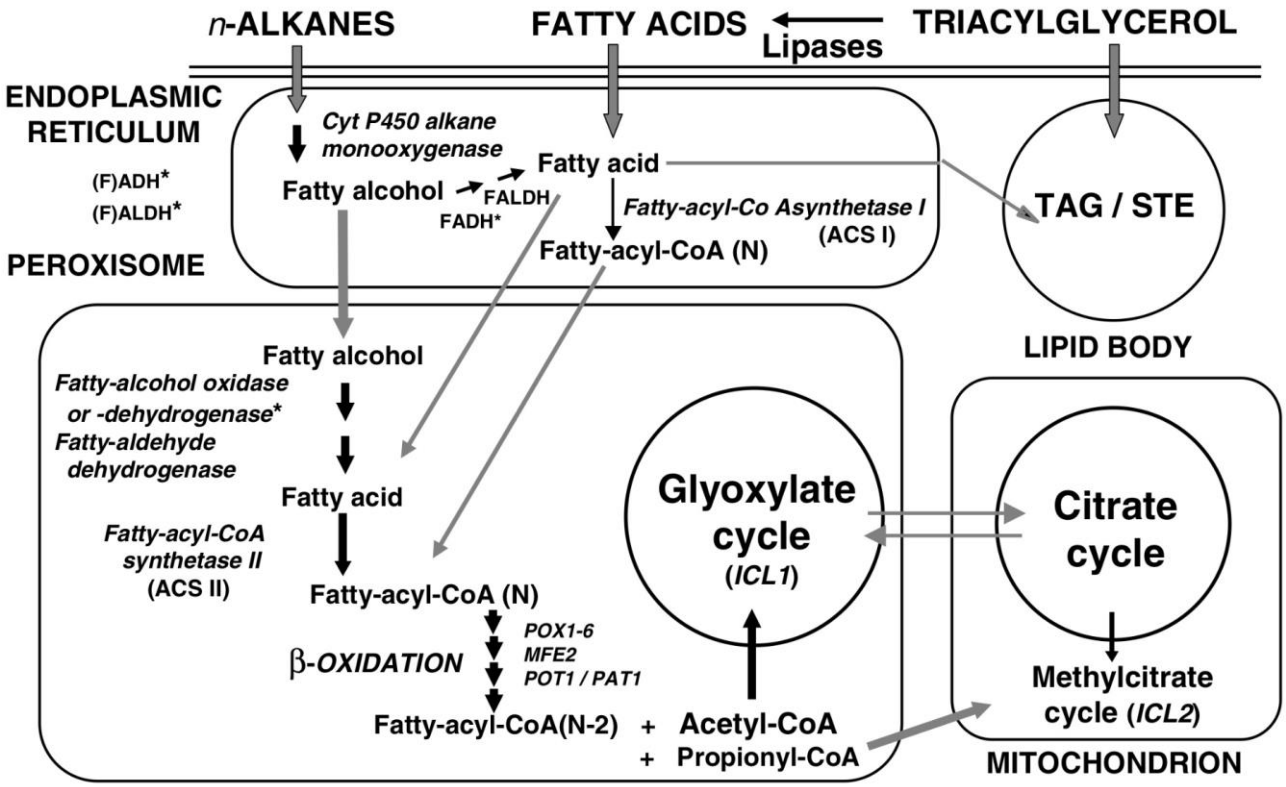


Figure 1. 22. Main metabolic pathways and cellular compartments involved in HS degradation (Fickers at al., 2005)

The catabolism of HS (such as alkanes, fatty acids and triglycerides) is a quite complex metabolism which involves several metabolic pathways taking place in different sub-cellular compartments (Fig. 1.22.). Since these substrates are not miscible with water, their uptake requires morphological and physiological modifications, notably in cell adhesion properties (surface hydrophobicity) or in the production of emulsifiers (surfactants).

Assimilation of n-alkanes

The emulsifying of hydrocarbons on the cell surface (in order small droplets to be created) is the first stage of their assimilation. An extracellular emulsifier called "liposan" (88% hydrocarbons and 12% proteins) is induced from yeast cells when using n-alkanes as substrate (Cirigliano and Carman, 1984; 1985). During alkane assimilation by yeasts, the metabolic flow of carbon from alkane substrates to the synthesis of all cellular components via fatty acids takes place, that is quite different from the case of conventional substrates (like carbohydrates). Alkanes are first oxidized, by monooxygenase of cytochrome P450 in endoplasmic reticulum (ER) and further converted by

fatty-alcohol-oxidising enzymes [fatty-alcohol oxidase (FAOD), fatty-alcohol-dehydrogenases (FADH), fatty-aldehyde dehydrogenases (FALDH)] into the corresponding fatty acids in the peroxisomes or in the ER. Following the fatty acids (FAs) are either oxidized in the peroxisomes or used for cellular material biosynthesis or finally are stored as lipid bodies (LB) in the forms of triglycerides or esters of sterols (Ilchenko *et al.*, 1980; Mauersberger and Matyashova, 1980; Delaisse *et al.*, 1981; Cirigliano and Carman, 1984; 1985; Luo *et al.*, 2002; Fickers *et al.*, 2005; Papanikolaou and Aggelis, 2010).

Assimilation of TAGs

As mentioned above, *Y. lipolytica* possesses the ability to utilize n-alkane, TAGs or fatty acids as carbon source. Concerning the utilization of TAGs, they should be initially modified into fatty acids (Fig. 1.23a.) in order to be used in various metabolic pathways. The microorganisms capable of breaking down TAGs or fatty esters obligatorily possess an active lipase system in their enzymatic arsenal. The free fatty acids (existing as initial substrate or produced after lipase-catalyzed hydrolysis of the employed TAG or fatty esters) will be incorporated by active transport inside the cells or the fungal mycelia. Following, the FAs will either be dissimilated for growth needs or will be accumulated as storage materials as they are, or after having been subjected to enzyme-catalyzed modifications (e.g. desaturation or elongation reactions) (Papanikolaou and Aggelis, 2010; 2011a). Fatty acids are activated into their corresponding acyl-CoA esters by fatty-acyl-CoA synthetase (ACS). ACS I and ACS II have been traced in *Y. lipolytica*; ACS I acts in ER giving direct incorporation of fatty-acyl moieties into cellular lipids after chain elongation and desaturation. The corresponding acyl-coA esters ACS II are subsequently degraded to acetyl-CoA

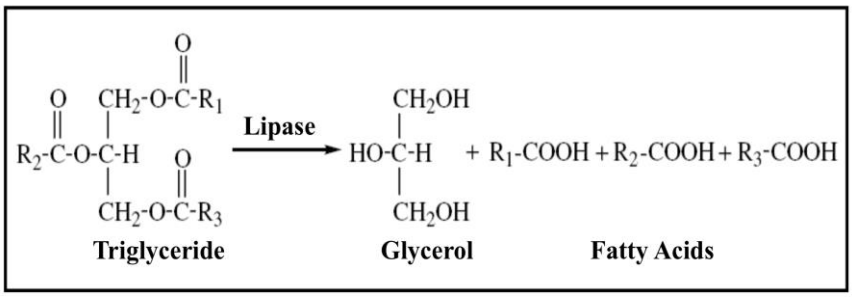


Figure 1. 23a. Lipase-catalyzed hydrolysis of triglyceride

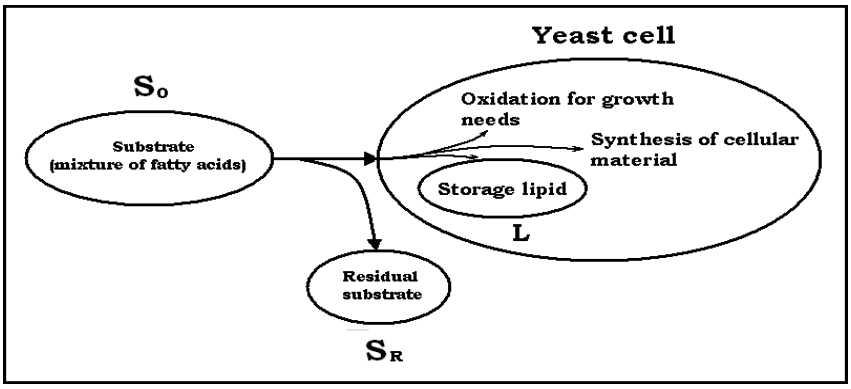


Figure 1. 23b. Fate of extracellular and intracellular fatty acids during growth of oleaginous yeasts on a fatty mixture (Papanikolaou and Aggelis, 2010)

and propionyl-CoA (in case of odd-chain alkanes) via peroxisomal β-oxidation by ACS II. The process of *ex novo* lipid accumulation is a primary anabolic process occurring simultaneously with the production of lipid-free material, being independent from the nitrogen exhaustion in the medium, but is critically influenced by the dissimilation and accumulation rates of the various fatty acids previously incorporated inside the cells or mycelia. Lipid accumulation is a dominant process when the rate of accumulation is significantly higher than the rate of dissimilation for growth needs (Papanikolaou and Aggelis, 2010). The fate of the extra-and intracellular fatty acids during growth of a yeast strain on fatty materials is depicted in Fig. 1. 23b..

β-Oxidation of the intracellular FAs (Fig. 1.24.) includes a cycle with four steps in the end of which two carbons are lost from the acyl-CoA ester chain. This cycle is repeated until the acetyl-CoA (and, thus, the FA) is fully degraded. Formed acetyl-CoA, or propionyl-CoA enter the glyoxylate-cycle pathway, located in peroxisomes, which interacts with the TCA and methylcitric-acid cycles, located in mitochondria. Specifically, the synthesis of TCA cycle intermediates from acetyl-CoA via the glyoxylate cycle is followed by gluconeogenesis and the methyl citrate cycle for propionyl-CoA utilization is activated when using odd-chain alkanes. Depending on environmental conditions, cells may accumulate FFA into lipid bodies as TAG or steryl esters (STE). It should be also mentioned that triglycerides are first hydrolyzed by lipolytic enzymes into FFA which are then taken up by the cell as mentioned above (Barth and Gaillardin, 1996; Fickers, 2005).

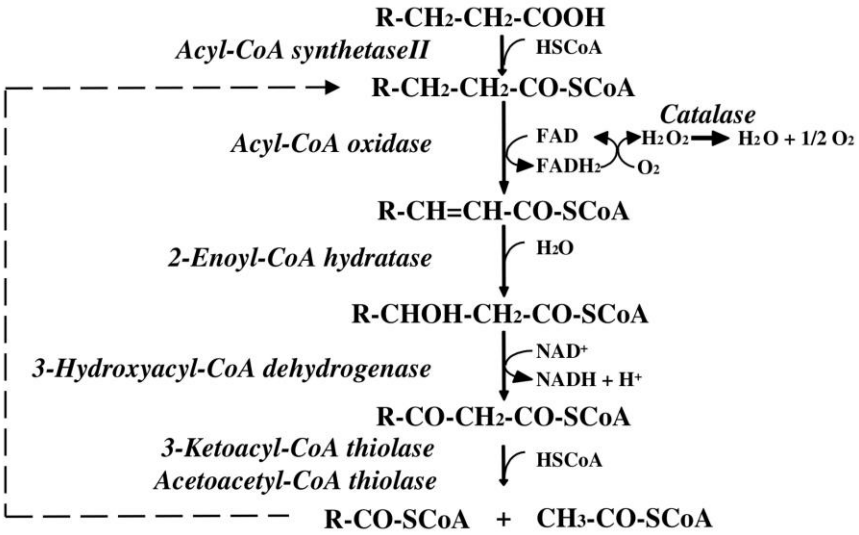


Figure 1. 24. β-oxidation of FA

which are converted into acyl-CoA esters (by ACS) and enter then the four-step β-oxidation sequence. Each cycle results in the loss of two carbons (acetyl-CoA) (Fickers *et al.*, 2005)

1.6.2.2. Lipid accumulation and storage

As mentioned above, depending on environmental conditions, yeast cells are able to mobilize FFA or to store inside them as triacylglycerols (TAG) (Mlíčková *et al.*, 2004) and steryl esters (STE) (Mlíčková *et al.*, 2004; Athenstaedt *et al.*, 2006) into structures known as lipid bodies (LB), lipid particles (LP), oil bodies or oleosomes (Fig. 1.25.). The structure of those particles is simple and common in all cells and looks like lipoproteins in mammals. LB consist of a hydrophobic core formed from neutral lipids (mainly TAG and to a lesser extent STE) which is surrounded by a phospholipid monolayer with a few embedded proteins (Mlíčková *et al.*, 2004; Athenstaedt *et al.*, 2006). LB role was initially thought to be as energy source and/or as FA and sterols source for membrane biogenesis. Later studies have indicated that LB are part of certain lipophilic components (like steroid hormones and prostaglandins) while various LB proteins are categorized as enzymes that take place in fatty acid metabolism. For instance, LB proteins of *Saccharomyces cerevisiae* contribute to the FA activation, to the synthesis and degradation of TAG, in the metabolism of sterols and in the synthesis of phosphatidic acid (PtdOH) (Athenstaedt *et al.*, 2006). Furthermore, the concentration of lipids and FAs in the LB is affected by the synthesis of acyl-CoA oxidase A (Mlíčková *et al.*, 2004). Several hypotheses to explain the mechanism of LB formation have been proposed. LB biogenesis probably results from budding of the ER (Murphy, 2001; Mlíčková *et al.*, 2004). Enzymes in the ER synthesize neutral lipids that are deposited between the two leaflets of the ER membrane to form a pre-LB. When this structure reaches a certain size, it buds off to form a mature LB (Zweytick *et al.*, 2000; Mlíčková *et al.*, 2004). Finally, LB content and composition depend on growth conditions and substrate composition (Papanikolaou and Aggelis, 2003a; Fickers *et al.*, 2005). It was also demonstrated that yeast genotype could improve LB accumulation (Fickers *et al.*, 2005).

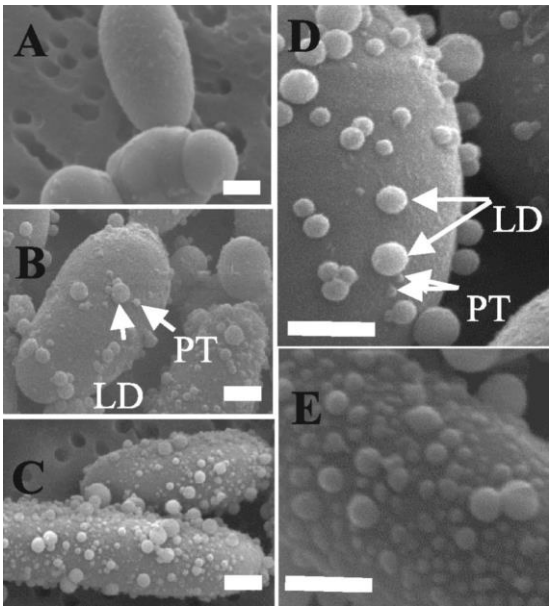


Figure 1. 25. Cryo-scanning Electron Microscopy of lipid droplets (LD) on *Y. lipolytica* cells (Mlíčková *et al.*, 2004)

The oleaginous microorganisms (as a general rule), consume their own lipid reserves after exhaustion or decrease in the upatake rate of the carbon source in the culture medium. The action of TAG lipase(s) and steryl-esters (STEs) hydrolase(s) is required for the utilization of TAGs and STEs (onset of storage lipid turnover), while carbon starvation conditions are also required. An

intra-cellular lipase's and hydrolase's system is responsible for esters cleavage and the generation of fatty acids that will be subsequently catabolized via the process of β-oxidation and the produced acetyl-CoA will be further converted via the TCA cycle and the anaplerotic by-pass of glyoxylic acid. It should be stressed out that the phenomenon of cellular lipid degradation in the oleaginous microorganisms is independent of the type of the carbon source assimilated in order to proceed with reserve lipid accumulation, since such turnover has been observed in both *de novo* and *ex novo* SCO accumulation processes (Aggelis and Sourdis, 2007; Papanikolaou *et al.*, 2004a; Fakas *et al.*, 2007; Makri *et al.*, 2010; Vamvakaki *et al.*, 2010). During lipid turnover period, in the case of *de novo* lipid accumulation, (potentially remaining in the medium) sugar, is no longer assimilated and the function of TCA cycle through the utilization of NAD^+-(and potentially $NADP^+$)-isocitrate dehydrogenase had already been suppressed (due to extra-cellular nitrogen limitation). In the case of *ex novo* lipid accumulation, the degradation of lipid occurs principally through the glyoxylic acid anaplerotic by-pass pathway. The reserve lipid turnover occurs generally when considerable decrease of extra-cellular flow rate of the substrate aliphatic chains is realized. This is either due to almost absence of substrate fat from the culture medium or due to the presence of a fat that is not easily assimilable by the microorganisms (i.e. a substrate fat substantially rich in the FA C18:0 that is not easily catabilized by yeast strains due to discrimination against it) (Papanikolaou *et al.*, 2001; 2002a; Papanikolaou and Aggelis, 2003a).

It should be also mentioned that in *ex novo* lipid accumulation, since extra-cellular nitrogen may be present in the culture medium, culture conditions could be still favorable for microbial growth even during reserve lipid degadation. Finally, utilization of multiple-limited media is of great industrial interest for the process of SCO fermentation as such media do not negatevily influence the process of storage lipid accumulation (Papanikolaou *et al.*, 2004a; Papanikolaou and Aggelis, 2011a).

1.6.3. Biotechnological uses of the yeast *Yarrowia lipolytica*

Due to the availability of its complete genome sequence and of efficient genetic tools, *Y. lipolytica* has emerged as a good model amongst non-conventional yeasts. Accordingly, the growing scientific interest in *Y. lipolytica* is illustrated by the increasing number of articles and book chapters devoted to this yeast (Papanikolaou and Aggelis, 2010; Nicaud, 2012). This yeast demonstrated the ability, when grown on various substrates, to produce high added value products, such as single cell protein (SCP), single cell oil (SCO), enzymes and organic acids.

1.6.3.1. Single cell protein (SCP) production

Yeasts were used as SCP source during World War I from Germany, where cells of *S. cerevisiae* were given for human consumption as meat substitute (Buzzini and Vaughan-Martini, 2006). Later on, SCP capable producing yeasts were used as animal food additives in former socialistic countries of Eastern Europe (Burden and Eveleigh, 1990; Buzzini and Vaughan-Martini, 2006). From the mid 60s to the 80s, *Y. lipolytica* (or strains of *Candida*, of the former genus *Hansenula* and of *Saccharomycopsis*) was used in large-scale fermentation for production of SCP from n-alkanes (Burden and Eveleigh, 1990; Buzzini and Vaughan-Martini, 2006) [or in general on lipid substrates of plant (Montet *et al.*, 1983) or animal (Papanikolaou *et al.*, 2001) origin], allowing process development for this yeast in very large fermenters with high cell densities. Despite the fact that a large amount of money was invested for the evolution of a financially feasible process using hydrocarbons (petroleum derivatives) as substrate, the industrial adaptation of this plan failed (Tusé and Miller, 1984; Demain *et al.*, 1998; Buzzini and Vaughan-Martini, 2006). Thus, the use o methanol as substrate for the production of SCP was studied as alternative (Tusé and Miller, 1984; Burden and Eveleigh, 1990; Buzzini and Vaughan-Martini, 2006).

Finally, the interest was focused on agro-industrial by-products and wastes and their adequacy as SCP substrates was studied. Potato starch was used as substrate for the co-culture of amylolytic (*Saccharomycopsis fibuligera*) and non-amyolytic (*C. utilis*) yeasts, while milk serum (whey) could be a promising substrate for lactose positive yeasts (Buzzini and Vaughan-Martini, 2006).

As mentioned before, *Y. lipolytica* when grown in various substrates presents the ability to produce metabolites in large quantities. The most widespread application of strains of this species is focused on the production of SCP (Barth and Gaillardin, 1996). The ability of *Y. lipolytica* to grow on industrial derivative of animal fat (Papanikolaou *et al.*, 2002a; 2007), rapeseed (Montet *et al.*, 1983) and stickwater (Green *et al.*, 1976) (a by-product of the fish meal and oil industry) was studied, resulting in the increase of SCP content of those wastes. Finally kinetic modeling approaches of SCP and lipases production when *Y. lipolytica* grown on animal fat derivatives were successfully performed (Papanikolaou *et al.*, 2007).

1.6.3.2. Single cell oil (SCO) production

Some lipophilic compounds known as microbial oil or single cell oil (SCO) present vast industrial interest the latest years. Oleaginous bacteria accumulate mainly specific lipids whereas oleaginous yeasts accumulate TAGs rich in polyunsaturated fatty acids. Various hydrophobic compounds were used as substrates for the production of SCO, such as vegetable oils, esters, soaps and hydrocarbons (for more information see paragraphs 1.4.3. and 1.4.4.). A number of yeasts of genus *Candida*, *Cryptococcus*, *Endomycopsis* as also some species of former genus *Hansenula* like *Lipomyces*, *Rhodosporidium*, *Rhodotorula*, *Trigonopsis* and *Yarrowia*, tend to accumulate cellular lipid in small droplets (Fig. 1.26.). The production of the aforementioned lipid many times exceeds 70% of total biomass and is synthesized almost exclusively from TAGs and from mainly C18 saturated and unsaturated fatty acids (mainly oleic and to lesser extent stearic and linoleic) and C16 saturated (palmitic) fatty acids.

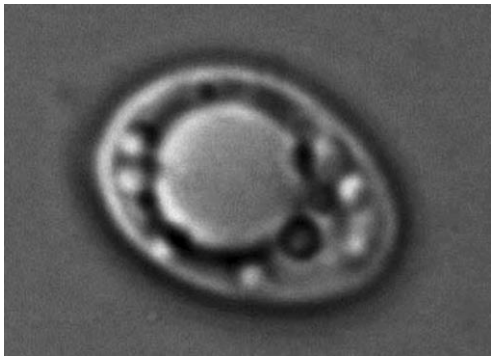


Figure 1. 26. Lipid Bodies in *Y. lipolytica* strain W29 (Fickers *et al.*, 2005)

As mentioned above, from mid 1960's till 1980 *Y. lipolytica* was vastly used for the production of SCP growing on n-alkanes and other lipophilic substrates. Recently, these technologies were applied for SCO production including a new cocoa-butter substitute (Papanikolaou and Aggelis, 2010; 2011b). Thus, the lipid accumulation by *Y. lipolytica* when grown on industrial fats (stearin) and vegetable oils (rapeseed oil) was studied. Finally, it has been demonstrated that lipid accumulation could also be obtained by genetic manipulation of *Y. lipolytica*. Potential application of lipids and lipid fractions might include the production of cerebrosides from the sphingolipid fraction of hexadecane-grown yeast cells (Matsuo *et al.*, 1981; Montet *et al.*, 1983; Burden and Eveleigh, 1990; Bati *et al.*, 1984; Papanikolaou *et al.*, 2001, 2002 and 2003b; Papanikolaou and Aggelis, 2003b; Fickers *et al.*, 2005; Buzzini and Vaughan-Martini, 2006).

1.6.3.3. Enzymes production

In comparison with some fungi (*Aspergillus niger*) and bacteria (*Bacillus* sp.), yeasts are not an important source of industrially useful enzymes. Nevertheless, the ability of various yeast strains for the production of enzymes and their use in industry has been studied lately (Burden and Eveleigh, 1990; Ratledge and Tan, 1990; Buzzini and Vaughan-Martini, 2006). Microbial proteases

have been thoroughly studied for their application in food and beverage industry (Nelson and Young, 1986; Dizi and Bisson, 2000).

For a large period of time, the study of amylolytic enzymes producing yeasts has been focused only on *S. fibuligera*. Due to the use of amylases in the industrial production of confectionary goods and marmalades, the interest on finding species which degrade starch increased. As a result, the synthesis of α-amylases and glucoamylases from yeast species of genus *Saccharomyces*, *Candida*, *Filobasidium*, *Lipomyces* and *Schwanniomyces* was studied (Buzzini and Vaughan-Martini, 2006). The production of lipases is also of great importance. The production of lipases from strains of species *Y. lipolytica*, *Cryptococcus (Candida) curvatus (curvata)*, *Y. lipolytica*, *Candida deformans* and *Rhodotorula glutinis* and their application in oil and fats industry, in detergents industry and in food industry has been studied (Montet *et al.*, 1983; Ratledge and Tan, 1990; Hantzinikolaou *et al.*, 1999; Papanikolaou *et al.*, 2007).

In contrast with yeasts that produce lipases and proteases, yeasts that produce other enzymes have not been studied in such extent. For the production of esterase *Y. lipolytica* and *C. guilliermondii*, for the production of cellulase *Aureobasidium pullulans*, for β-glucosidase *Dekkera intermedia* και *C. ıntermedia* and for the production of lyase *Cryptococcus* spp. and *Trichosporon* spp, have been studied (Buzzini and Vaughan-Martini, 2006). Lately, genetic modified yeast strains which possess desirable enzymatic activity have been studied. For instance, genetic modified strains of *C. rugosa* and *Y. lipolytica* have been used for further production of lipases whereas strains of *S. cerevisiae* for the production of invertase (Fickers *et al.*, 2005; Buzzini and Vaughan-Martini, 2006; Beopoulos *et al.*, 2009).

Specifically, *Y. lipolytica* strains have been reported to secrete (when grown on rich YPD type medium at pH=6.8) large amounts of alkaline extracellular protease (AEP) and acid extracellular protease (induction of this enzyme occurs under conditions very similar to the ones used for secreting AEP, except for the pH of the medium which should be 4.0). Moreover, extracellular RNase activity is detected when *Y. lipolytica* strains grow on cultures with conditions leading to AEP secretion. Following, if *Y. lipolytica* strains grow on media depleted of inorganic phosphate sources, biosynthesis of a cell-bounded acid phosphatase activity is induced. Finally, *Y. lipolytica* strains display lipase activities when grown of TAG of fatty acid-enriched media. Specifically, growth on TAG-type substrates is accompanied by secretion of an extra-cellular lipase called Lip2p, encoded by the *LIP*2 gene (Pignède *et al.*, 2000). This gene is encoded for the biosynthesis of a precursor pre-mature protein with Lys-Arg cleavage site. The secreted lipase was reported to be a 301-amino-acid glycosylated polypeptide which belongs to the TAG hydrolase family (EC 3.1.1.3) (Pignède *et al.*, 2000; Beopoulos *et al.*, 2009). The Lip2p precursor protein was

processed by the KEX2-like endoprotease encoded by the gene *XPR*6, whereas deletion of the above gene resulted in the secretion of an active but fewer stable pro-enzyme (Pignède *et al*., 2000). Simultaneously, other intra-cellular lipases (Lip7p, Lip8p) may also be produced and secreted into the culture medium, that present different fatty acid specificities, with maximum activity being displayed against $^{\Delta 9}$C18:1 (oleic), C6:0 (capronic) and C10:0 (caprinic) fatty acids (Fickers *et al.*, 2005; Beopoulos *et al*., 2009). Moreover, lipase secretion into the culture medium can be conducted as a physiological response of the strains in the presence of long-chain free-fatty acids found into the medium (Sharma *et al*., 2001). Microbial lipases can be used for various purposes (e.g. utilization as biocatalysts, use in food technology and food processing, leather industry, cosmetology etc), while their production is also substantially enhanced by the addition into the culture medium of organic nitrogen (e. g. peptone, urea etc) (Barth and Gaillardin, 1997; Fickers *et al*., 2005; Beopoulos *et al*., 2009; Nicaud, 2012).

1.6.3.4. Organic acids production

Yeasts, as they transcend in cellular growth in comparison to fungi forming mycelia, could be candidates for study of organic acids production (such as citric, α-ketoglutaric, gluconic, oxalic, 2-hydroxyglutaric, 2-ketoglutaric and isopropylmalic) many of which present great importance in food and pharmaceutical industry (Burden and Eveleigh, 1990; Buzzini and Vaughan-Martini, 2006). Various species like *Y .lipolytica*, *C. zeylanoides*, *C. boidinii* and *C. tropicalis* have been studied for the production of citric acid (Papanikolaou *et al.*, 2002b).

The yeast *Y. lipolytica* possesses the unique ability to produce, accumulate and excrete into the medium a broad range of organic acids, including the TCA-cycle intermediates citric, isocitric and 2-ketoglutaric, as well as pyruvic acid. These acids can be produced from low-cost carbon sources (alkanes, vegetables oils, fats, ethanol, molasses, starch hydrolysates) by growth limitation caused by different nutrition factors, like nitrogen source, thiamine, phosphate, or mineral salt compounds. As mentioned before, nitrogen exhaustion triggers secretion of citric and and isocitric acids, while limitation of thiamine at low pH values causes secretion of mainly 2-ketoglutaric and pyruvic acid (Fickers *et al.*, 2005).

Despite the fact that until recently, fungus *Aspergillus niger* was predominantly used for the production of citric acid using molasses as substrate, *Y. lipolytica* offers an alternative as it has an advantage of growing on various substrates and exhibit a higher maximal product formation rate and a higher substrate-related yield. Moreover, it shows advantages in sugar tolerance, product isolation, waste and sewage minimization, and the possibility to develop a process with closed circuits. Therefore, processes are under development for *Y. lipolytica* using renewable low-cost

substrates such as plant oils, fatty acids and glycerol. For instance, *Y. lipolytica* strains have been cultivated on glucose, n-paraffins, n-alkanes, ethanol, date coats, tapioca starch hydrolyzates and glucose hydrol for the production of citrate and isocitrate (Barth and Gaillardin, 1997). Likewise, *Y. lipolytica* strains have been cultivated on n-alkanes (in the presence of a limited thiamine supply) (Barth and Gaillardin, 1997; Finogenova *et al.*, 2002), glucose and ethanol-containing media for the secretion of large amounts of α-ketoglutarate, or, finally, on media containing glucose and glycerol (under the conditions of thiamine deficiency) for the accumulation of pyruvic acid (Finogenova *et al.*, 2002; Fickers *et al.*, 2005).

The simultaneous production of citric and isocitric acid (ratio depending on the carbon source and strain) by *Y. lipolytica* is a disadvantage of citric acid production bioprocess. Wild-type strains produce citric acid/isocitric acid in a ratio of about 90:10 on glucose, glycerol or ethanol, while this ratio is 60:40 on alkanes or triglycerides. However, this ratio can be changed by selecting mutant strains or by gene amplification. Production levels and processes developments like 2-ketoglutaric acid accumulated up to 195 g L^{-1} by selected *Y. lipolytica* strains grown on alkanes in fed-batch conditions (resulting into yield 90%) demonstrate that this species may be used for 2-ketoglutaric production (Fickers *et al.*, 2005).

AIM OF THESIS

OMWs are the principal residues deriving from the olive fruit processing by mechanical means, being considered as one of the strongest and most difficult to treat industrial effluents. In general, these effluents are discharged directly to the environment without any other treatment. On the other hand, recent developments have indicated that OMWs should be considered as fermentation medium to be valorized rather than a waste to discharge, being a potential substrate for various fermentation processes. Therefore research should focus on both the waste bioremediation and the production of high-added value products simultaneously under cost-effective technologies.

The aim of the present study was dual; to investigate the potential of yeast strains to grow on OMW-based media so as to produce added value products and simultaneously to reduce the color and the phenol content of the residue. OMWs were used as simultaneous substrate and process water of the fermentations employed. Thus, addition of low-cost sugars (such as commercial glucose or sugars contained in low-cost by-products like molasses) supplementing already existing OMWs sugar content for the enhancement of added value compounds production, is of great importance. Molasses, which were blended with OMWs in some experiments of current investigation, are the viscous by-product of the sugar cane or sugar beet processing and pose one of the main environmental threats for soil and water, as they resist to microbial degradation and to conventional biological treatment processes. In order to evaluate the physiological behavior of the tested strains, the impact of several factors (such as initial sugars concentration and C/N ratio, initial phenolic compounds quantities, different aeration regimes, scale up of methods employed to laboratory bioreactors) upon the biogenesis of biomass, ethanol, citric acid, cellular lipids and the fatty acid composition of cellular lipids produced by the strains were investigated. Besides the physiological aspect of the study, a second aim concerning the technological development of OMW fermentation rarely applied in the past, namely the potential of fermentation under either pasteurized or even completely non-aseptic conditions, was developed. Application of pasteurized or non-aseptic process in large-scale operations can significantly reduce the overall cost of the proposed bioprocesses, rendering the whole concept completely eco-friendly and cost-effective.

The originality of the present study is based upon certain points: There are a few reports in literature suggesting the simultaneous bioremediation of OMWs and production of added-value products. Moreover, there are a couple of reports presenting citric acid production from OMW-based media, whereas in very few reports OMW-based media are used as substrate for the production of single cell oil or ethanol. Following, yet only a very limited number of reports refer to the use of pasteurized and/or non-aseptic OMW-based media in several types of microbial processes for the production of added-value compounds. Finally, none report is included at

international bibliography presenting the simultaneous presence of molasses and OMWs (not pre-treated) as blended substrates. Concerning the aforementioned blended substrates, it should be stressed out that the strain that was used in order to produce biomass and bioethanol was grown in completely non-aseptic conditions, without contamination problems that could risk decreasing the efficiency of the fermentation. It should be also highlighted that the sole external addition of nutrients was that of yeast extract and $(NH_4)_2SO_4$ while in the current study, the phenolic content of OMWs was much higher than the typical values found in the literature, indicating that OMWs can partly or even completely substitute tap water in the bioethanol fermentation in which molasses are usually used as carbon substrates, without significant negative effect in the performed bioprocess.

2. MATERIALS & METHODS

2.1. Characteristics and specifications of Olive Mill Wastewaters and Molasses

OWMs used in this study were obtained from a three-phase decanter olive mill in the region Kalentzi (Corinthia, Peloponnisos – Hellas) and were immediately frozen at T= – 20 ± 2 °C in order to preserve their characteristics. To be used in the experiments, OMWs were de-frozen and the solids removed by centrifugation (9000 rpm, 15 min, T=21 ± 1°C – Hettich Universal 320R). OMW phenolic content expressed as gallic acid equivalent was 10.0 ± 0.5 g L^{-1}, while the sugar concentration of this residue was 30.0 ± 3.0 g L^{-1}, expressed as glucose equivalent. Glucose to a quantity of 90% *w/w*, was the principal sugar quantified in the OMWs (analysis performed by HPLC – see below). Fructose was also found, but its concentration was significantly lower than that of glucose (~10% *w/w*) whereas no other sugars were identified. The OMWs pH was c. 5.5. The OMWs used, contained negligible quantities of olive oil (0.4 ± 0.1 g L^{-1} – determination of oil conducted after triple extraction with hexane). Organic acids were also present in small quantities. The principal organic acids detected were acetic acid (2.0 ± 0.5 g L^{-1}) and gluconic acid (2.0 ± 0.5 g L^{-1}). Raw beet molasses used, were obtained from Hellenic Industry of Sugar S.A. (Orestiada, Greece) and kept at 4 °C. The concentration of total sugars (analysis performed by DNS method – see below) was 573.0 ± 10.0 g L^{-1}, expressed as glucose equivalent. The molasses density was 1.38-1.42 g $(cm^3)^{-1}$.

2.2. Microorganisms and culture conditions

Three strains of the yeast *Yarrowia lipolytica* [W29 (ATCC 20460), ACA-YC 5028 and ACA-YC 5033] and *Saccharomyces cerevisiae* strain MAK-1 were used. *Y. lipolytica* strains ACA-YC 5028 and ACA-YC 5033 were isolated from traditional Greek sourdoughs and identified in the Laboratory of Dairy Research-Department of Food Science and Human Nutrition, Agricultural University of Athens (Paramythiotis *et al.*, 2000). *S. cerevisiae* strain MAK-1 (Sarris *et al.* 2009) was provided by the National Agricultural Research Foundation (Laboratory of Oenology, Athens, Greece). The microorganisms were conserved in PDA (Potato Dextrin Agar) (T=6 ± 1 °C). In order to maintain the viability of the strains, the microorganisms were sub-cultured every three months.

Prior to any inoculation in liquid growth medium (pre-culture and main culture) the strains were regenerated (under sterile conditions) so as the inoculum would be three days old. Following, three 250-mL Erlenmeyer flasks (pre-culture) filled with 50 ± 1 mL of mineral salts medium (for composition of mineral salts solution see Table 2.1.) were aseptically inoculated from the principal freshly regenerated strain and were incubated in orbital shaker (for 24 ± 2 hours at 180 ± 5 rpm, 28 ± 1 °C). Gram tests were carried out in order to verify the purity of the strain. The pre-culture was consisted by 35.0 g L^{-1} of commercial glucose (with 95% *w/w* purity and impurities composed of

maltose (2% *w/w*), malto-dextrines (0.5% *w/w*), water (1.5% *w/w*) and salts (0.5% *w/w*) - provided by the Hellenic Industry of Sugar S.A., Thessaloniki Greece), 0.5 g L^{-1} $(NH_4)_2SO_4$ and 0.5 g L^{-1} yeast extract. Finally, the inoculation of the main culture (from the principal pre-culture) took place. A flow chart of the aforementioned procedure is shown in Fig. 2.1. The inoculation of the culture was done under aseptic conditions only at the trials which included sterile media (sterilization performed at c. 115 °C, 20 min).

In fermentations that were carried out in 250-mL Erlenmeyer flasks filled with 50 ± 1 mL of growth medium, the inoculation volume was 1 mL (2% *v/v* inoculum - 10^5-10^7 cfu) of the exponential pre-culture. Flasks were incubated in an orbital shaker (New Brunswick Scientific, USA) at an agitation of 180 ± 5 rpm and incubation temperature 28 ± 1 °C. The correction of the medium pH was maintained at the desired value by periodically (and aseptically concerning the trials performed under sterile conditions) adding into the flasks, quantities of KOH 5 mol L^{-1} or 1 mol L^{-1} HCl. The exact base or acid solution volume needed for pH correction was evaluated by measuring the volume of the solution required for pH correction in one (at least) flask. Following, the appropriate volume of base or acid was (aseptically) added in the remaining flasks and the value of the pH reached was verified to be the desired one. Aseptic and non-aseptic batch fermentations were also conducted in a laboratory scale bioreactor (MRB Bioreactor, AG Switzerland), with total volume 3.5 L and working volume 3.0 L, fitted with four probes and two six-bladed turbines. The culture vessel was inoculated with 60 mL (2% *v/v* inoculum) of exponential pre-culture (see above). The incubation temperature was controlled automatically at 28 ± 1 °C. Agitation rate was adjusted to 300 rpm. The pH was automatically controlled at the desired value by adding base quantities of KOH 5 mol L^{-1} or 1 mol L^{-1} HCl. Various aeration regimes were created by sparging the media with air, passing through a bacteriological filter with 0.2 μm pore size. In order to avoid loses of valuable volatile metabolites (such as ethanol) an evaporating cooling tower operated with circulation of water was also attached on the bioreactor.

Blank experiments (no OMW addition; commercial glucose or molasses added as the sole carbon source) were also carried out. All trials were conducted in duplicate (each experimental point of the kinetics was the mean value of two independent determinations). Finally, in order to investigate whether possible reduction of phenolic compounds concentration and/or color reduction occurred due to agitation (and thus aeration) alone (no presence of *Y. lipolytica* or *S. cerevisiae* cells), sterile shake flasks were subjected to agitation (180 ± 5 rpm) for 200 h without the presence of microorganisms. OMWs and glucose were added to the growth medium giving initial phenolic compounds concentration (in g L^{-1}) 1.20, 2.00 and 2.90 and Glc_0~40.0 g L^{-1}.

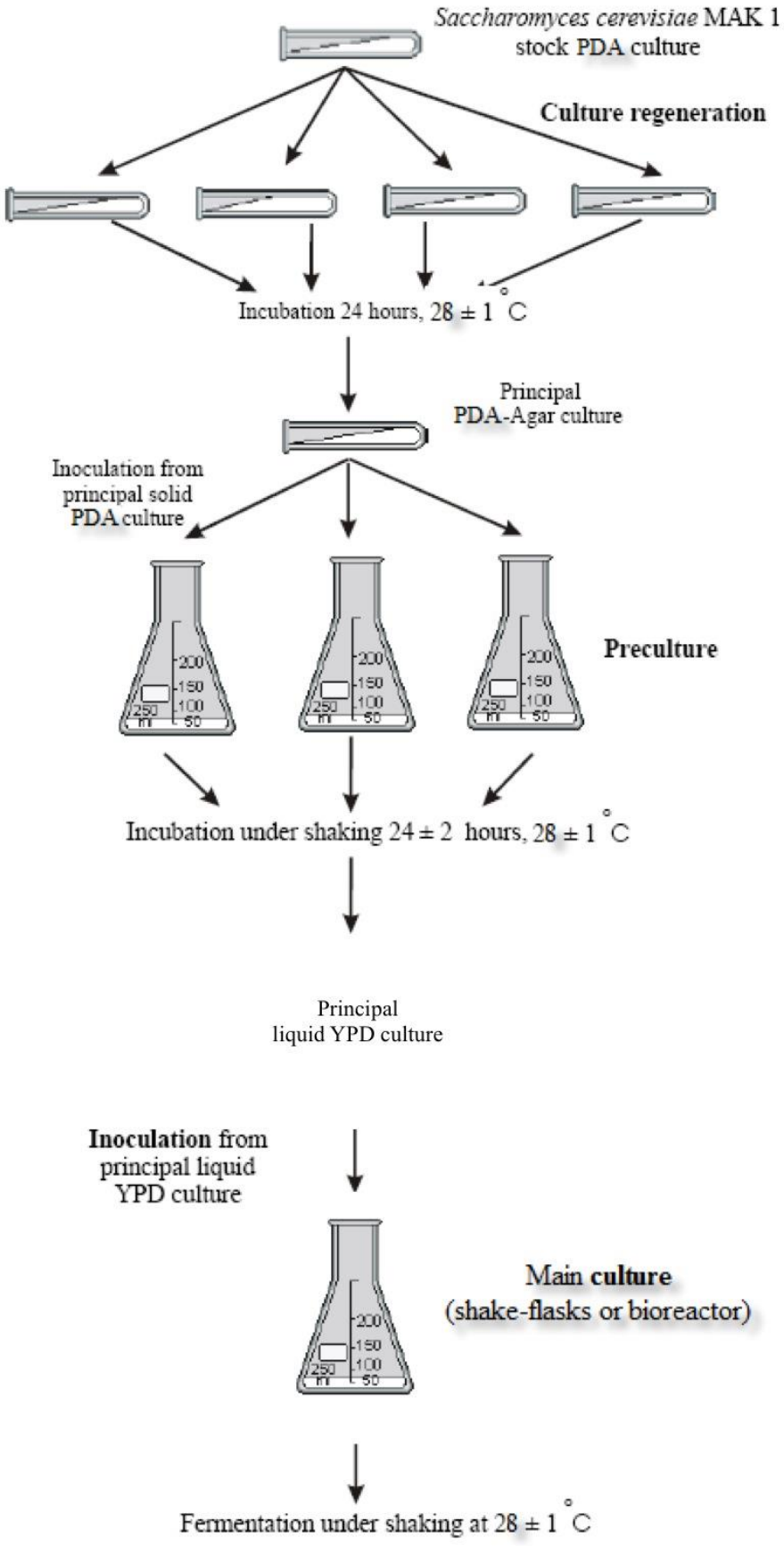


Figure 2. 1. Flow chart of culture regeneration, pre-culture inoculation and main culture inoculation

2.2.1. OMW-based growth media enriched with commercial glucose for the production of citric acid and ethanol as main metabolic products

The essential elements for the growth and the continuation of life of microorganisms are amongst others carbon, nitrogen and various mineral salts. There was an addition (whenever needed) of commercial glucose, ammonium sulphate, yeast extract and mineral salts solution in the OMW-based media for the enhancement of the production of the metabolic products. The composition of mineral salts solution is shown in Table 2.1.. Inexpensive commercial-type glucose was used as supplementary substrate (instead of analytical-grade glucose in order to further reduce the cost of the bioprocess) so as to support considerable production of the metabolic products. In all cases, glucose was added in order to offer the typical initial concentration of reducing sugars in most OMWs (Crognale *et al.*, 2006).

Table 2. 1. Composition of mineral salts solution

Compound	Concentration (g L^{-1})
KH_2PO_4	7.00
$Na_2HPO_4 \cdot 2H_2O$	2.50
$MgSO_4 \cdot 7H_2O$	1.50
$FeCl_3 \cdot 6H_2O$	0.15
$CaCl_2 \cdot 2H_2O$	0.15
$ZnSO_4 \cdot 7H_2O$	0.02
$MnSO_4 \cdot H_2O$	0.06

(Papanikolaou *et al.*, 2010)

2.2.1.1. Growth of Yarrowia lipolytica *strains on OMW-based media for the production of citric acid, biomass and lipids*

For the production of citric acid, cellular lipids and biomass from OMW-based media, the strains ACA-YC 5028, ACA-YC 5033 και W29 (ATCC 20460) of the yeast *Y. lipolytica* were used (see Fig. 2.2.). OMWs were diluted in several ratios to yield in liquid media presenting various initial phenolic compound concentrations, in order to study the effect of the effluent (and specifically of the phenolic compounds) on the metabolism of the yeast as also the reduction of the phenolic content and the percentage of the medium decolorization. The initial concentrations of phenolic compounds correspond to quantities that can be found in typical OMWs (Crognale *et al.*,

2006). In all OMW-diluted media, salts were added having the final concentration mentioned in Table 2.1..

To investigate the biochemical response of the *Y. lipolytica* strains grown on OMW-based media, aseptic fermentations in nitrogen-limited conditions (Glc_0~35 g L^{-1}, $(NH_4)_2SO_4$ = 0.5 g L^{-1}, yeast extract = 0.5 g L^{-1}; initial C/N~85) for the production of citric acid and carbon-limited conditions (Glc_0~30.0 g L^{-1}, $(NH_4)_2SO_4$ = 4.0 g L^{-1}, yeast extract = 2.0 g L^{-1}; initial C/N~13) for the production of biomass, were carried out. OMW was added into the synthetic medium in various ratios. The initial concentration of phenolic compounds in the growth media (in g L^{-1}) were as follows: 0.0 (control experiment without OMW addition), ~1.15 and ~1.55 (see Fig. 2.3.). The pH medium was maintained between 5.0 and 6.0. For more information about pH correction and maintenance see paragraph 2.2..

Following, at the second experimental part of this study, *Y. lipolytica* strain ACA-YC 5033 selected to grow on OMW-based media, under carbon-limited (Glc_0~30.0 g L^{-1}; initial C/N~13) and nitrogen-limited (Glc_0~35.0 g L^{-1}; initial C/N~85) aseptic conditions. OMW was added into the synthetic medium in various ratios giving initial concentration of phenolic compounds ~2.00 g L^{-1} and ~2.90 g L^{-1}. Moreover, two sterile trials with Glc_0~35.0 g L^{-1} and initial phenolic compounds concentration ~4.50 g L^{-1} and ~5.50 g L^{-1}, a pasteurized fermentation [thermal treatment of the medium in c. 80 °C for 5 min, inoculation with 3 mL (6% *v/v* inoculum) of exponential pre-culture] with Glc_0~35.0 g L^{-1} and initial phenolic compounds concentration ~2.90 g L^{-1} and a sterile trial with Glc_0~80.0 g L^{-1} and initial phenolic compounds concentration ~2.90 g L^{-1} under nitrogen limited conditions were carried out. Finally, aseptic and pasteurized bioreactor experiments (T=28 ± 1 °C, 300 rpm, D.O.>20%, aeration at 1 vvm, pH=5.2 ± 0.02, working volume 3.0 L) were carried out under nitrogen limited conditions with Glc_0~35.0 g L^{-1} and initial phenolic compounds concentration ~2.90 g L^{-1} in nitrogen limited conditions (see Fig. 2.3.).

2.2.1.2. Growth of Saccharomyces cerevisiae *strain MAK-1 on OMW-based media for the production of ethanol and biomass*

At the third experimental part of this study, the ability of *S. cerevisiae* strain MAK-1 to grow on and convert OMW-based media for the production of ethanol and biomass, was assessed. OMWs were diluted in several ratios to yield in liquid media presenting various initial phenolic compounds concentrations. Inexpensive commercial-type glucose was added in various amounts (resulting in the creation of media with different initial glucose concentrations) into the effluent so as to support considerable ethanol production (see Fig. 2.2. and Fig. 2.4.). In all OMW-diluted media, salts were added having the final concentration mentioned in Table 2.1.. Finally, $(NH_4)_2SO_4$

= 0.5 g L^{-1} and yeast extract = 0.5 g L^{-1} were also added. The initial concentrations of phenolic compounds and glucose correspond to quantities that can be found in typical OMWs (Crognale *et al.*, 2006). The pH medium was maintained around 3.5±0.1. For more information about pH correction and maintenance see paragraph 2.2.

The fermentations were carried out under aseptic and non aseptic conditions in 250-mL Erlenmeyer flasks as also in laboratory scale bioreactor (total volume 3.5 L; working volume 3.0 L) by *Saccharomyces cerevisiae* strain MAK-1. Concerning shake-flasks experiments, to investigate the biochemical response of *S. cerevisiae* strain MAK-1 grown on OMW-based media, fermentations in sterile (axenic) and non-sterile shake-flask cultures were carried out. OMWs and glucose were added, and media presenting various initial concentrations of phenolic compounds and Glc were created (see Fig. 2.4.). Initial phenolic compounds concentration of 0.00 g L^{-1} corresponded to the control experiment (without OMW addition), whereas two non-sterilized trials were also performed (Glc_0~40.0 g L^{-1} with initial phenolic compounds concentration at 0.00 g L^{-1} and Glc_0~75.0 g L^{-1} with initial phenolic compounds concentration at ~2.90 g L^{-1}). Maximum Glc concentration selected (~75.0 g L^{-1}) corresponded to Glc quantity that can usually be found in OMWs derived from press extraction systems (Crognale *et al.*, 2006). Equally, the initial concentrations of phenolic compounds correspond to quantities that can be found in typical OMWs (Crognale *et al.*, 2006). Regarding bioreactor experiments, fermentations were carried out under aseptic and non-aseptic conditions. Initially, no phenolic compounds were added into the growth medium (control experiment without OMW addition) and the influence of sterilization or non-sterilization on the bioprocess was quantified. Thereafter, the impact of the aeration rate on the fermentation efficiency was studied, and finally OMWs were added to the medium to yield an initial phenolic compound concentration of ~2.80 g L^{-1} in order to study the impact of OMWs addition on the conversion. Glucose was added into the media giving an initial Glc concentration of ~75.0 g L^{-1}. In order to further increase the maximum ethanol level achieved, a supplementary batch bioreactor non-sterilized trial was performed, in which OMWs were added in a (relatively concentrated) glucose-based medium, and the initial Glc and phenolic compounds concentrations of the fermentation medium were ~115.0 and ~2.90 g L^{-1}, respectively.

2.2.2. Growth of *Saccharomyces cerevisiae* strain MAK-1 on blends of molasses and OMWs for the production of ethanol and biomass

At the fourth - and final - experimental part of this study, fermentations were carried out under sterile and non-sterile conditions in 250-mL Erlenmeyer flasks as also in non-sterile aerated and non-aerated conditions in laboratory scale bioreactor (total volume 3.5 L; working volume 3.0 L) by *Saccharomyces cerevisiae* strain MAK-1 (see Fig. 2.2. and Fig. 2.5.).

To investigate the biochemical response of *S. cerevisiae* strain MAK-1 grown on media composed of mixtures of molasses and OMWs, fermentations in aseptic and non-aseptic flask cultures and non-aseptic batch bioreactor cultures were carried out. Firstly, sterile control (without OMW addition; initial total sugars TS_0~100.0 g L^{-1}) flask cultures with salts added compared to cultures without salts addition. Previously sterilized (without salts addition) mixtures of molasses and OMWs (10% *v/v*; initial total phenolics at ~3.90 g L^{-1}; TS_0~100.0 g L^{-1}) were subjected to shake-flask fermentations and were compared with non-aseptic shake-flask cultures containing the same initial quantities of phenolic compounds and TS. Finally, OMW was added into the non-sterile molasses flask cultures in various ratios (% *v/v*): 0 (control experiment, no OMW addition), 10, 20, 30, 40 and 50 giving respectively initial concentration of phenolic compounds (in g L^{-1}): ~2.62, ~3.94, ~4.48, ~5.20, ~5.54 and ~6.26. The initial total sugars concentration was TS_0~100.0 g L^{-1} (see Fig. 2.5.).

Non-aseptic batch fermentations were conducted in a laboratory scale bioreactor (MBR bioreactor, AG Switzerland), with total volume 3.5 L and working volume 3.0 L. The culture vessel was inoculated with 60 mL (2% *v/v* inoculum) of exponential pre-culture. The incubation temperature was controlled at T=28 ± 1 °C. Agitation rate was 300 rpm. The pH was automatically controlled at 3.50 ± 0.02 by adding 1 M HCl. Bioreactor fermentations were performed by sparging the media with air (passing through a bacteriological filter with 0.2 μm pore size) at a constant flow rate of 1.2 vvm as also under no aeration conditions (0.0 vvm). In fermentations carried out under non-aerated conditions, OMW was added into the medium in ratio 20% *v/v* giving initial phenolic compounds concentration ~4.67 g L^{-1}. Molasses added in various amounts giving initial total sugars concentration (in g L^{-1}): ~100.0, ~135.0, ~150.0 and ~200.0. A non-sterile aerated bioreactor trial with aeration imposed 1.2 vvm (initial phenolic compounds concentration ~4.72 g L^{-1}; TS_0~100.0 g L^{-1} was also performed. The flow charts of all aforementioned experimental procedures are shown in the figures following:

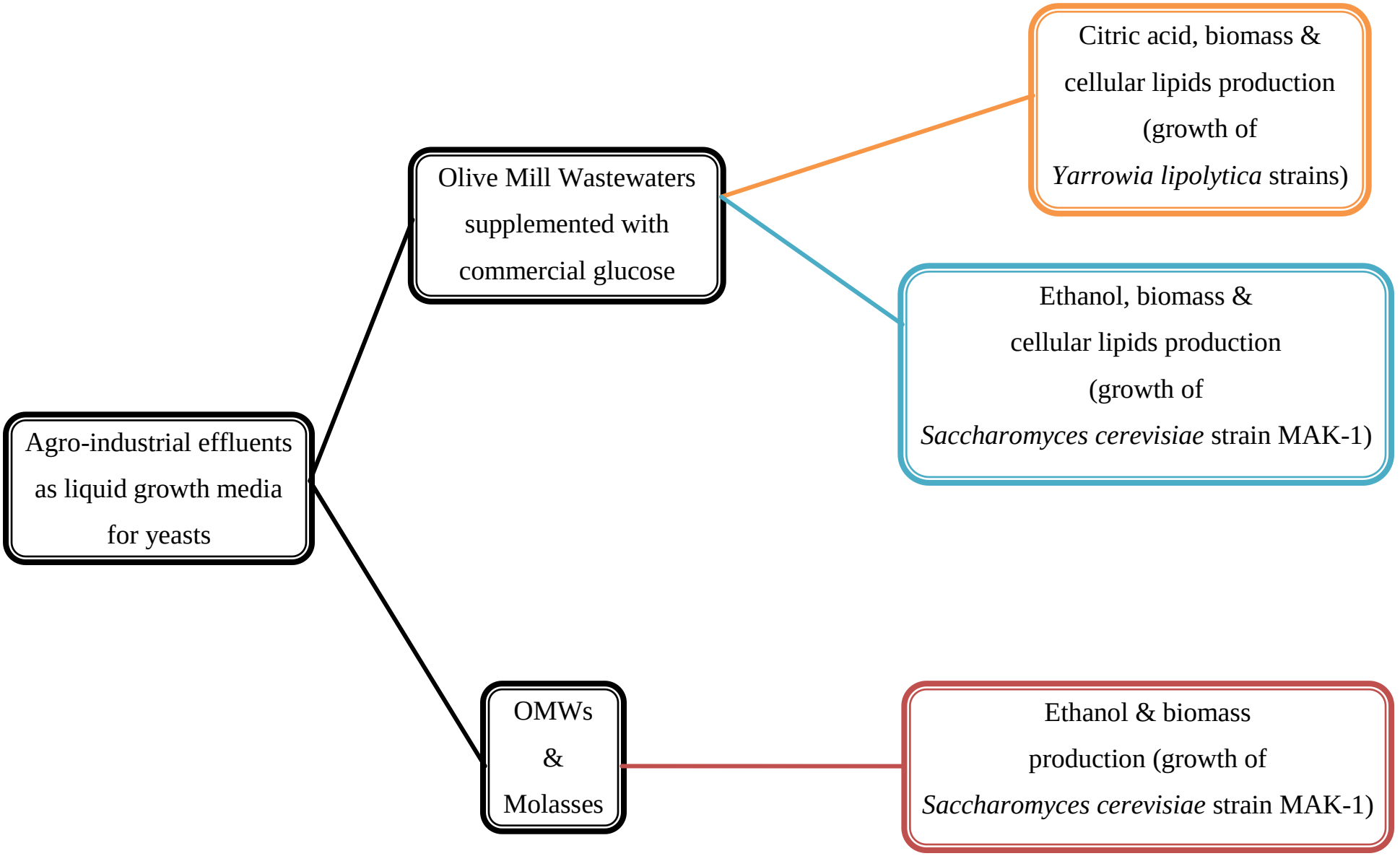


Figure 2. 2. Flow chart of overall experiments

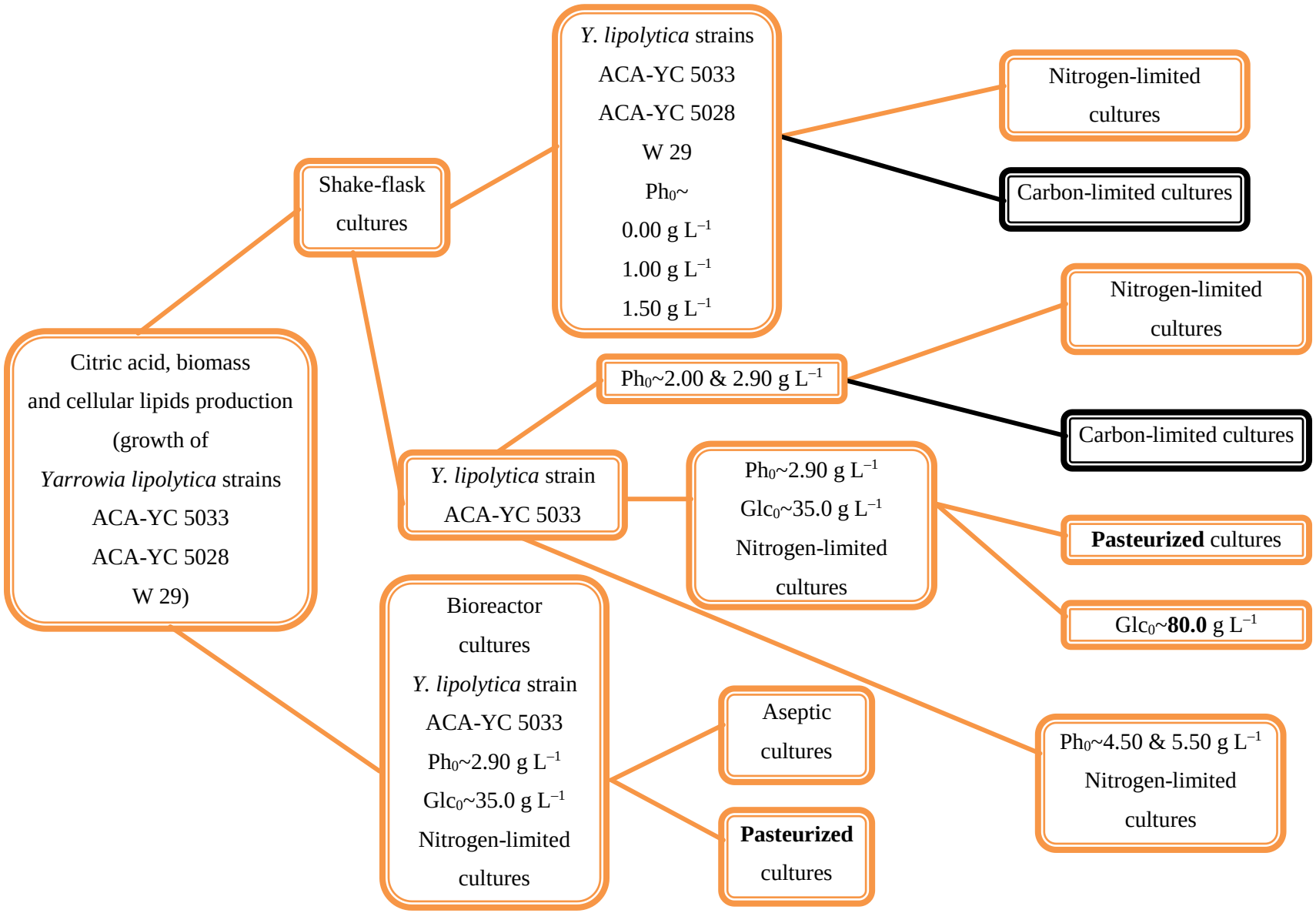


Figure 2. 3. Flow chart of experiments for the production of citric acid, biomass and cellular lipids by *Yarrowia lipolytica* strains cultivated on OMW-based media

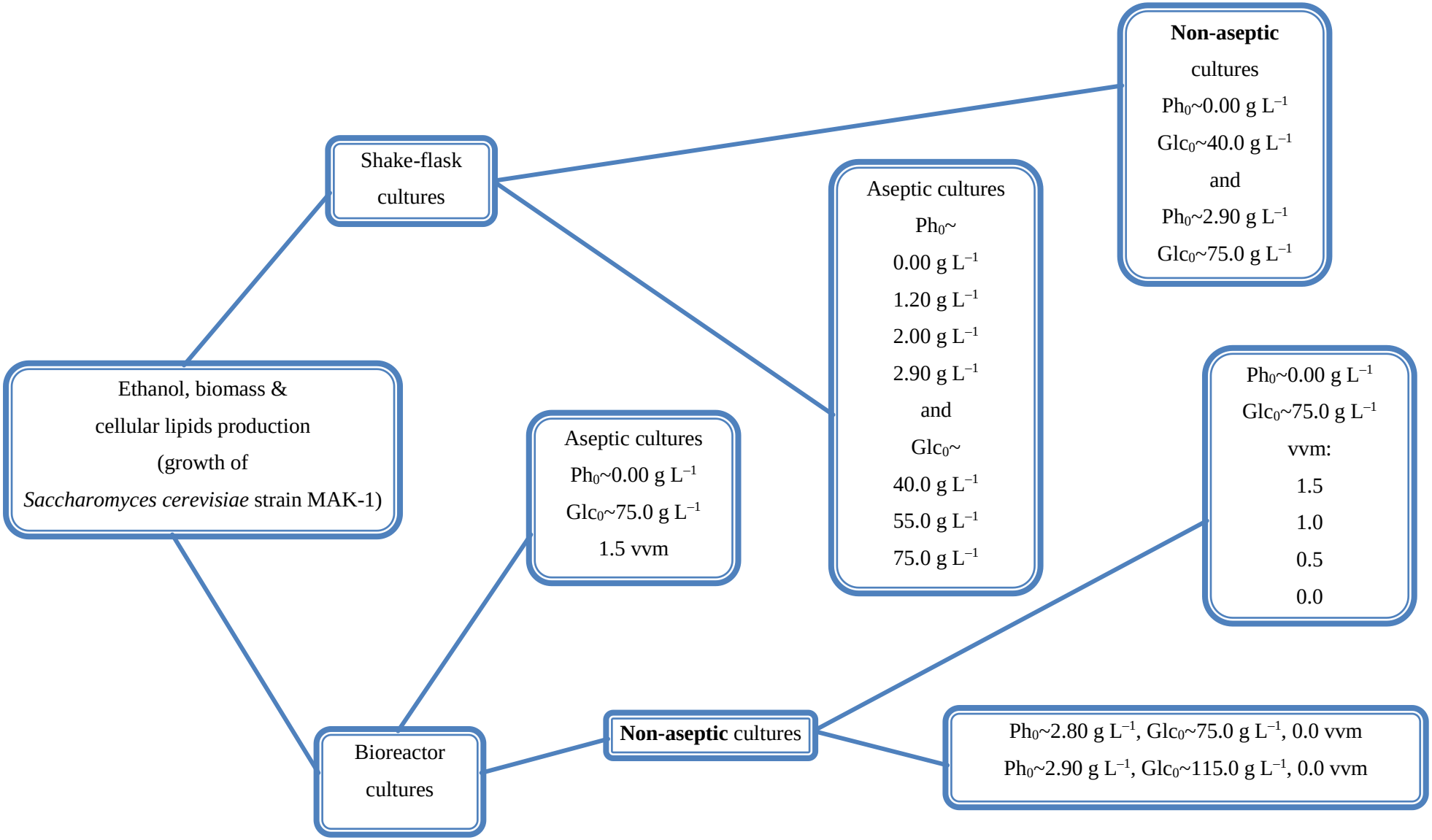


Figure 2. 4. Flow chart of experiments for the production of ethanol, biomass and cellular lipids by *Saccharomyces cerevisiae* strain MAK-1 cultivated on OMW-based media under sterile and non-sterile conditions

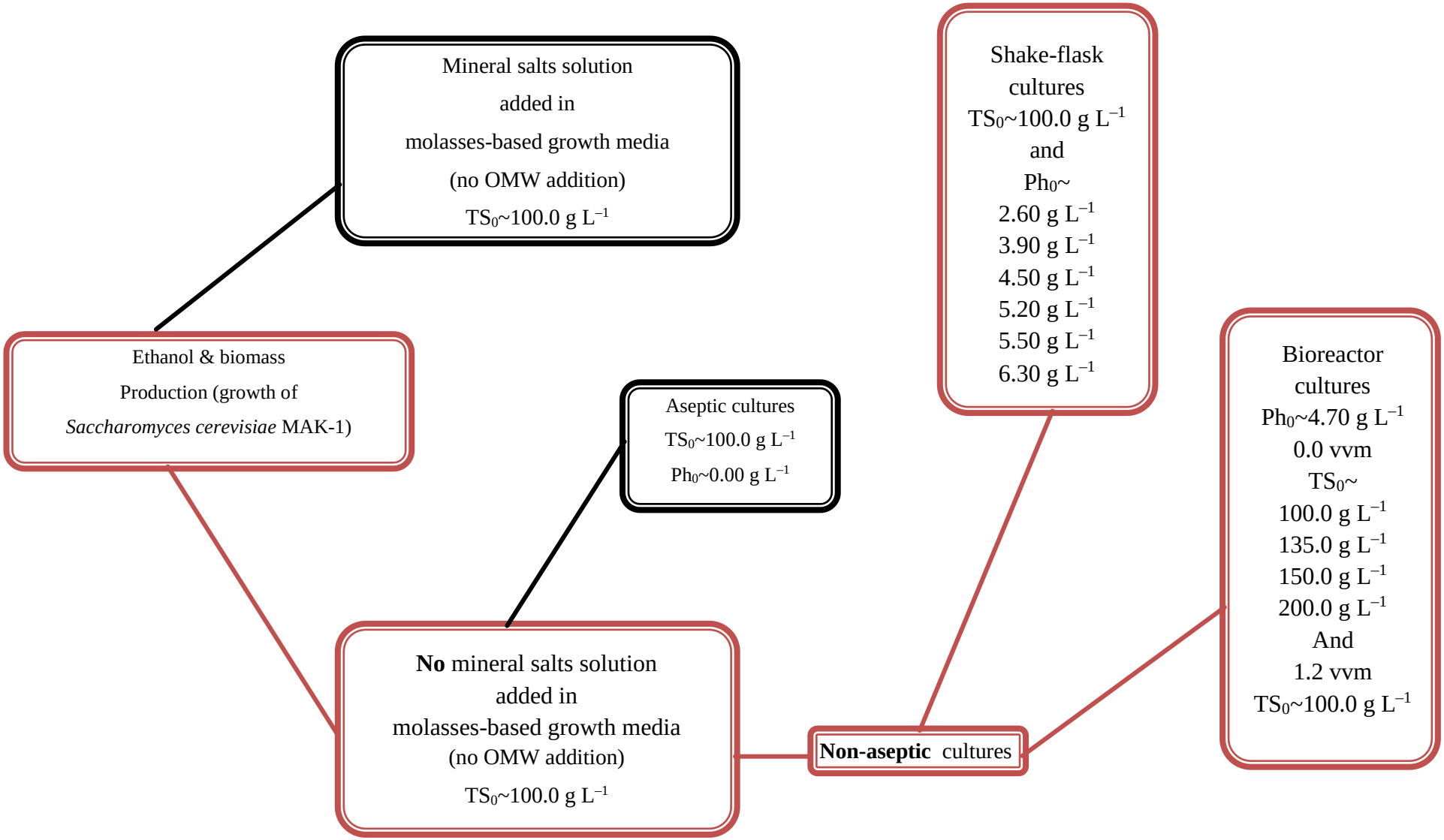


Figure 2. 5. Flow chart of experiments for the production of ethanol, biomass and cellular lipids by *Saccharomyces cerevisiae* strain MAK-1 cultivated on mixtures of molasses and OMWs under sterile conditions and non-sterile aerated and non-aerated conditions

2.3. Analyses

The flow chart of experimental analyses performed in this study (described as follows) is presented schematically in Fig. 2.12..

2.3.1. Determination of pH

For the determination of the medium pH (for the shake-flask experiments) a Jenway pHmeter 3020 Germany apparatus was used. The correction of the medium pH was maintained at the desired value by periodically (and aseptically concerning the trials performed under sterile conditions) adding into the flasks, quantities of KOH 5 mol L^{-1} or 1 mol L^{-1} HCl. The exact base or acid solution volume needed for pH correction was evaluated by measuring the volume of the solution required for pH correction in one (at least) flask. Following, the appropriate volume of base or acid was (aseptically) added in the remaining flasks and the value of the pH reached was verified to be the desired one.

2.3.2. Biomass determination

Yeast cell mass was harvested by centrifugation at Universal 320R-Hettich centrifuge at 9000 rpm for 10 min at 21 ± 1 °C, washed once with distilled water and centrifuged again. Biomass concentration (X g L^{-1}) was determined gravimetrically from dry weight at temperature T=100 °C until constant weight (usually within ~24 h).

2.3.3. Dissolved oxygen (DO) determination

For the determination of DO of the shake-flask cultures, a Lonibond Sensodirect OXI 200 (Dortmund, Germany) oxygen meter was used. While the shaker being in operation the electrode was set in a flask [and under stable (agitation and temperature) conditions after ~10 min] giving a % DO value. Following, the shaking was terminated and for 30 sec the value of DO was noted every 5 sec showing reduction.

2.3.4. Glucose, citric acid and ethanol determination

Glucose, ethanol and organic acids (mainly total citric acid) were determined with the aid of High Performance Liquid Chromatography (HPLC) analysis. The supernatant of the fermentation medium was centrifuged and filtered with 0.2 μm filter. The inject volume was 20 μL. The HPLC apparatus (Waters Association 600E) was equipped with a UV (Waters 486) and RI (Waters 410) detector. The column used for the separation of compounds was Aminex HPX - 87*H* (Biorad) (30 cm x 7.8 mL), the mobile phase was 0.005 mol L^{-1} H_2SO_4, the column temperature was 65 °C and

the flow rate was 0.6 ml min^{-1}. Citric and iso-citric acid were not sufficiently separated and the reported concentration corresponds to the sum of these acids, expressed as total citric acid. For a more precise determination of iso-citric acid, an enzymatic method, based on the measurement of the $NADPH_2$ produced during conversion of the iso-citric to α-ketoglutaric acid, reaction catalyzed by iso-citrate dehydrogenase, was employed. The chromatographic HPLC software used was Empower. Calibration curves for the analyzed compounds are presented in the following figures.

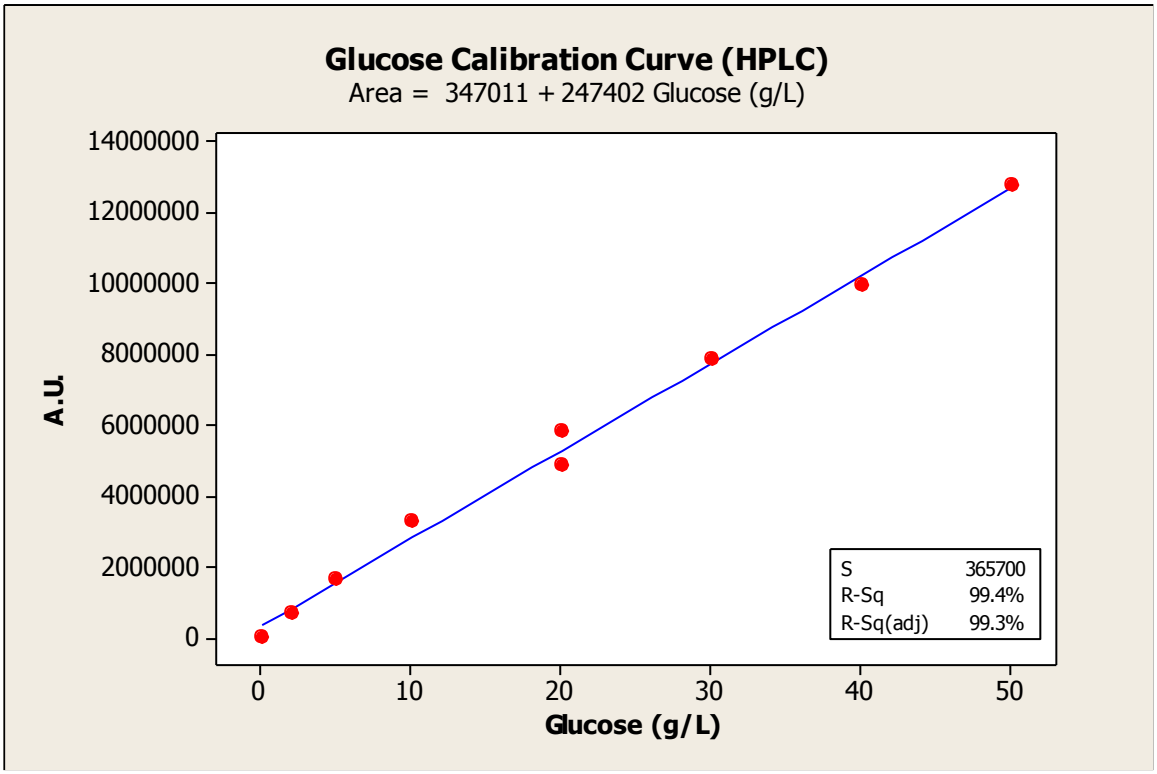


Figure 2. 6. Calibration curve of glucose (HPLC analysis)

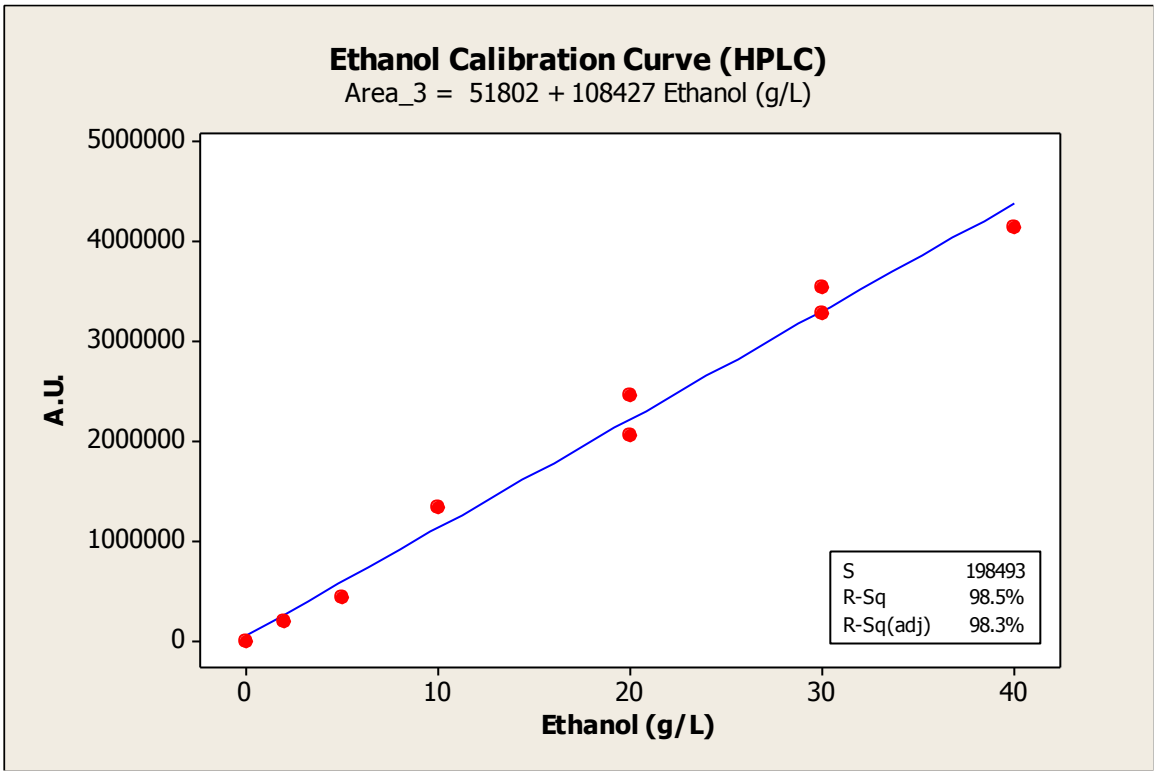


Figure 2. 7. Calibration curve of ethanol (HPLC analysis)

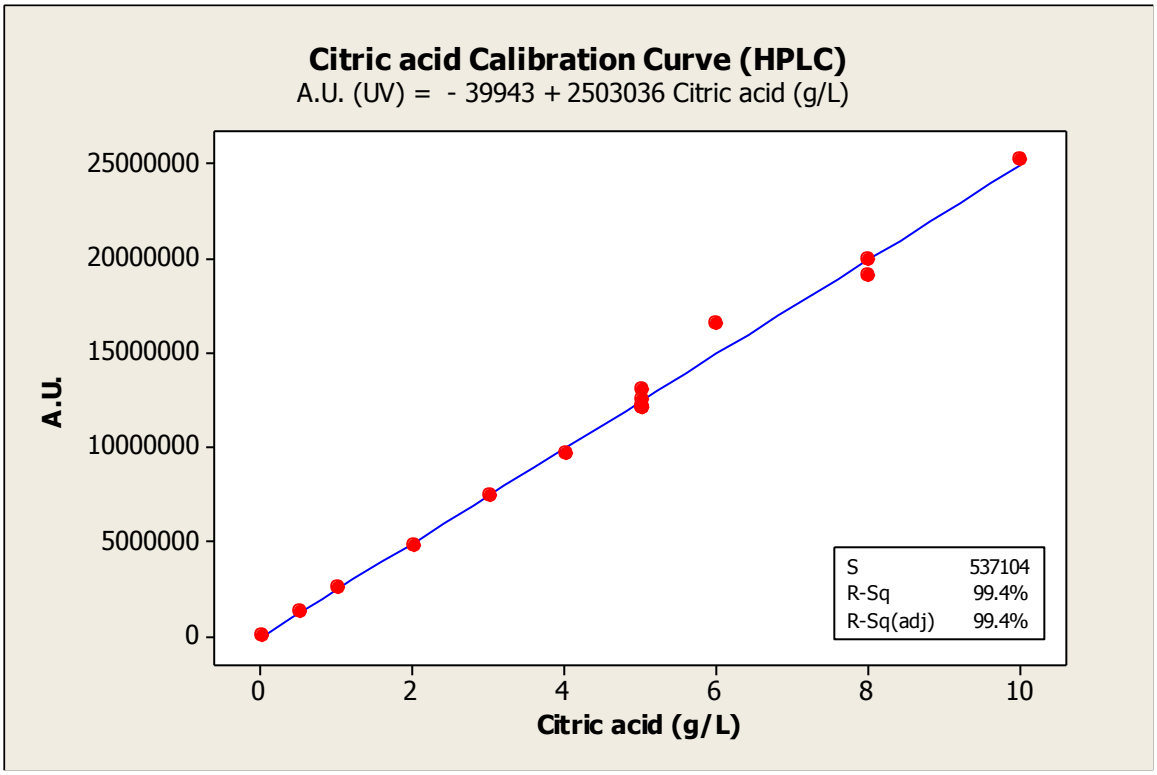


Figure 2. 8. Calibration curve of citric acid (HPLC analysis)

2.3.5. Total sugars determination

For the hydrolysis of sucrose into glucose and fructose [at the experiments including molasses (as they consist of 30-40% sucrose; see Table 1.3.)], 4.5 mL of HCl 1M were added in a test tube with 1 mL of sample. The test tube was led to water bath (100 °C; 30 minutes) and finally 4.5 mL of 1M KOH were added (Roukas, 1996a). The reducing sugars concentration was determined according to DNS method (Miller, 1959) and was expressed as glucose equivalent. The method is based on the reduction of dinitro 3,5 salicilic acid to 3-amino-5-nitro salicylic acid when NaOH present and on the simultaneous oxidization of glucose towards gluconic acid.

In mild heated 500 mL distilled water were added 200 mL NaOH (2 mol L^{-1}), dinitro 3,5 salicilic acid (DNS) and potassium sodium tartrate until dilution. The liquid mixture was placed in a 1000 mL volumetric flask and filled with distilled water up to final volume. 0.5 mL of the sample was mixed with 0.5 mL DNS reagent and were placed in a test tube and stirred at the vortex mixer. Afterwards the whole mixture was placed in boiling water for exactly 5 minutes and left to cool down. Finally, 5 mL distilled water were added to the tube and after stirring, absorbance was measured at 540 nm (Hitachi U-2000 spectrophotometer). The calibration curve in g L^{-1} of glucose is show at the next figure.

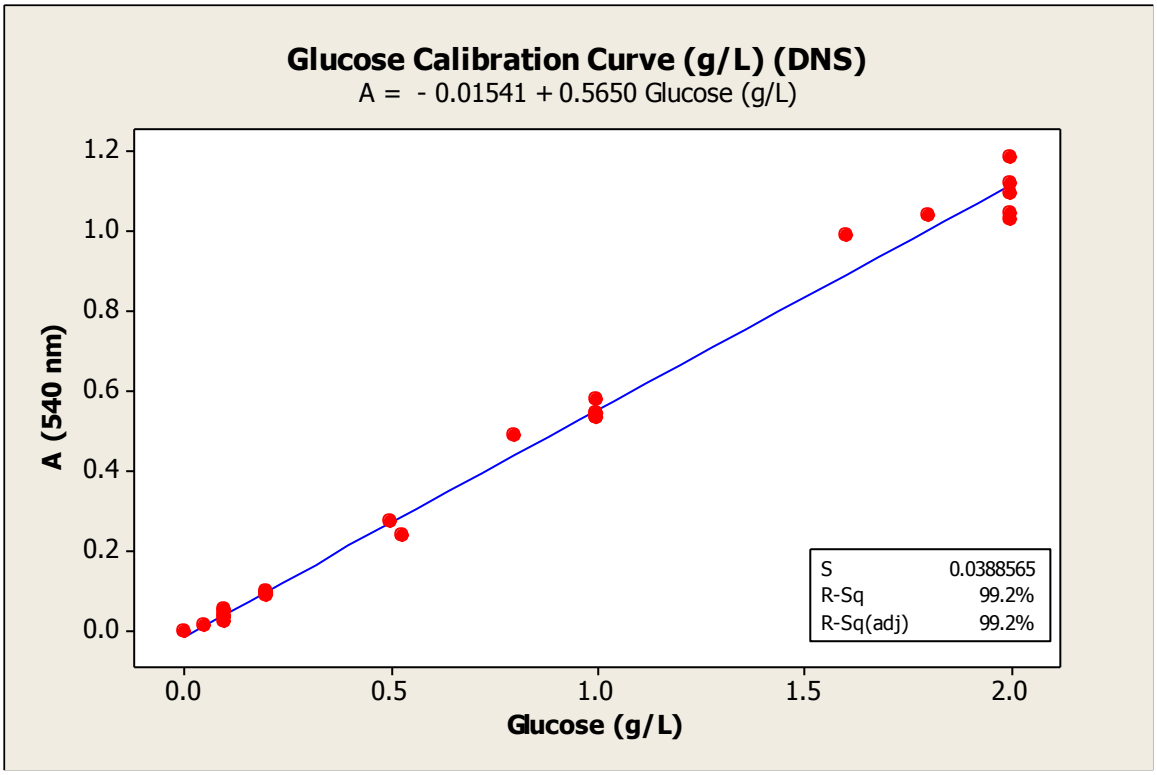


Figure 2. 9. Calibration curve of glucose (DNS)

2.3.6. Total cellular lipids and FAMEs determination

Quantitative and qualitative analysis of total intracellular lipids was done. Total intracellular lipids were extracted with a mixture of chloroform and methanol 2:1 (*v/v*) (Folch *et al*., 1957; Papanikolaou *et al*., 2001). Solvents were removed at reduced pressure (Büchi Rotavapor R-114) and lipids were determined gravimetrically (quantitative analysis). Lipids were converted to their fatty acid methyl-esters (FAMEs) and analyzed (qualitative analysis) in gas chromatograph-flame ionization detector (GC-FID) apparatus (Fisons 8000 series) according to Fakas et al. (2006). FAMEs were identified by comparison with authentic standards.

Because of the fact that fatty acids are not highly volatile and in order to be detected in GC analysis, their transformation to the respective volatile methyl esters is necessary. The procedure of free fatty acids transformation into the respective methyl-esters is called transesterification and at the present study was done according to AFNOR (1959) method and as described in Fakas *et al.* (2006). This procedure includes two stages (Fig. 2.10.). The first stage is under alkaline environment whereas the second stage is under acidic environment. During the first stage the nucleophilic substitution at the molecule of triglycerides and phospholipids takes place, resulting in the formation of the respective methyl-esters of fatty acids. Simultaneously the, already existing, free fatty acids are transformed to the respective soaps reacting with sodium methoxide solution. At the second stage, soaps are converted into the respective methyl-esters.

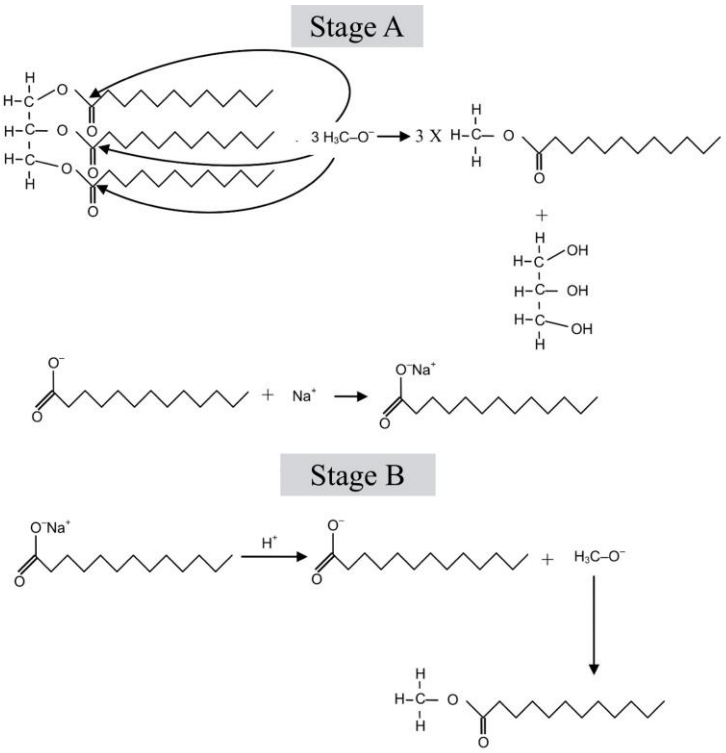


Figure 2. 10. Transesterification of free fatty acids

Stage A: under alkaline environment; Stage B: under acidic environment

(Μακρή, 2011)

At the evaporation/boiling flask - including the gravimetrically determined total lipids - 10 mL of sodium methoxide solution [$CH_3O^-Na^+$; 1% of sodium metal in mixture of methanol and benzene (70:30 *v/v*) and phenolphthalein] and some boiling chips are added. The flask is then placed on a heating mantle with a vertical condenser attached at 100 °C for 20 min. Following, an amount of hydrocloride methanol (CH_3OH-HCl; 20 mL acetyl chloride mixed with 250-mL of methanol) is added until the solution solvent turns from pink to white. Boiling continues for 20 more minutes. The addition of an amount of deionized water terminates the reaction. Finally the solution is transferred into a separator funnel where 6 mL of (n-) hexane is added and methyl-esters are extracted into hexane after strong stirring. Finally, the water phase is discarded and a small amount of sodium sulfate anhydrous (Na_2SO_4) is added to the organic phase (for the absorption of any remaining water).

For the analysis of FAMEs at the GC with an FID detector, 1 μL of methyl-esters sample was injected. The carrier gas (He) flow was 1.38 mL min^{-1}, H_2 60 kPa and O_2 110 kPa. The stationary phase was Fused silica WCOT: CP-Sill 88 (50 m x 0.32 mm and DF=0.20 μm film thickness). The column temperature was stable at 200 °C for 20 min.

2.3.7. Phenolic compounds concentration determination

Phenolic compounds concentration in the culture media was determined according to Folin-Ciocalteu (FC) method measured at 750 nm and expressed as gallic acid equivalent (Slinkard and Singleton, 1997) (the calibration curve in g L^{-1} of gallic acid is show at Fig. 2.11.). This method is based on the breakup of total phenols under alkaline environment with the addition of FC reagent which is a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$). The FC reagent is simultaneously reduced towards the respective oxides WsO_{23} και Mo_8O_{23} which are responsible for the blue colour resulting. In a test tube, 0.2 mL of supernatant (after appropriate dilution), 10.8 mL of distilled water, 8 mL of anhydrous sodium carbonate (Na_2CO_3; 75 g L^{-1}) solution and 1 mL of FC reagent. After two hours at room temperature, the absorbance was measured at 750 nm (Hitachi U-2000 spectrophotometer). In all trials, initial phenolic compounds concentration was determined after sterilization (when sterilization occurred).

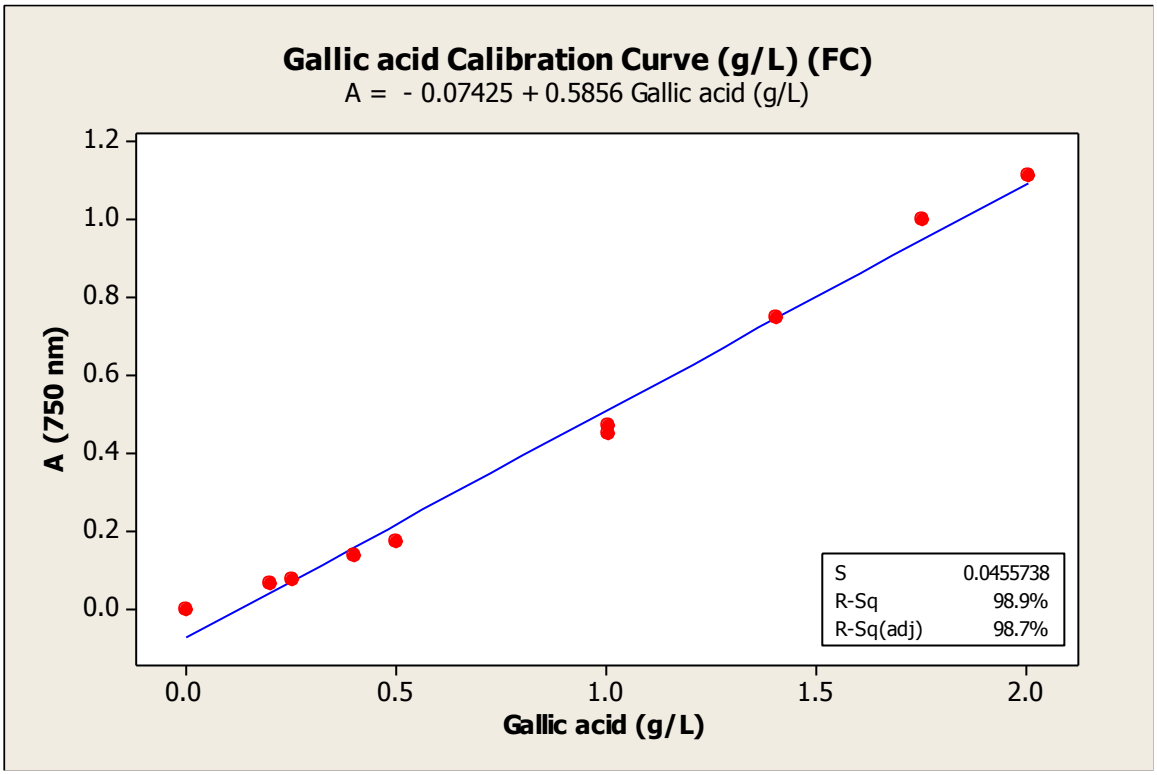


Figure 2. 11. Calibration curve of gallic acid (FC)

2.3.8. Decolorization determination

The decolorization assay of the treated residue was performed according to Sayadi and Ellouz (1992). The supernatant samples were 30-fold diluted, their pH was adjusted between 6.0 and 6.3 and the absorbance was measured at 395 nm (Hitachi U-2000 spectrophotometer). In all trials, initial color content was determined after sterilization (when sterilization occurred). The percentage of decolorization was calculated according to:

$$\% \text{ Decolorization} = [(A_0 - A_t) / A_0] \times 100$$

A_0: absorption of the supernatant at 0 hours (right before inoculation)

A_t: absorption of the supernatant at *t* hours after inoculation

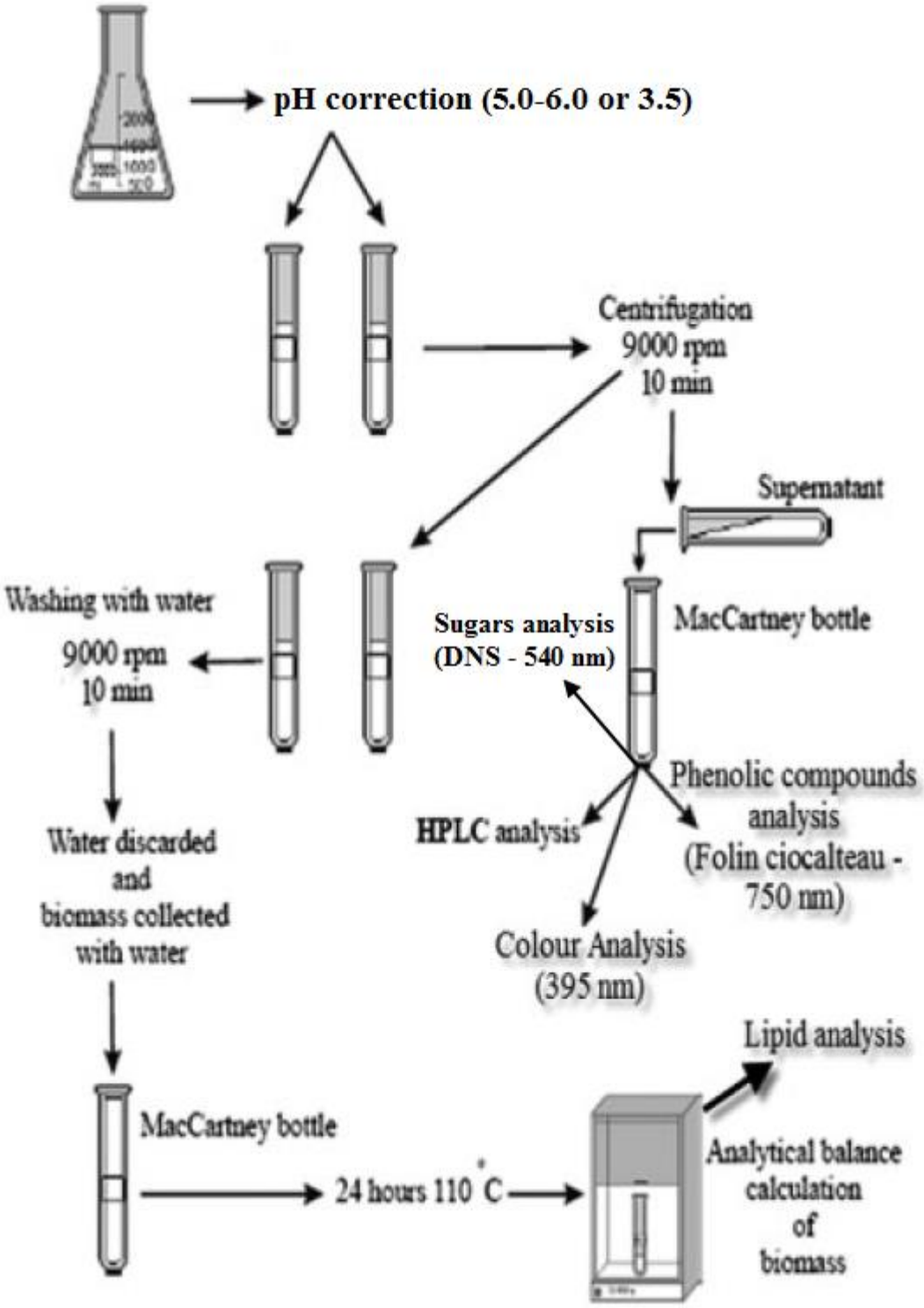


Figure 2. 12. Flow chart of experimental analytical methods.

Biomass harvesting by centrifugation and gravimetrical determination from dry weight, supernatant photometric (color, phenolic compounds and sugars) and chromatographic (total citric acid, glucose and FAMEs) analysis.

3. RESULTS

The ability of two yeasts, *Yarrowia lipolytica* [strains ACA-YC 5028, ACA-YC 5033 and W29 (ATCC 20460)] and *Saccharomyces cerevisiae* strain MAK-1, to simultaneous bioremediate (reduce phenolic content and colour) OMW-based media and produce high-added value products (biomass, citric acid, ethanol, cellular lipids) was assessed at the present study.

During the fermentation kinetics, the following parameters were evaluated:

- pH of the medium
- Glucose – total sugars consumption (Glc – T.S., g L^{-1})
- Biomass (in dry cell weight) and total lipid production (X and L, g L^{-1})
- Citric acid and ethanol production (Cit and EtOH, g L^{-1})
- Removal of phenolic compounds (Ph, g L^{-1})
- Reduction of medium colour

During the microbial growth, the following parameters were calculated:

- Biomass yield on glucose consumed (g formed g^{-1} glucose consumed –$Y_{X/Glc}$)
- Biomass yield on total sugars consumed (g formed g^{-1} total sugars consumed – $Y_{X/TS}$)
- Total lipid yield on biomass (g formed g^{-1} biomass formed – $Y_{L/X}$)
- Total citric acid yield on glucose (g formed g^{-1} glucose consumed – $Y_{Cit/Glc}$)
- Ethanol yield on glucose (g formed g^{-1} glucose consumed – $Y_{EtOH/Glc}$)
- Ethanol yield on total sugars (g formed g^{-1} total sugars consumed – $Y_{EtOH/TS}$)

The present study is divided in four main parts. At the first part, the ability of three *Yarrowia lipolytica* strains [ACA-YC 5028, ACA-YC 5033 and W29 (ATCC 20460)] to grow on and convert glucose-enriched OMWs into biomass, cellular lipids and citric acid in carbon- and nitrogen-limited sterile shake-flask cultures was assessed. The FAMEs profile of produced cellular lipids, the removal of phenolic compounds from the medium and the decolorization of the medium were also tested.

At the second part, the ability of *Y. lipolytica* strain ACA-YC 5033 (a yeast strain that was revealed capable of resisting in high concentrations of phenolic compounds of the medium) to grow on glucose-enriched OMW-based media with higher ratios of OMWs added was tested. Fermentations under pasteurized conditions and also trials in bioreactors were performed. As previously, the removal of phenolic compounds from the medium and the decolorization of the medium were also tested.

At the third part, the ability of *Saccharomyces cerevisiae* strain MAK-1 to grow on and convert glucose-enriched OMWs into biomass, cellular lipids and ethanol in aseptic and non-aseptic shake-flask and in batch bioreactor cultures was assessed. The FAMEs profile of the produced cellular lipid, the removal of phenolic compounds from the medium and the decolorization of the medium were also tested.

Finally at the fourth part, the ability of *Saccharomyces cerevisiae* strain MAK-1 to grow on and convert blends of OMWs and molasses into biomass and ethanol under (principally) non-aseptic shake-flask and non-aseptic aerobic and non-aerated batch bioreactor cultures was assessed. The removal of phenolic compounds from the medium and the decolorization of the medium were also tested.

3.1. Citric acid, biomass and cellular lipid production by *Yarrowia lipolytica* strains cultivated on olive mill wastewater-based media

3.1.1. General presentation

The fermentations were carried out in 250-mL Erlenmeyer flasks under aseptic conditions by *Yarrowia lipolytica* strains ACA-YC 5028, ACA-YC 5033 and W29 (ATCC 20460). To investigate the biochemical response of the *Y. lipolytica* strains grown on OMW-based media, fermentations in nitrogen-limited (Glc_0~35.0 g L^{-1}; initial C/N~85) and carbon-limited (Glc_0~28.0 g L^{-1}; initial C/N~13) conditions were carried out. OMW was added into the synthetic medium in various ratios. The initial concentration of phenolic compounds in the growth media were as follows: 0.00 g L^{-1} (control experiment without OMW addition), 1.15±0.10 g L^{-1} and 1.55±0.15 g L^{-1} (see Tables 3.1. and 3.2.).

Table 3. 1. Experimental data of *Yarrowia lipolytica* strains in nitrogen-limited glucose-based media with olive-mill waste-waters added in various amounts.
Representations of total biomass (X, g L^{-1}), total cellular lipid (L, g L^{-1}), total citric acid (Cit, g L^{-1}) and consumed substrate (Glc_{cons}, g L^{-1}) concentrations at different fermentation points of each trial:

Strain	Initial phenolics (g L^{-1})	Fermentation time (h)	X (g L^{-1})	L (g L^{-1})	Cit (g L^{-1})	Glc_{cons} (g L^{-1})	$Y_{X/Glc}$ (g g^{-1})	$Y_{L/X}$ (g g^{-1})	$Y_{Cit/Glc}$ (g g^{-1})
ACA-YC 5028	0.00	144	7.0±0.5[a,b]	1.20±0.10	7.80±0.50	29.9±2.0	0.23	0.18	0.26
		120	6.6±0.5[c]	0.80±0.10	9.40±1.00	29.8±2.0	0.22	0.12	0.32
	1.17±0.10	144	5.5±0.4[a,c]	0.20±0.05	8.40±0.50	34.2±2.5	0.16	0.04	0.25
		96	4.9±0.4[b]	0.90±0.10	7.40±0.50	24.8±2.0	0.20	0.18	0.30
	1.50±0.15	187	4.8±0.4[a,b,c]	0.80±0.10	8.40±0.50	34.8±2.5	0.14	0.17	0.24
W29	0.00	144	5.9±0.4[a,c]	0.50±0.05	18.50±1.50	27.3±2.0	0.22	0.08	0.68
		72	5.6±0.4[b]	0.60±0.05	4.10±0.05	13.8±1.0	0.41	0.11	0.30
	1.17±0.10	144	5.1±0.4[a,c]	1.30±0.10	15.80±0.10	34.0±2.5	0.15	0.25	0.46
		48	5.0±0.4[b]	1.70±0.10	6.00±0.50	17.0±1.5	0.29	0.34	0.35
	1.64±0.15	72	6.8±0.5[a,b]	1.90±0.15	0.90±0.05	26.2±2.0	0.26	0.28	0.03
		120	6.7±0.5[c]	0.60±0.05	3.70±0.50	34.2±2.5	0.20	0.09	0.11
ACA-YC 5033	0.00	120	5.5±0.4[a]	0.40±0.05	13.80±1.00	23.7±2.0	0.23	0.07	0.58
		24	4.2±0.3[b]	0.50±0.05	0.00	5.4±0.5	0.78	0.12	0.00
		144	5.5±0.4[c]	0.30±0.05	18.90±1.50	25.8±2.0	0.21	0.05	0.73
	1.01±0.10	144	5.1±0.4[a,b,c]	0.50±0.05	18.10±1.50	35.7±2.5	0.14	0.10	0.51
	1.50±0.15	165	4.6±0.4[a,b]	0.50±0.05	15.40±1.00	30.4±2.0	0.15	0.11	0.51
		187	4.5±0.4[c]	0.40±0.05	17.20±1.00	34.5±2.5	0.13	0.09	0.50

[a] when X_{max} concentration was achieved; [b] when L_{max} concentration was achieved; [c] when Cit_{max} concentration was achieved. Fermentation time, conversion yield of biomass produced per glucose consumed ($Y_{X/Glc}$, g g^{-1}), total lipid in dry biomass ($Y_{L/X}$, g g^{-1}) and conversion yield of total citric acid produced per glucose consumed ($Y_{Cit/Glc}$, g g^{-1}) are presented for all points of the trials. Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, Glc_0~35.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, D.O.T.>20% *v/v*, incubation temperature *T*=28 °C. Each point is the mean value of two independent measurements.

Table 3. 2. Experimental data of *Yarrowia lipolytica* strains in carbon-limited glucose-based media with olive-mill waste-waters added in various amounts.

Representations of total biomass (X, g L^{-1}), total cellular lipid (L, g L^{-1}) and consumed substrate (Glc_{cons}, g L^{-1}) concentrations at different fermentation points of each trial:

Strain	Initial phenolics (g L^{-1})	Fermentation time (h)	X (g L^{-1})	L (g L^{-1})	Glc_{cons} (g L^{-1})	$Y_{X/Glc}$ (g g^{-1})	$Y_{L/X}$ (g g^{-1})
ACA-YC 5028	0.00	72	10.0±0.8[a]	1.10±0.10	30.8±2.5	0.32	0.11
		96	9.4±0.7[b]	1.20±0.10	30.9±2.5	0.30	0.13
	1.23±0.10	48	12.7±1.0[a]	1.20±0.10	22.7±1.5	0.45	0.05
		144	10.3±0.8[b]	1.70±0.10	27.3±2.0	0.38	0.17
	1.57±0.15	96	11.6±0.8[a]	0.20±0.05	29.3±2.5	0.40	0.02
		48	10.1±0.8[b]	0.40±0.05	24.5±2.0	0.41	0.04
W29	0.00	120	8.4±0.6[a]	0.30±0.05	27.6±2.0	0.30	0.04
		144	8.1±0.6[b]	0.30±0.05	27.8±2.0	0.29	0.04
	1.23±0.10	48	12.0±0.9[a]	0.60±0.05	27.5±2.0	0.44	0.05
		24	6.7±0.5[b]	0.80±0.05	12.3±1.0	0.54	0.12
	1.57±0.15	120	10.4±0.8[a,b]	0.40±0.05	28.5±2.5	0.36	0.04
ACA-YC 5033	0.00	96	10.1±0.8[a]	0.60±0.05	23.4±1.5	0.43	0.06
		144	9.1±0.8[b]	1.00±0.10	24.5±2.0	0.37	0.11
	1.04±0.10	72	12.2±0.9[a,b]	0.50±0.05	28.9±2.5	0.42	0.04
	1.57±0.15	96	11.3±0.8[a,b]	0.40±0.05	28.9±2.5	0.39	0.04

[a] when X_{max} concentration was achieved;

[b] when L_{max} concentration was achieved.

Fermentation time, conversion yield of biomass produced per glucose consumed ($Y_{X/Glc}$, g g^{-1}) and total lipid in dry biomass ($Y_{L/X}$, g g^{-1}) are presented for all points of the trials. Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, Glc_0~28.0 g L^{-1}, $(NH_4)_2SO_4$=4.0 g L^{-1}, yeast extract=2.0 g L^{-1} initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, D.O.T.>20% *v/v*, incubation temperature *T*=28 °C. Each point is the mean value of two independent measurements.

3.1.2. Biomass and lipid production

In the nitrogen-limited fermentations performed, biomass production seemed to be affected by the addition of OMW into the medium (Table 3.1.); compared with the control experiment (no OMW addition), X_{max} concentration was reduced proportionally to the phenolic content for strains ACA-YC 5028 and ACA-YC 5033. Kinetics of biomass production as affected by the addition of OMW into the medium in nitrogen-limited experiments [strain ACA-YC 5028 grown on glucose-enriched OMW-based media with initial phenolic compounds at 0.00 g L^{-1} (no OMW addition), 1.17±0.10 g L^{-1} and 1.50±0.15 g L^{-1}] is shown in Fig.3.1..

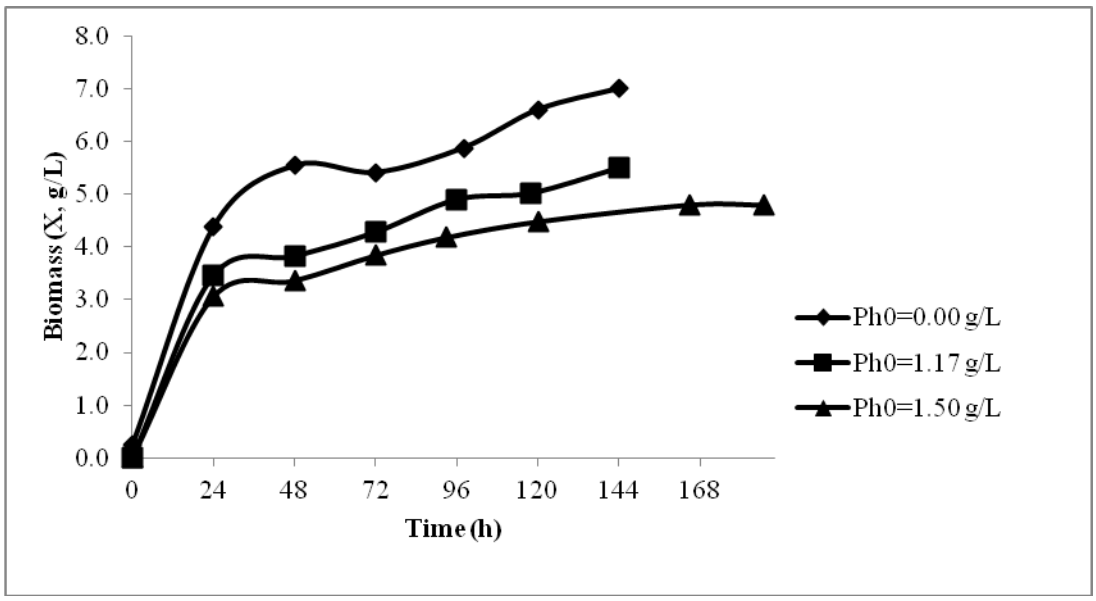


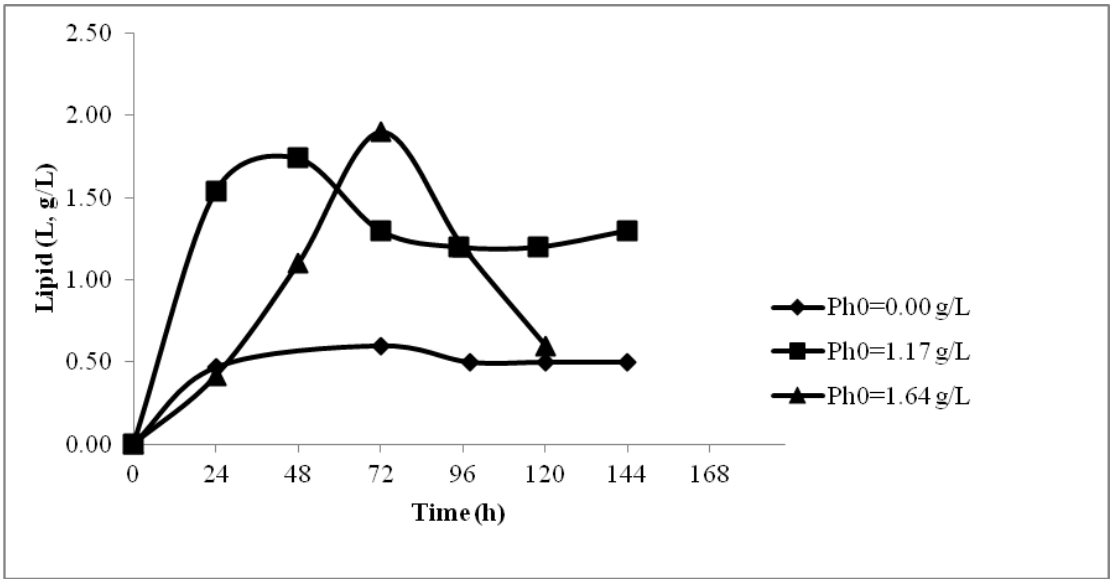
Figure 3. 1. Biomass (X, g L^{-1}) evolution during growth of *Yarrowia lipolytica* strain ACA-YC 5028 on OMW-based media [initial phenolic compounds concentration (Ph_0) 0.00 g L^{-1} (no OMW addition), 1.17±0.10 g L^{-1} and 1.50±0.15 g L^{-1}) enriched with commercial glucose in nitrogen-limited conditions. Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, Glc_0~35.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% *v/v*, incubation temperature *T*=28 ºC. Each point is the mean value of two independent measurements.

On the other hand, high X_{max} concentration was observed in media presenting increased initial phenolic compounds concentration for the strain W29 (Table 3.1.), suggesting that OMWs seemed to stimulate biomass production. Yield $Y_{X/Glc}$ was also increased for strain ACA-YC 5028 but insignificantly reduced for strain ACA-YC 5033. Finally, for the strain W29, biomass production (expressed as X_{max} and $Y_{X/Glc}$) was slightly reduced when the initial phenolic content was 1.17±0.10 g L^{-1} but, surprisingly enough, it increased again in fermentation with phenolic compounds concentration 1.64±0.15 g L^{-1}, compared with the control experiment (Table 3.1.). In general, the strain ACA-YC 5028 showed highest X_{max} production (~7.0 g L^{-1} for the control experiment and ~5.5 g L^{-1} for trial with initial phenolic compounds at 1.15±0.10 g L^{-1}), excluding fermentation with the strain W29 at initial phenolics concentration 1.55±0.15 g L^{-1} where X_{max} as ~6.8 g L^{-1} (Table 3.1.).

Total cellular lipids were quantified in all growth phases for all strains and in some cases lipid quantities >25% *w/w* of lipid in dry matter were found (Table 3.1.), indicating that lipid accumulation occurred in some of the trials performed. An interesting result obtained was that the presence of OMWs in the medium seemed to favor the accumulation of storage lipids for the strain W29, suggesting that OMWs seemed to be a "lipogenic" medium. Specifically, L_{max} quantities up to 1.9 g L^{-1}, corresponding to lipid in dry weight ($Y_{L/X}$) values up to 0.28 g g^{-1} (or even higher) were

obtained when OMW was added into the medium, suggesting stimulation of a reserve lipid accumulation process, since in the control experiment clearly lower $Y_{L/X}$ and L values were obtained [Table 3.1. and Fig. 3.2. (a); (b)]

(a)



(b)

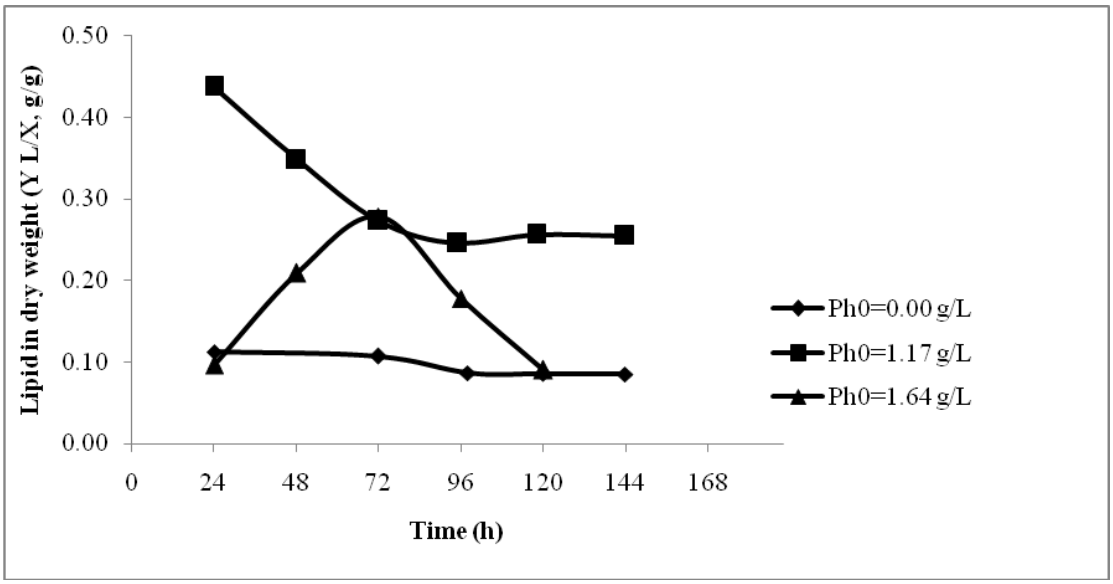
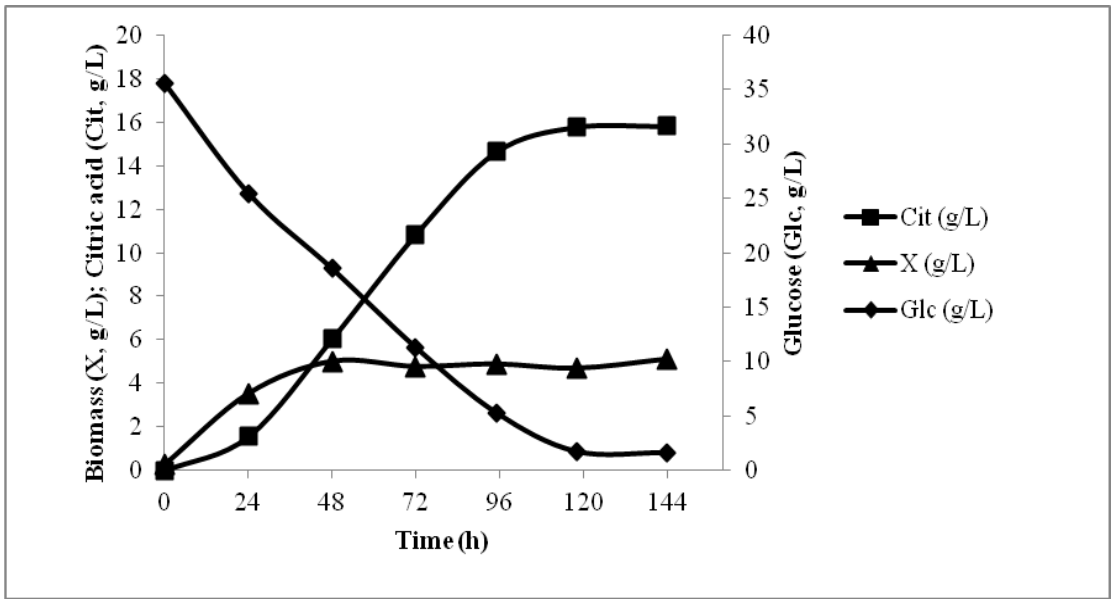


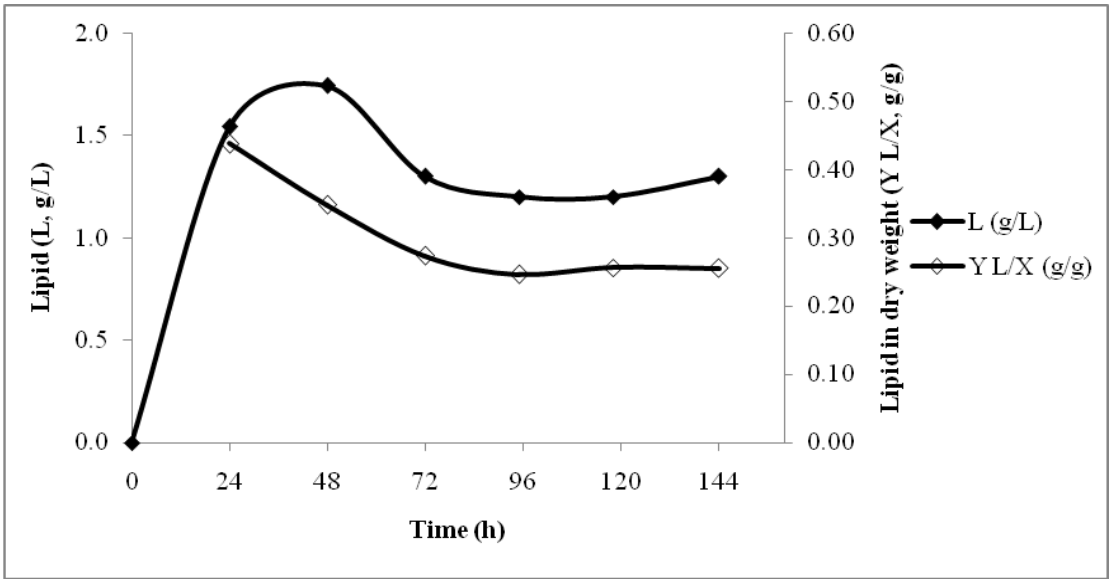
Figure 3. 2. (a) Cellular lipids (L, g L^{-1}) and (b) total cellular lipid in dry weight ($Y_{L/X}$, g g^{-1}) evolution during growth of *Yarrowia lipolytica* strain W29 on OMW-based media [initial phenolic compounds concentration (Ph_0) 0.00 g L^{-1} (no OMW addition), 1.17±0.10 g L^{-1} and 1.64±0.15 g L^{-1}] enriched with commercial glucose in nitrogen-limited conditions. Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, Glc_0~35.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% v/v, incubation temperature *T*=28 ºC. Each point is the mean value of two independent measurements.

In contrast, in the strain ACA-YC 5028, no such correlation of onset of lipid accumulation with increment of initial phenolic compounds concentration in the medium could be established. Moreover, it should be noted though that in similar features with the strain W29, for the strain ACA-YC 5033 cellular lipid production (expressed as L_{max} and $Y_{L/X}$) was, significantly enhanced again in fermentation with phenolic compounds concentration 2.00±0.20 g L^{-1} and 2.90±0.25 g L^{-1}, compared with the control experiment (see paragraph 3.1.2, Table 3.7. and Fig. 3.9.). In all trials, insignificant quantities of glucose remained unconsumed at the end of the fermentation (Table 3.1.). Interestingly, in most of the trials performed, nitrogen-limited fermentations showed some reduction of lipids concentration (both L and $Y_{L/X}$ values) in the early stationary growth phase of the microorganism before significant amounts of citric acid begin to be secreted into the medium. A representative kinetics indicating all the above-mentioned observations is shown in Fig. 3.3. (a) and 3.3. (b) (culture of the strain W 29 in nitrogen-limited OMW-based media with initial phenolic compounds at 1.17±0.10 g L^{-1}). Even in the early growth step (*T*=24 h) lipids were found in significant quantities inside the yeast cells ($Y_{L/X}$~0.45 g g^{-1}) suggesting that lipid accumulation was a partially growth-associated process in contrast with more of the literature reports (for reviews see: Fakas *et al.* 2009; Papanikolaou and Aggelis 2011a) whereas as fermentation proceeded, some cellular lipid biodegradation occurred coinciding with significant secretion of citric acid into the culture medium. Moreover, increment of lipid in dry cell weight values ($Y_{L/X}$) that were observed at the early fermentation steps, coincided with relatively decreased DOT (in %, v/v) values into the medium (see Figs 3.3. b and c), suggesting that lipid synthesis is a highly oxygen dependent process, in agreement with the literature (Ratledge, 2004; Wynn and Ratledge, 2006).

(a)



(b)



(c)

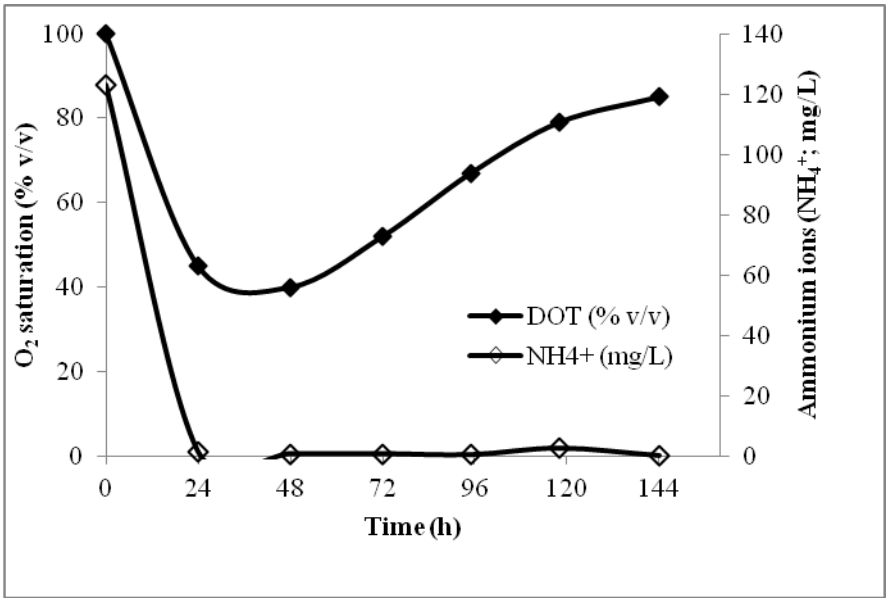


Figure 3. 3. (a) Biomass (X, g L^{-1}), total citric acid (Cit, g L^{-1}) and Glucose (Glc, g L^{-1}), (b) cellular lipids (L, g L^{-1}) and total cellular lipid in dry weight ($Y_{L/X}$, g g^{-1}) evolution and (c) ammonium ions assimilation (mg L^{-1}) and dissolved oxygen tension (DOT, % v/v) during growth of *Yarrowia lipolytica* strain W29 on OMW-based media (initial phenolic compounds concentration 1.17±0.10 g L^{-1}) enriched with commercial glucose in nitrogen-limited conditions. Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, Glc0~35.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% v/v, incubation temperature *T*=28 ºC. Each point is the mean value of two independent measurements.

In carbon-limited fermentations, biomass production was (remarkably) enhanced by OMW addition to the medium for all the three strains tested (Table 3.2.). Biomass evolution, enhanced by the addition of OMW into the medium in carbon-limited experiments (strain W29 grown on glucose-enriched OMW-based media with initial phenolic compounds at 0.00 g L^{-1} (no OMW addition), 1.23±0.10 g L^{-1} and 1.57±0.15 g L^{-1}), is shown in Fig.3.4..

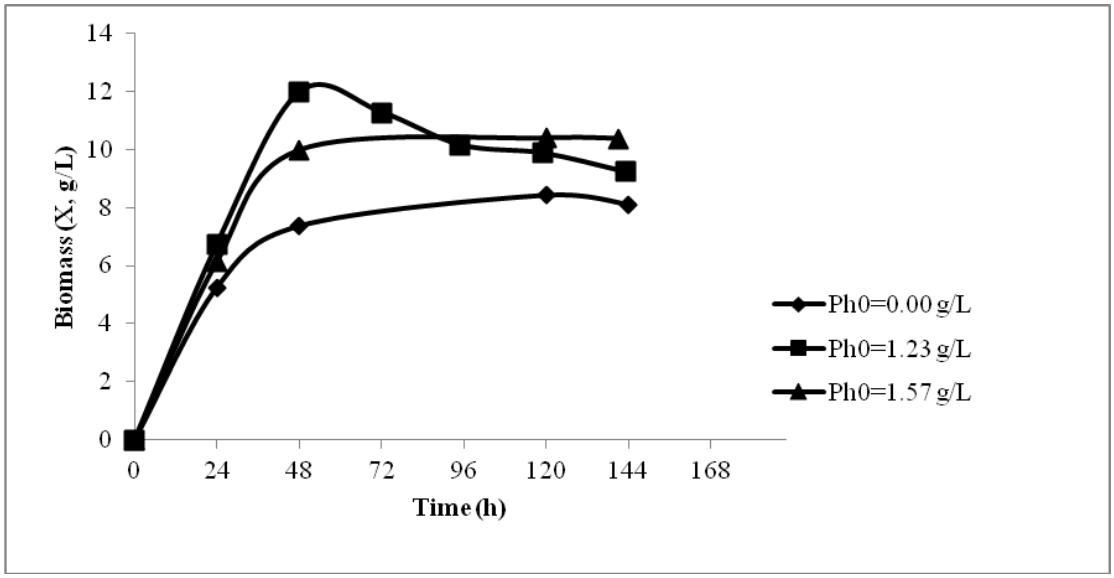


Figure 3. 4. Biomass (X, g L^{-1}) evolution during growth of *Yarrowia lipolytica* strain W29 on OMW-based media (initial phenolic compounds concentration 0.00 g L^{-1} (no OMW addition), 1.23±0.10 g L^{-1} and 1.57±0.15 g L^{-1}) enriched with commercial glucose in carbon-limited conditions. Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, Glc_0~28.0 g L^{-1}, $(NH_4)_2SO_4$=4.0 g L^{-1}, yeast extract=2.0 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% *v/v*, incubation temperature *T*=28 ºC. Each point is the mean value of two independent measurements.

The kinetics of biomass and glucose evolution for a carbon- and a nitrogen-limited experiment presenting similar initial concentrations of glucose (strain ACA-YC 5028 grown on glucose-enriched OMW-based media with initial phenolic compounds at 1.15±0.10 g L^{-1}) is shown in Fig. 3.5.. Generally (significantly) higher biomass values were obtained in comparison with the nitrogen-limited trials (Tables 3.1. and 3.2. and also Fig. 3.5.). Insignificant quantities of glucose remained unconsumed at the end of all carbon-limited fermentations. The strain ACA-YC 5028 grown in carbon-limited cultures, showed some slight reduction of biomass concentration in the stationary growth phase (Fig. 3.5.) suggesting possible cell autolysis at the end of fermentation. Also, glucose consumption was almost linear for both carbon- and nitrogen-limited experiments, with glucose consumption rate (r'''_{Glc}, in g L^{-1} h^{-1}) higher in the carbon-limited culture than the nitrogen-limited culture (r'''_{Glc}~0.34 g L^{-1} h^{-1}against 0.23 g L^{-1} h^{-1}, respectively).

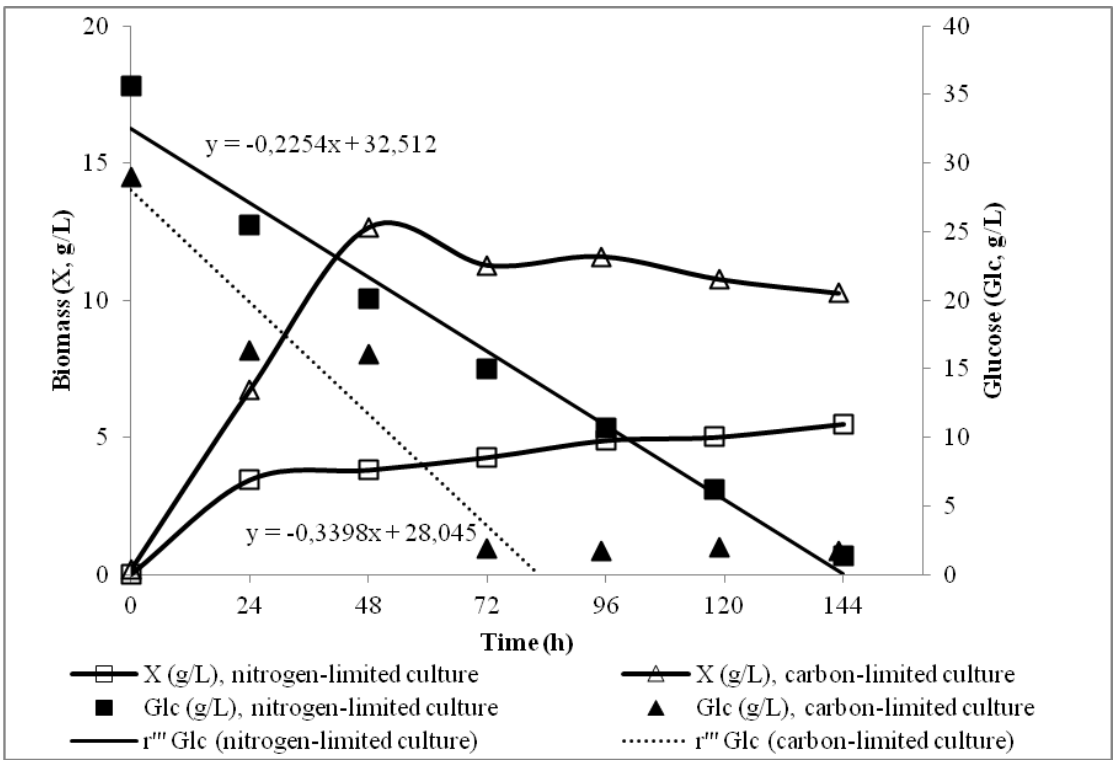


Figure 3. 5. Biomass (X, g L^{-1}) and Glucose (Glc, g L^{-1}) evolution during growth of *Yarrowia lipolytica* strain ACA-YC 5028 on OMW-based media enriched with commercial glucose, with initial phenolic compounds concentration 1.15±0.10 g L^{-1} in nitrogen-limited (in g L^{-1}: Glc_0~35.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}) and carbon-limited (in g L^{-1}: Glc_0~28.0 g L^{-1}, $(NH_4)_2SO_4$=4.0 g L^{-1}, yeast extract=2.0 g L^{-1}) conditions. Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% *v/v*, incubation temperature *T*=28 ºC. Each point is the mean value of two independent measurements.

3.1.3. Citric acid production

In nitrogen-limited experiments *Y. lipolytica* strains produced (in some cases in non-negligdible quantities) total citric acid. The overall maximum citric acid concentration achieved was 18.9 g L^{-1} (yield $Y_{Cit/Glc}$=0.73 g g^{-1}), during growth of the strain ACA-YC 5033 in the control experiment (no OMW addition). For the strains ACA-YC 5033 and ACA-YC 5028, Cit_{max} concentration was insignificantly reduced regardless of OMW addition to the medium (see Table 3.1.). In contrast, the yield $Y_{Cit/Glc}$ decreased remarkably with increased initial phenolic content in the medium specifically for the strain W29 (Table 3.1.); this strain presented remarkable citric acid production in the control experiment (Cit_{max}=18.5 g L^{-1}, $Y_{Cit/Glc}$=0.68 g g^{-1}), was strongly affected by OMW addition into the medium, since indeed significant reduction of citric acid production was observed in OMW-added media (i.e. Cit_{max}<4.0 g L^{-1} and $Y_{Cit/Glc}$=0.11 g g^{-1} values were observed in the trial with initial phenolic compounds of 1.64±0.15 g L^{-1} - see Table 3.1.). Finally, strain ACA-YC5028 produced lower amounts of citric acid compared with the other strains, but, as

previously stressed, in this strain citric acid production remained almost unaffected by the addition of OMW to the medium (Table 3.1.). Finally, in carbon-limited fermentations, in full accordance with the literature (Papanikolaou and Aggelis, 2009; Papanikolaou *et al.*, 2013), insignificant citric acid quantities were produced (Cit<1.5 g L^{-1}). It should be highlighted that iso-citric acid represented a quantity of 5 - 8% *w/w* of total citric acid produced, regardless of the strain or the culture conditions employed.

3.1.4. Decolorization - removal of phenolic compounds

Significant color removal was performed in both nitrogen and carbon-limited fermentations with all strains. The overall maximum decolorization achieved was within the range 45-63% (Table 3.3.). On the other hand, the overall maximum reduction of phenolic compounds ranged between 13 and 34% *w/w*. The maximum decolorization achieved was 62.9% (with initial phenolic compounds 1.50±0.10 g L^{-1} - results concerning the strain ACA-YC 5028) and the maximum reduction in phenol compounds obtained was 33.5% *w/w* (at initial phenolic compounds 1.64±0.15 g L^{-1} - strain W29) (Table 3.3.). In carbon-limited fermentations, the maximum decolorization achieved was 57.3% (at initial phenolic compounds 1.57±0.15 g L^{-1} – strain W29) and the maximum reduction in phenolic compounds was 22.1% *w/w* (at initial phenolic compounds 1.04±0.10 g L^{-1} – strain ACA-YC 5033). The removal of phenolic compounds and the decolorization process seemed to be increased by the addition of waste (excluding strain ACA-YC 5033). The kinetics of color and phenolic compounds removal from the culture medium in one case (strain ACA-YC 5033 on OMW-based media with initial phenolics at 1.15±0.10 g L^{-1}) is shown in Fig. 3.6..

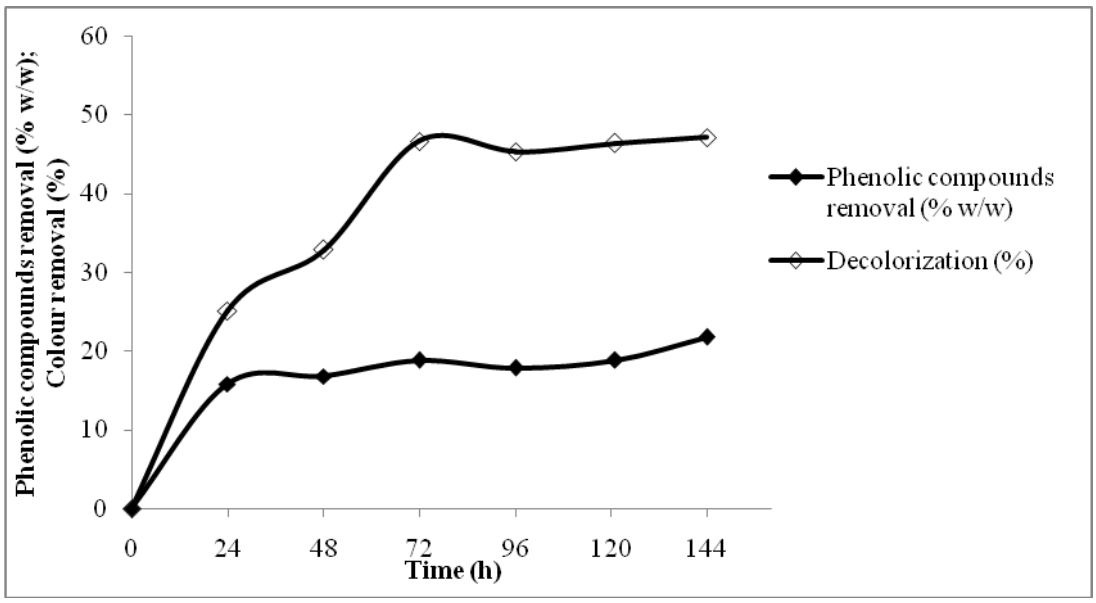


Figure 3. 6. Phenolic compounds removal (% *w/w*) and color removal (%) during growth of *Yarrowia lipolytica* strain ACA-YC 5033 on O.M.W-based media enriched with commercial glucose, with initial phenolic compounds concentration 1.15±0.10 g L^{-1} in nitrogen limited conditions. Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, Glc_0~35.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% *v/v*, incubation temperature *T*=28 ºC. Each point is the mean value of two independent measurements.

Comparing nitrogen- with carbon-limited trials per strain, one can conclude that concerning phenol compounds removal, in trials with initial phenolics at 1.15±0.10 g L^{-1}, such values are slightly higher in nitrogen-limited fermentations, excluding strain ACA-YC 5033. In cultures with initial phenolics at 1.55±0.15 g L^{-1}, phenolic removal values are almost equal for all trials excluding strain W29, where the values are significantly higher in the nitrogen-limited experiments (Table 3.3.). Concerning color removal, in trials with initial phenolic compounds 1.15±0.10 g L^{-1}, values present no significant differences, whereas in trials with initial phenolics at 1.55±0.15 g L^{-1}, decolorization is higher in nitrogen-limited fermentations, excluding strain ACA-YC 5033.

Table 3. 3. Experimental data of *Yarrowia lipolytica* strains ACA-YC 5028, W29 and ACA-YC 5033 concerning removal of phenol compounds and color obtained from kinetics in media containing commercial glucose and various initial O.M.W. concentrations. Representation of initial and final phenol compounds concentration in the culture medium, phenol compounds removal (% *w/w*) and color removal (%) from the medium in nitrogen-limited (a) and carbon-limited (b) experiments. Each point is the mean value of two independent measurements.

Strain		Initial phenolics ($g\ L^{-1}$)	Final phenolics ($g\ L^{-1}$)	Phenol removal (%, *w/w*)	Color removal (%)
ACA-YC 5028		1.17±0.10	0.99±0.10	15.4±1.0	45.1±4.0
	a	1.50±0.15	1.26±0.10	15.9±1.0	62.9±4.5
		1.23±0.10	1.07±0.10	13.0±1.0	49.4±4.0
	b	1.57±0.15	1.35±0.15	14.0±1.0	54.7±4.0
W29		1.17±0.10	0.97±0.10	17.1±1.0	45.1±4.0
	a	1.64±0.15	1.09±0.10	33.5±2.0	62.4±4.5
		1.23±0.10	1.07±0.10	13.0±1.0	45.3±4.0
	b	1.57±0.15	1.31±0.15	16.6±1.0	57.3±4.0
ACA-YC 5033		1.01±0.10	0.79±0.10	21.8±1.5	47.1±4.0
	a	1.50±0.15	1.29±0.10	14.0±1.0	55.6±4.0
		1.04±0.10	0.81±0.10	22.1±1.5	45.3±4.0
	b	1.57±0.15	1.29±0.10	17.8±1.0	55.8±4.0

3.1.5. Fatty acid composition analysis

Fatty acid composition of intra-cellular lipids was analyzed for all *Y. lipolytica* strains in all trials at various growth phases (Tables 3.4. and 3.5.). The fatty acid composition for the various strains presented similarities irrespective of the presence or absence of nitrogen from the culture medium. The principal fatty acids (FAs) detected were with C16 and C18 aliphatic chains. The fatty acid composition changed (in some cases notably) with fermentation time and the addition of OMWs into the medium, since the concentration of the FA C18:0 seemed to decrease with time in both carbon- and nitrogen-limited experiments, whereas the concentration of $^{\Delta 9}$C18:1 increased (this trend is clearer in the nitrogen-limited experiments - see Table 3.4.). What is clearer for all cultures grown is that the addition of phenolic compounds increased the concentration of cellular fatty acid $^{\Delta 9}$C18:1, decreasing that of C18:0, regardless of the carbon- or nitrogen-limited conditions imposed (Tables 3.4. and 3.5.). In several cases, addition of OMWs resulted in concentration of cellular $^{\Delta 9}$C18:1>60% *w/w*, while in one case (growth of the strain ACA-YC 5028 in carbon-limited media enriched with OMWs) the concentration of this fatty acid was ~75% *w/w* (Table 3.5.).

Table 3. 4. Fatty acid composition in the total cellular lipid (% *w/w*) of *Yarrowia lipolytica* strains ACA-YC 5028, W29 and ACA-YC 5033, cultivated on media containing commercial glucose and various initial O.M.W. concentrations in nitrogen limited fermentations.

Each experimental point presented is the mean value of two determinations.

Strain	Initial Phenolic Compounds ($g\ L^{-1}$)	Fermentation time (h)	16:0	$^{\Delta 9}$C16:1	18:0	$^{\Delta 9}$C18:1	$^{\Delta 9,12}$C18:2
ACA-YC 5028	0.00	24	7.7	8.2	11.1	38.2	24.8
		72	9.5	1.0	6.1	39.4	24.0
		144	0.7	2.7	6.3	35.7	22.7
	1.17±0.10	24	8.7	7.3	0.5	46.0	17.5
		72	6.5	5.3	5.6	55.8	16.8
		144	4.9	5.5	4.6	42.6	22.3
	1.50±0.15	24	5.9	4.9	1.1	50.1	15.1
		72	5.8	4.2	4.2	58.6	16.1
		144	3.1	4.0	3.9	55.2	14.9
W29	0.00	24	9.2	3.9	7.0	49.0	10.0
		72	4.5	7.1	5.8	54.1	17.0
		144	3.1	8.0	7.9	49.1	17.9
	1.17±0.10	24	9.3	5.9	6.8	49.4	18.6
		72	2.5	5.1	5.8	58.1	16.0
		144	8.1	6.4	6.7	50.7	18.1
	1.64±0.15	24	9.1	5.8	2.1	58.0	14.4
		72	3.5	5.0	1.8	59.9	15.0
		144	2.7	4.1	1.5	57.5	16.1
ACA-YC 5033	0.00	24	7.1	1.9	6.2	50.5	12.1
		72	6.0	1.7	6.0	54.1	11.0
		144	3.4	10.1	4.4	57.3	9.7
	1.01±0.10	24	9.5	3.2	4.1	55.1	14.9
		72	9.4	2.9	3.4	55.9	14.6
		144	0.9	6.5	3.1	61.7	12.8
	1.50±0.15	24	3.0	3.8	4.2	61.8	17.2
		93	3.0	8.4	4.0	58.5	15.4
		147	5.1	9.9	1.3	55.5	15.3

Table 3. 5. Fatty acid composition in the total cellular lipid (% *w/w*) of *Yarrowia lipolytica* strains ACA-YC 5028, W29 and ACA-YC 5033, cultivated on media containing commercial glucose and various initial O.M.W. concentrations in carbon limited fermentations.

Each experimental point presented is the mean value of two determinations.

Strain	Initial Phenolic Compounds ($g\ L^{-1}$)	Fermentation time (h)	16:0	$^{\Delta 9}$C16:1	18:0	$^{\Delta 9}$C18:1	$^{\Delta 9,12}$C18:2
ACA-YC 5028	0.00	24	3.9	11.1	15.5	35.1	23.0
		48	3.8	5.9	12.1	41.5	21.2
		144	9.9	5.4	7.9	56.6	20.1
	1.23±0.10	24	10.3	11.2	13.7	38.9	25.8
		48	12.3	12.3	6.1	48.4	20.9
		144	14.7	1.1	3.4	60.1	20.5
	1.57±0.15	24	17.4	7.9	12.1	49.6	12.2
		48	17.0	3.8	4.4	58.1	16.7
		144	6.5	0.5	6.3	77.0	6.1
W29	0.00	24	17.6	5.1	15.1	44.5	12.1
		48	16.5	5.5	15.0	45.3	10.7
		144	8.2	8.3	5.3	56.1	22.0
	1.23±0.10	24	20.0	10.4	5.9	45.1	18.6
		48	12.8	7.5	3.0	47.8	21.0
		144	13.1	2.4	7.0	58.8	12.3
	1.57±0.15	24	15.0	2.0	6.2	59.9	15.9
		48	18.7	2.7	9.8	51.8	16.9
		144	12.2	4.0	1.0	59.5	22.4
ACA-YC 5033	0.00	24	15.1	8.7	7.9	53.5	13.6
		48	14.8	9.9	5.1	48.2	12.1
		144	10.1	8.8	5.3	54.5	12.2
	1.04±0.10	24	11.9	5.6	8.1	49.7	24.7
		48	10.9	5.0	8.0	56.1	19.5
		144	10.4	3.8	4.1	59.2	17.3
	1.57±0.15	24	6.9	6.5	4.1	65.1	16.4
		48	9.2	4.5	3.2	73.2	9.4
		144	11.3	0.7	3.2	74.9	8.0

The results of the current chapter have been published in a peer-reviewed journal as follows:

Sarris, D.; Galiotou-Panayotou, M.; Koutinas, A. A.; Komaitis, M.; Papanikolaou, S., Citric acid, biomass and cellular lipid production by *Yarrowia lipolytica* strains cultivated on olive mill wastewater-based media. *Journal of Chemical Technology and Biotechnology* **2011,** 86, 1439-1448.

3.2. Citric acid, biomass and cellular lipid production by *Yarrowia lipolytica* strain ACA-YC 5033 cultivated on olive mill wastewater-based media

3.2.1. General presentation

According to results obtained from the previous chapter, it was found that as far as the strain ACA-YC 5033 was concerned, with the addition of ascending amounts of OMWs into the synthetic glucose-based medium, dry cell weight production was insignificantly reduced, total cellular lipid production seemed to remain unaffected (whereas for *Y. lipolytica* strain ACA-YC 5028 was reduced) and citric acid production was enhanced (whereas for *Y. lipolytica* strain W29 was significantly reduced). Thus the aforementioned strain was selected for further investigation.

In order to further study the behavior of *Yarrowia lipolytica* strain ACA-YC 5033, fermentations were carried out under aseptic and pasteurized conditions in 250-mL Erlenmeyer flasks as also in a laboratory-scale bioreactor (total volume 3.5 L; working volume 3.0 L) in batch experiments, again under aseptic and pasteurized conditions, with the addition of higher amounts of OMWs into the medium.

As in the previous chapter, to investigate the kinetic behavior of the *Y. lipolytica* strain ACA-YC 5033 grown on OMW-based media, fermentations in carbon-limited (Glc_0~30.0 g L^{-1}; initial C/N~13) and nitrogen-limited (Glc_0~35.0 g L^{-1}; initial C/N~85) conditions were carried out. OMW was added into the synthetic medium in higher quantities giving initial concentration of phenolic compounds 2.00±0.20 g L^{-1} and 2.90±0.25 g L^{-1} (Tables 3.6. and 3.7.). Moreover, two trials with Glc_0~35.0 g L^{-1} and initial phenolic compounds concentration 4.50±0.35 g L^{-1} and 5.50±0.40 g L^{-1}, a pasteurized fermentation [heat treatment of the medium at T=80 °C for 5 min, followed by inoculation with 3 mL (6% inoculum) of exponential pre-culture] with Glc_0~35.0 g L^{-1} and initial phenolic compounds concentration 2.90±0.25 g L^{-1} (Table 3.7.) and an aseptic trial with Glc_0~80.0 g L^{-1} and initial phenolic compounds concentration 2.90±0.25 g L^{-1}, under nitrogen limited conditions were carried out (Table 3.7.). Finally, aseptic and pasteurized batch bioreactor experiments were carried out under nitrogen limited conditions with Glc_0~35.0 g L^{-1} and initial phenolic compounds concentration 2.90±0.25 g L^{-1} under nitrogen-limited conditions (Table 3.7.).

Table 3. 6. Experimental data of *Yarrowia lipolytica* strain ACA-YC 5033 in carbon-limited glucose-based media with olive-mill waste-waters added in various amounts.

Representations of total biomass (X, g L^{-1}), total cellular lipid (L, g L^{-1}), total citric acid (Cit, g L^{-1}) and consumed substrate (Glc_{cons}, g L^{-1}) concentrations at different fermentation points of each trial:

Initial Phenolic Compounds (g/L)	Fermentation time (h)	X (g L^{-1})	L (g L^{-1})	Glc_{cons} (g L^{-1})	$Y_{X/Glc}$ (g g^{-1})	$Y_{L/X}$ (g g^{-1})
2.00±0.20	70	10.9±0.8[a]	0.70±0.05	23.3±1.5	0.47	0.07
	46	9.2±0.7[b]	0.90±0.05	22.3±1.5	0.41	0.10
2.90±0.25	72	13.3±1.0[a,b]	0.50±0.05	25.8±2.0	0.52	0.04

[a] when X_{max} concentration was achieved;

[b] when L_{max} concentration was achieved.

Fermentation time, conversion yield of biomass produced per glucose consumed ($Y_{X/Glc}$, g g^{-1}) and total lipid in dry biomass ($Y_{L/X}$, g g^{-1}) are presented for all points of the trials. Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, Glc_0~28.0 g/L, $(NH_4)_2SO_4$=4.0 g L^{-1}, yeast extract=2.0 g L^{-1} initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% *v/v*, incubation temperature *T*=28 °C. Each point is the mean value of two independent measurements.

Table 3. 7. Experimental data of *Yarrowia lipolytica* strain ACA-YC 5033 in nitrogen-limited glucose-based media with olive-mill waste-waters added in various amounts.

Representations of total biomass (X, g L^{-1}), total cellular lipid (L, g L^{-1}), total citric acid (Cit, g L^{-1}) and consumed substrate (Glc_{cons}, g L^{-1}) concentrations at different fermentation points of each trial:

Initial Phenolic Compounds (g L^{-1})		Fermentation time (h)	X (g L^{-1})	L (g L^{-1})	Cit (g L^{-1})	Glc_{cons} (g L^{-1})	$Y_{X/Glc}$ (g g^{-1})	$Y_{L/X}$ (g g^{-1})	$Y_{Cit/Glc}$ (g g^{-1})
2.00±0.20	Shake-flasks Aseptic	120	4.1±0.3[a]	0.70±0.10	13.5±1.0	23.3±1.5	0.18	0.16	0.58
		96	4.0±0.3[b]	0.80±0.05	12.2±1.0	15.8±1.0	0.25	0.19	0.77
		168	3.7±0.3[c]	0.50±0.05	18.2±1.5	23.7±1.5	0.15	0.15	0.77
2.90±0.25	Shake-flasks Aseptic	121	3.7±0.3[a]	0.60±0.05	17.2±1.0	22.5±1.5	0.16	0.16	0.76
		96	3.6±0.3[b]	1.00±0.10	15.1±1.0	18.7±1.5	0.20	0.27	0.81
		144	3.5±0.3[c]	0.70±0.05	19.0±1.5	25.6±2.0	0.14	0.20	0.74
	Shake-flasks Pasteurised	120	3.3±0.2[a,b]	0.90±0.10	11.6±1.0	17.0±1.0	0.19	0.26	0.68
		178	3.2±0.2[c]	0.60±0.05	15.5±1.0	22.8±1.5	0.14	0.19	0.68
	Shake-flasks Glc_0~80.0 g L^{-1} Aseptic	288	4.6±0.3[a]	1.20±0.10	43.3±3.5	70.7±5.0	0.07	0.26	0.61
		240	4.3±0.3[b]	1.40±0.10	38.3±3.5	57.3±4.0	0.08	0.33	0.67
		312	4.3±0.3[c]	0.90±0.10	47.1±4.0	70.8±5.0	0.06	0.20	0.67
	Bioreactor Aseptic	188	4.7±0.3[a,c]	0.90±0.10	15.2±1.0	25.0±2.0	0.19	0.19	0.61
		138	4.3±0.3[b]	1.10±0.10	13.4±1.0	21.7±1.5	0.20	0.26	0.62
	Bioreactor Pasteurized	192	4.8±0.3[a,c]	0.80±0.05	13.9±1.0	23.9±1.5	0.20	0.17	0.58
		144	4.4±0.3[b]	1.00±0.10	12.1±1.0	19.7±1.5	0.22	0.22	0.61
4.50±0.35	Shake-flasks Aseptic	243	2.1±0.1[a,c]	0.40±0.05	4.0±0.5	7.4±0.5	0.29	0.18	0.55
		48	1.7±0.1[b]	0.80±0.05	1.0±0.5	4.3±0.5	0.40	0.45	0.24
5.50±0.40		216	3.0±0.3[a,c]	0.90±0.10	2.2±0.5	6.1±0.5	0.50	0.30	0.36
		48	2.6±0.1[b]	1.20±0.10	0.4±0.5	3.6±0.5	0.72	0.48	0.11

[a] when X_{max} concentration was achieved;

[b] when L_{max} concentration was achieved;

[c] when Cit_{max} concentration was achieved.

Fermentation time, conversion yield of biomass produced per glucose consumed ($Y_{X/Glc}$, g g^{-1}), total lipid in dry biomass ($Y_{L/X}$, g g^{-1}) and conversion yield of total citric acid produced per glucose consumed ($Y_{Cit/Glc}$, g g^{-1}) are presented for all points of the trials. Culture conditions: growth on aseptic and pasteurized 250-mL flasks at 180 ± 5 rpm, Glc_0~35.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, D.O.T.>20% *v/v*, incubation temperature *T*=28 °C; growth on aseptic and pasteurized batch bioreactor, 300 rpm, initial phenolic compounds concentration 2.90±0.25 g L^{-1}, initial pH=6.00 ± 0.02, incubation temperature *T*=28 °C and sparge of air 1.0 vvm. Each point is the mean value of two independent measurements.

3.2.2. Biomass and lipid production

In the nitrogen limited shake-flask fermentations performed, biomass production seemed to be affected by the addition of OMW into the synthetic medium. Comparing with the control experiment (no OMW addition; Table 3.1.), X_{max} concentration was reduced proportionally to the phenolic content (Table 3.7. and Fig. 3.7.). Yield $Y_{X/Glc}$ (excluding control experiment) was almost unaffected (Table 3.7.). Total cellular lipids were quantified in all growth faces for all trials and in some cases lipid quantities >25% *w/w* of lipid in dry matter were found (Table 3.7.), indicating that lipid accumulation occurred in the fermentations. Interestingly enough, the addition of OMWs in the medium seemed to favor the accumulation of storage lipids [Fig. 3.8. (a) and (b); Fig. 3.9.; Tables 3.1. and 3.7.]. Likewise, as mentioned in paragraph 3.1.2., similar results were obtained by *Y. lipolytica* strain W29 suggesting that OMWs seemed to be a "lipogenic" medium as the presence of OMWs in the medium seemed to stimulate a reserve lipid accumulation process [L_{max} quantities up to 1.9 g L^{-1}, corresponding to lipid in dry weight ($Y_{L/X}$) values up to 0.28 g g^{-1} (or even higher)], since in the control experiment clearly lower $Y_{L/X}$ and L values were obtained (Table 3.1. and Fig. 3.2.).

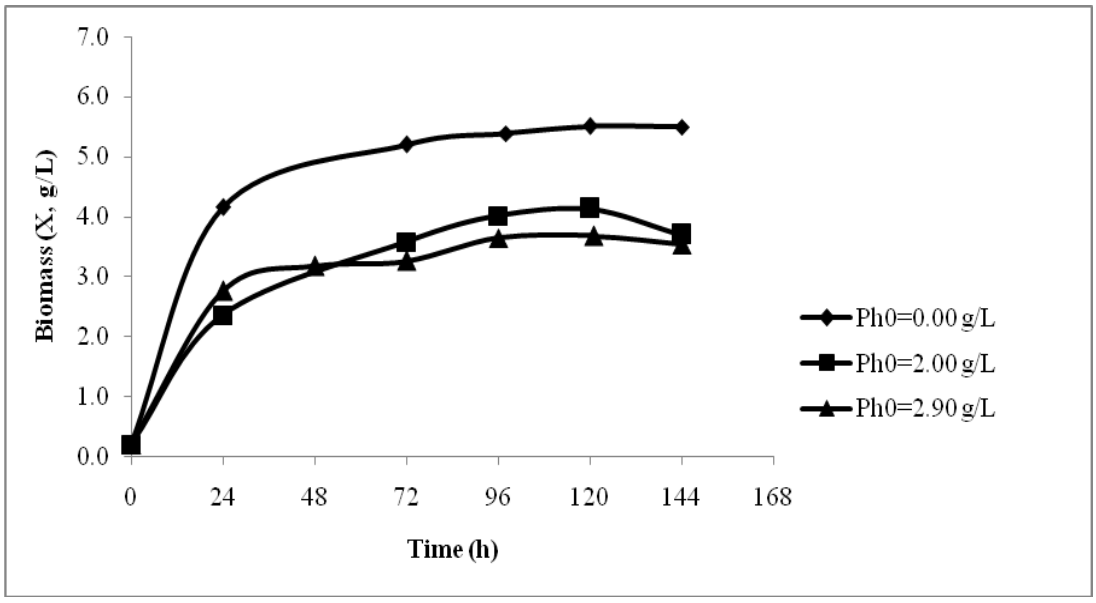
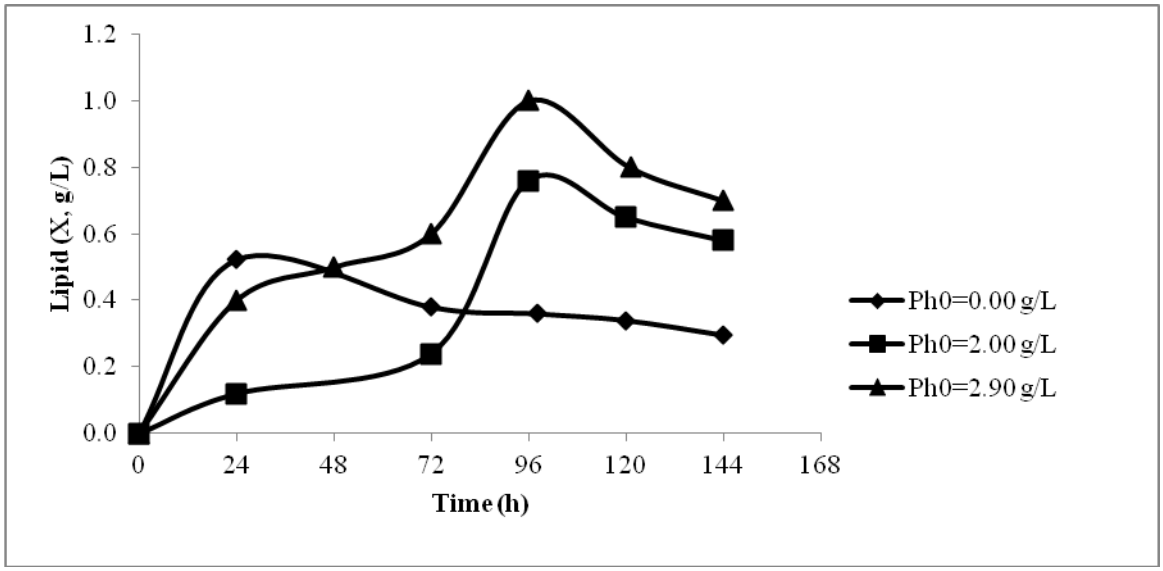


Figure 3. 7. Biomass (X, g L^{-1}) evolution during growth of *Yarrowia lipolytica* strain ACA-YC 5033 on OMW-based media (initial phenolic compounds concentration 0.00 g L^{-1} (no OMW addition), 2.00±0.20 g L^{-1} and 2.90±0.25 g L^{-1}) enriched with commercial glucose in nitrogen-limited conditions. Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, Glc_0~35.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% *v/v*, incubation temperature *T*=28 ºC. Each point is the mean value of two independent measurements.

L_{max} quantities up to 1.0 g L^{-1}, corresponding to lipid in dry weight ($Y_{L/X}$) values up to 0.27 g g^{-1} were obtained when OMW was added into the medium (Table 3.7.), suggesting stimulation of

a reserve lipid accumulation process, since in the control experiment clearly lower $Y_{L/X}$ and L values were obtained [Table 3.1. and Fig. 3.8. (a) and (b) and Fig. 3.9.]. In the trial with initial phenolic compounds concentration 2.00±0.20 g L^{-1} insignificant quantities of glucose remained unconsumed at the end of the fermentation whereas in the trial with initial phenolic compounds concentration 2.90±0.25 g L^{-1} the final Glc~10.0 g L^{-1}.

(a)



(b)

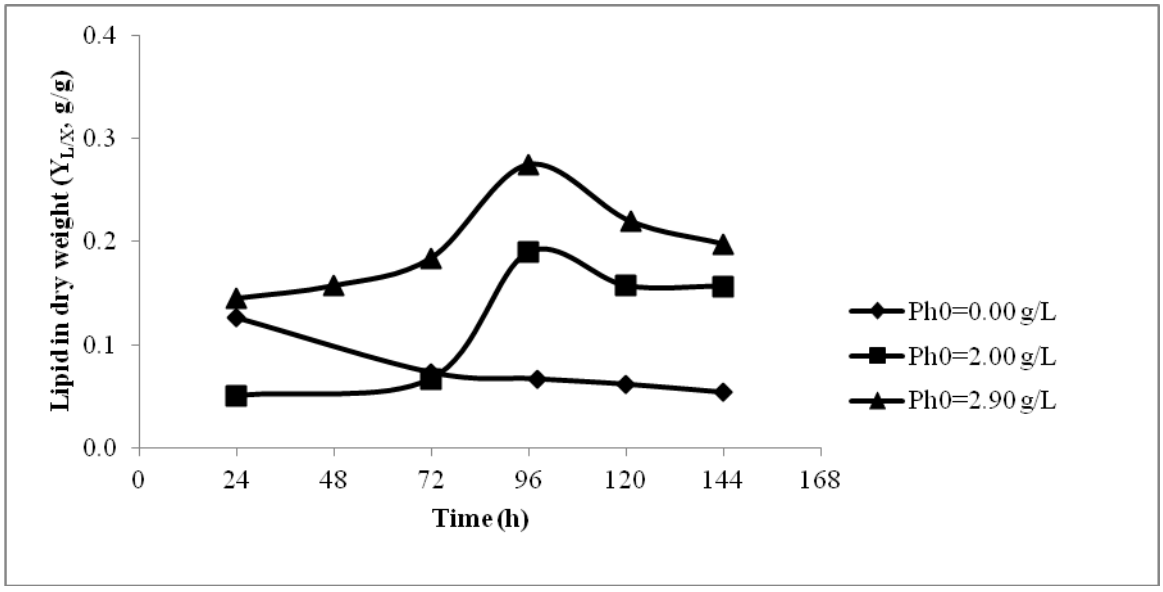


Figure 3. 8. (a) Cellular lipids (L, g L^{-1}) and (b) total cellular lipid in dry weight ($Y_{L/X}$, g g^{-1}) evolution during growth of *Yarrowia lipolytica* strain ACA-YC 5033 on OMW-based media (initial phenolic compounds concentration 0.00 g L^{-1} (no OMW addition), 2.00±0.20 g L^{-1} and 2.90±0.25 g L^{-1}) enriched with commercial glucose in nitrogen-limited conditions. Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, Glc_0~35.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% *v/v*, incubation temperature *T*=28 ºC. Each point is the mean value of two independent measurements.

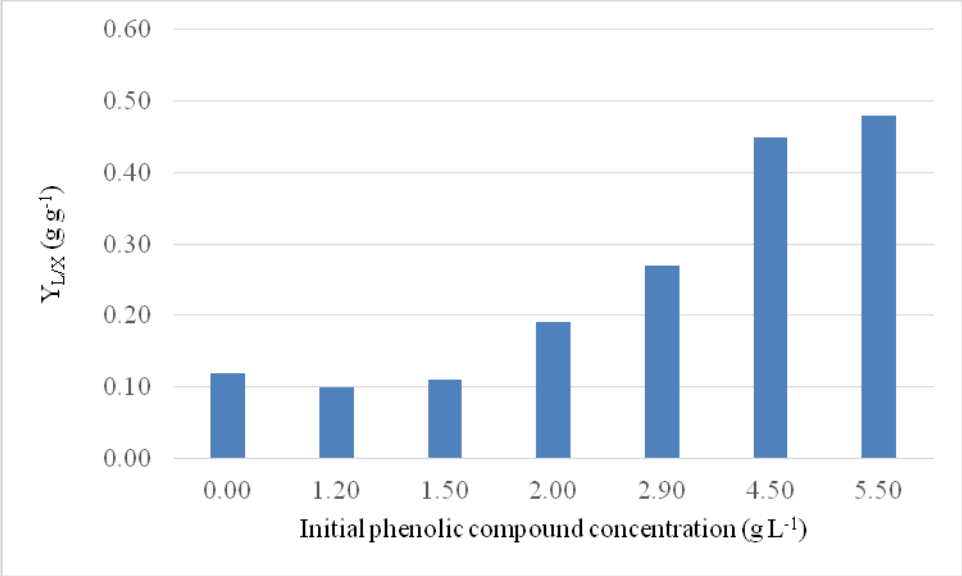
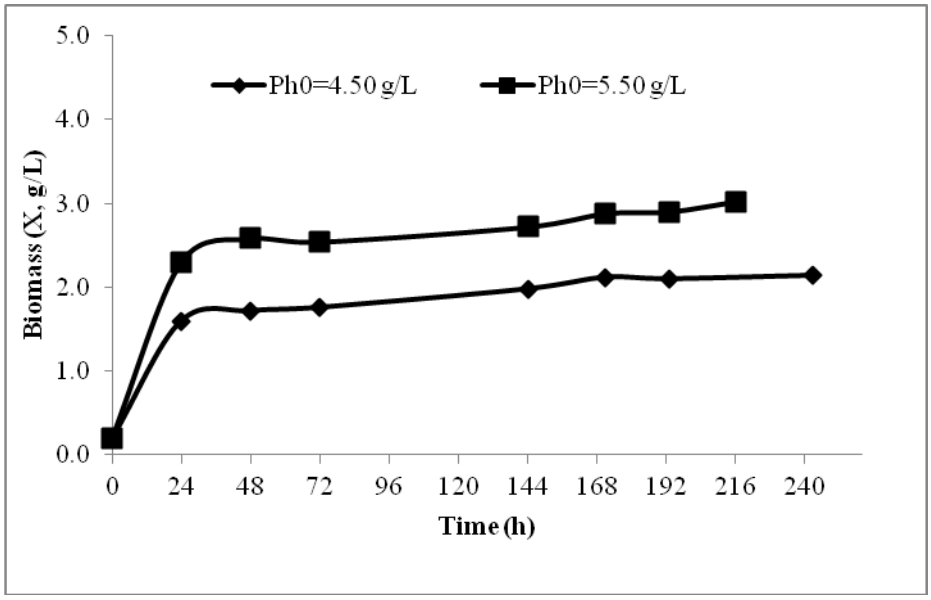


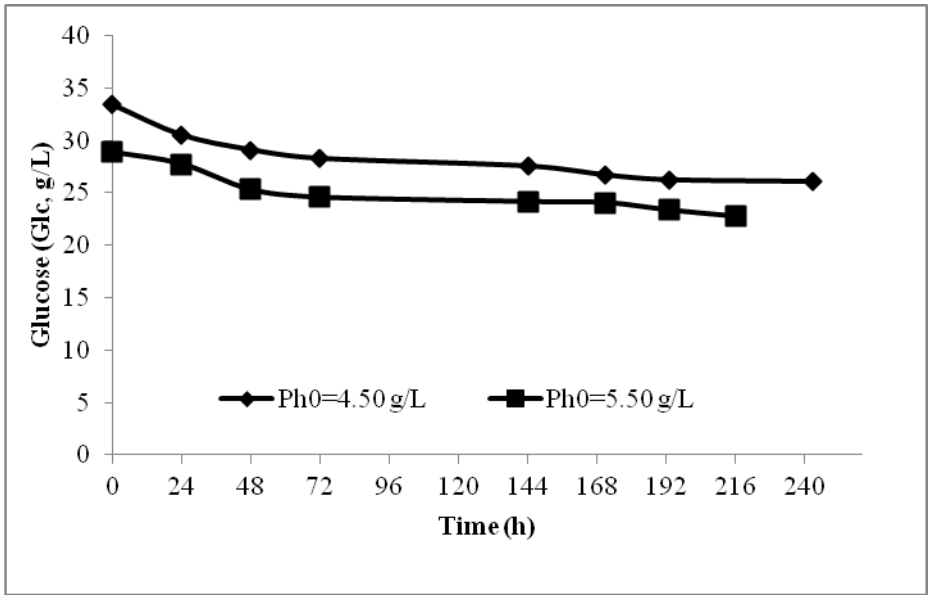
Figure 3. 9. Total cellular lipid in dry weight ($Y_{L/X}$, g g^{-1}) overall evolution during growth of *Yarrowia lipolytica* strain ACA-YC 5033 on OMW-based media (initial phenolic compounds concentration 0.00 g L^{-1} (no OMW addition), 1.20±0.10 g L^{-1}, 1.50±0.15 g L^{-1}, 2.00±0.20 g L^{-1}, 2.90±0.25 g L^{-1}, 4.50±0.35 g $L^{-1}$and 5.50±0.40 g L^{-1}) enriched with commercial glucose in nitrogen-limited conditions. Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, Glc_0~35.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% *v/v*, incubation temperature *T*=28 ºC. Each point is the mean value of two independent measurements.

Two additional sterile shake-flask trials with Glc_0~35.0 g L^{-1} and initial phenolic compounds concentration 4.50±0.35 g L^{-1} and 5.50±0.40 g L^{-1} were performed. The high phenol content clearly inhibited the growth of the microorganism. The production of biomass (by means of X_{max}) reached in both trials the values of ~2.0 g L^{-1} and of ~3.0 g L^{-1} respectively [Fig. 3.10 (a)]. Moreover, small quantities of glucose were consumed [~7.0 g L^{-1} and ~6.0 g L^{-1} respectively; Fig. 3.10 (b)]. Nevertheless, interestingly enough, even higher lipid accumulation compared with the previous trials was observed by the addition of OMWs at these ratios, giving L_{max} quantities up to 0.8 g L^{-1}, corresponding to lipid in dry weight ($Y_{L/X}$) values up to 0.45 g g^{-1} for the trial with initial phenolic compounds concentration 4.50±0.35 g L^{-1} and L_{max} quantities up to 1.2 g L^{-1} [Fig. 3.10 (c)], corresponding to lipid in dry weight ($Y_{L/X}$) values up to 0.48 g g^{-1} for the trial with initial phenolic compounds concentration 5.50±0.40 g L^{-1} [Fig. 3.10 (d)].

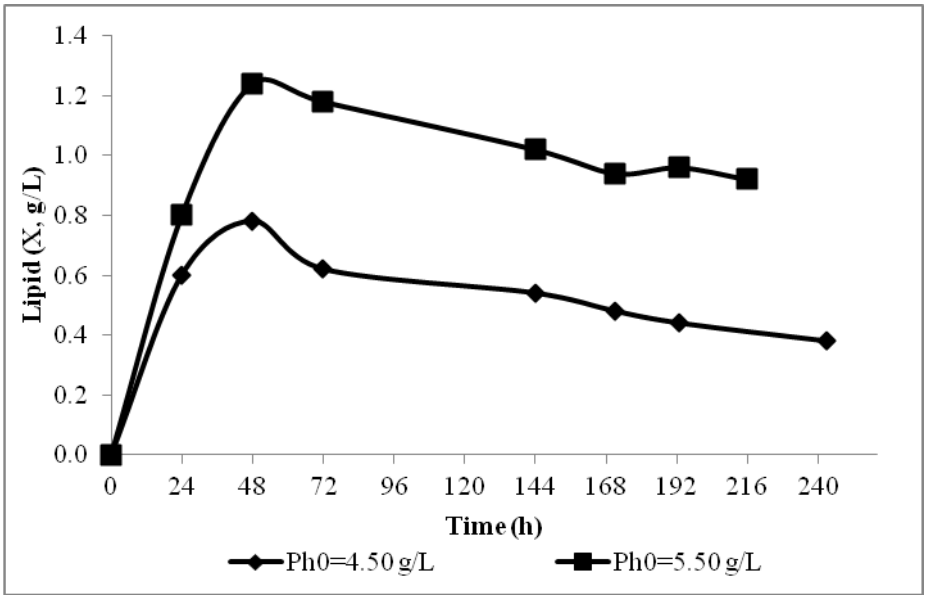
(a)


5.0
4.0
3.0
2.0
1.0
0.0
Biomass (X, g/L)
Ph0=4.50 g/L
Ph0=5.50 g/L
0
24
48
72
96
120
144
168
192
216
240
Time (h)

(b)


40
35
30
25
20
15
10
5
0
Glucose (Glc, g/L)
Ph0=4.50 g/L
Ph0=5.50 g/L
0
24
48
72
96
120
144
168
192
216
240
Time (h)

(c)



(d)

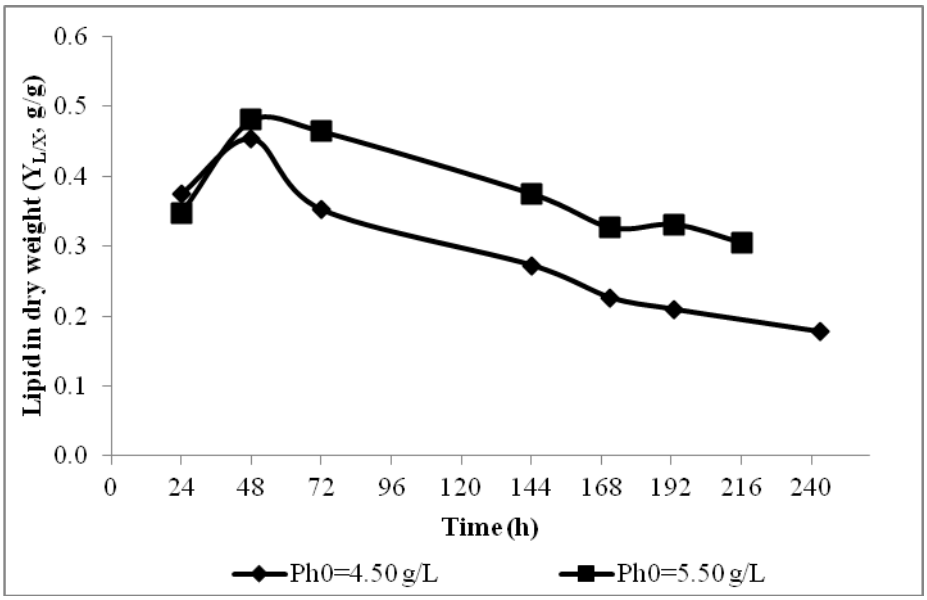


Figure 3. 10. (a) Biomass (X, g L^{-1}), (b) glucose (Glc, g L^{-1}), (c) cellular lipids (L, g L^{-1}) and (d) total cellular lipid in dry weight ($Y_{L/X}$, g g^{-1}) evolution during growth of *Yarrowia lipolytica* strain ACA-YC 5033 on OMW-based media (initial phenolic compounds concentration 4.50±0.35 g L^{-1} and 5.50±0.40 g L^{-1}) enriched with commercial glucose in nitrogen-limited conditions. Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, Glc_0~35.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% *v/v*, incubation temperature *T*=28 ºC. Each point is the mean value of two independent measurements.

In carbon-limited fermentations, biomass production was (remarkably) enhanced by OMW addition to the medium (Table 3.2. and 3.6.). The kinetics of dry cell weight evolution that was enhanced by the addition of OMWs into the medium in the carbon-limited experiments is shown in Fig.3.11. [see the trials with initial phenolic compounds at 0.00 g L^{-1} (no OMW addition), 2.00±0.20 g L^{-1} and 2.90±0.25 g L^{-1}].

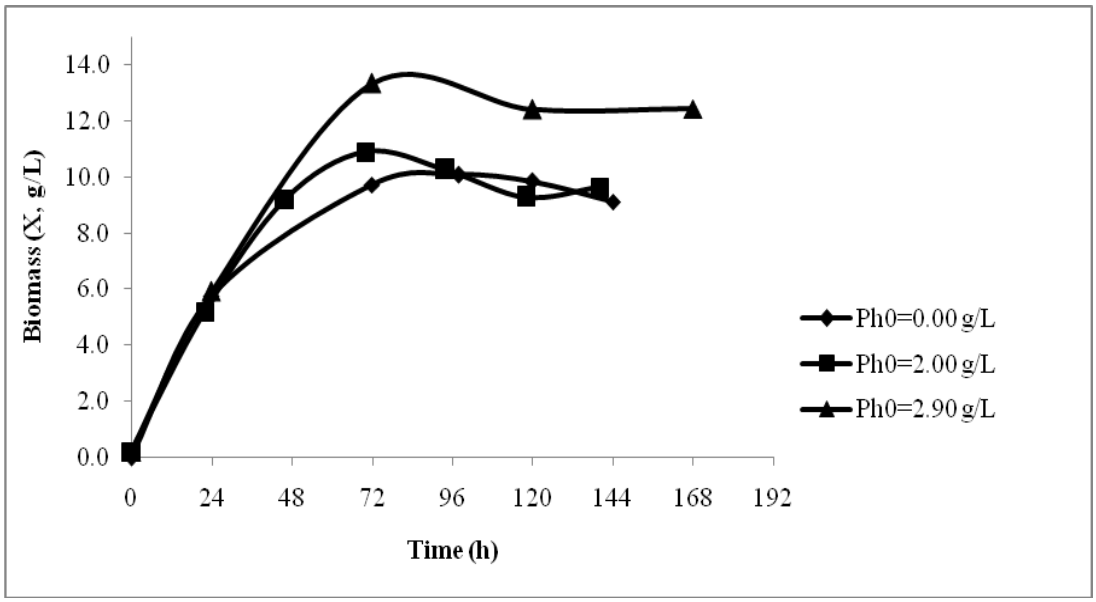


Figure 3. 11. Biomass (X, g L^{-1}) evolution during growth of *Yarrowia lipolytica* strain ACA-YC 5033 on OMW-based media [initial phenolic compounds concentration 0.00 g L^{-1} (no OMW addition), 2.00±0.20 g L^{-1} and 2.90±0.25 g L^{-1}] enriched with commercial glucose in carbon-limited media. Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, Glc_0~28.0 g L^{-1}, $(NH_4)_2SO_4$=4.0 g L^{-1}, yeast extract=2.0 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% *v/v*, incubation temperature *T*=28 ºC. Each point is the mean value of two independent measurements.

The kinetics of biomass and glucose evolution for a carbon- and a nitrogen-limited experiment (glucose-enriched OMW-based media with initial phenolic compounds at 2.90±0.25 g L^{-1}) is shown in Fig. 3.12.. Generally (significantly) higher dry cell weight values were obtained in comparison with the nitrogen-limited trials (Tables 3.6. and 3.7.; Fig. 3.12.). Insignificant quantities of glucose remained unconsumed at the end of all carbon-limited fermentations. Finally, total cellular lipids were quantified for all cultures and growth steps, without exceeding 10% *w/w* in dry matter for all trials. In most cases, cellular lipids in dry weight were within the range 4-10% *w/w*, suggesting, in accordance with the literature (Beopoulos *et al*., 2009; Fakas *et al*., 2009; Papanikolaou and Aggelis, 2009) that no significant lipid accumulation occurred in carbon-limited conditions (Table 3.6.).

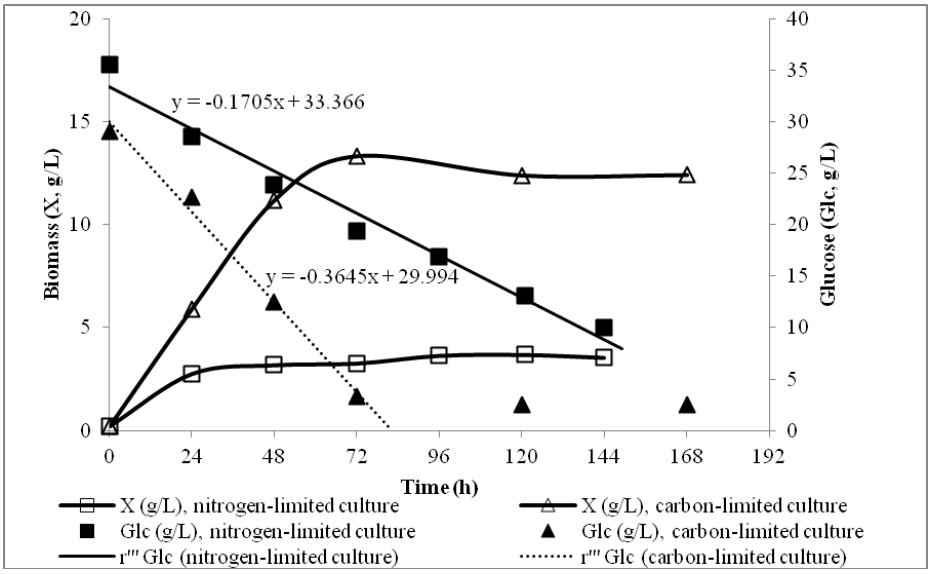


Figure 3. 12. Biomass (X, g L^{-1}) and glucose (Glc, g L^{-1}) evolution during growth of *Yarrowia lipolytica* strain ACA-YC 5033 on OMW-based media enriched with commercial glucose, with initial phenolic compounds concentration 2.90±0.25 g L^{-1} in nitrogen-limited (in g L^{-1}: Glc_0~35.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}) and carbon-limited (in g L^{-1}: Glc_0~28.0 g L^{-1}, $(NH_4)_2SO_4$=4.0 g L^{-1}, yeast extract=2.0 g L^{-1}). Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% *v/v*, incubation temperature *T*=28 ºC. Each point is the mean value of two independent measurements.

In order to attempt to reduce the cost of the proposed technology for a potential scale-up of the bioprocess, same pasteurized trials were performed and were compared to the similar experiments elaborated under axenic cultures. It should be stressed out that between aseptic and pasteurized (thermal treatment at *T*=80 °C for 5 min) shake-flasks cultures, no significant differences were observed in kinetics [for both biomass (by means of X_{max} and $Y_{X/Glc}$) and lipid (by means of L_{max} and $Y_{L/X}$, g g^{-1}) production; see Table 3.7.]. In the previously pasteurized culture media, the quantity of glucose remained unconsumed at the end was ~8.0 g L^{-1} while the assimilation rate of glucose was linear for both experiments, with glucose consumption rate (r'''_{Glc}, in g L^{-1} h^{-1}) being higher in aseptic than in pasteurized cultures (r'''_{Glc}~0.17 g L^{-1} h^{-1}against 0.13 g L^{-1} h^{-1}, respectively) (Fig. 3.13.).

In order to perform a process scale-up for several microbial conversions, an important aspect that is taken into consideration and studied is the comparison and the potential differences in the physiological and kinetic features between experiments performed in shake-flask and batch bioreactor cultures, given that agitation and aeration conditions may be different in these fermentation configurations (Aggelis *et al.*, 2003; Mantzavinos and Kalogerakis, 2005; Crognale *et al.*, 2006; Papanikolaou *et al.*, 2007). Comparison was performed between aseptic shake-flask and

aseptic bioreactor fermentations of OMW-based media that presented almost equal initial glucose (~35.0 g L^{-1}) and phenolic compounds (2.9±0.25 g L^{-1}) concentrations.

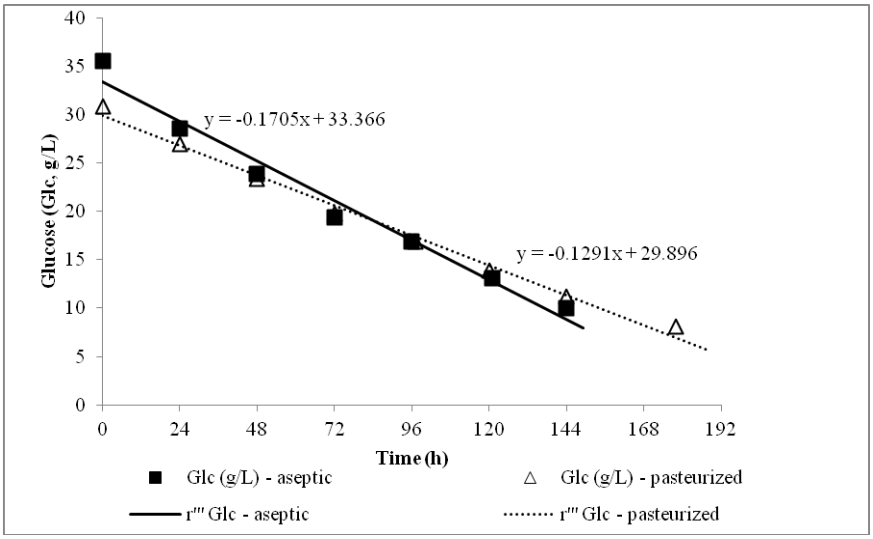
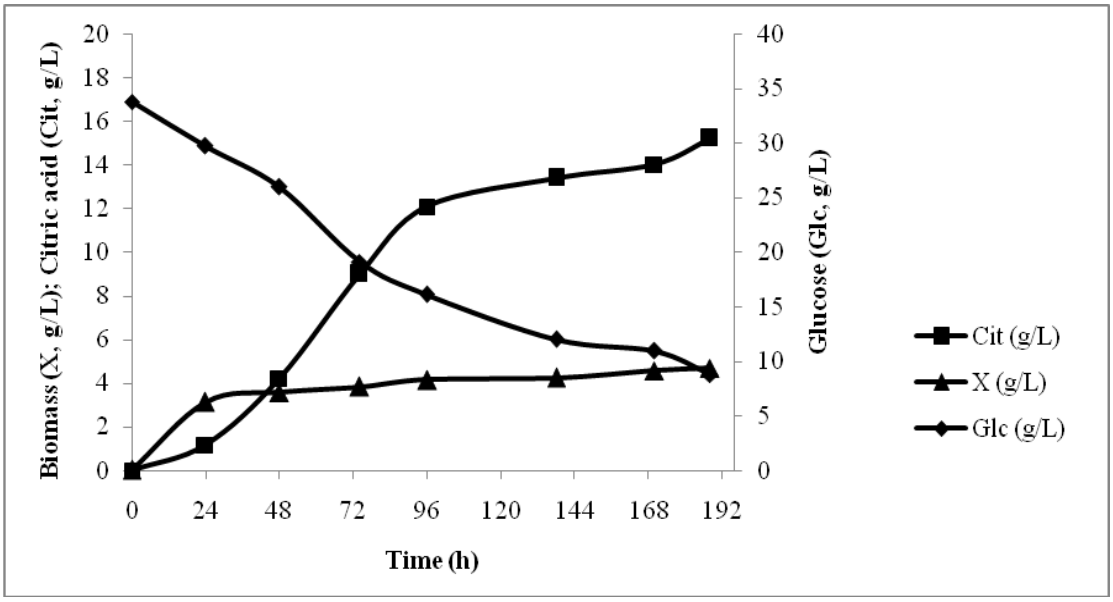


Figure 3. 13. Glucose (Glc, g L^{-1}) evolution during growth of *Yarrowia lipolytica* strain ACA-YC 5033 under aseptic and pasteurized conditions on OMW-based media (initial phenolic compounds concentration 2.90±0.25 g L^{-1}) enriched with commercial glucose in nitrogen-limited conditions. Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, Glc_0~35.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% *v/v*, incubation temperature *T*=28 ºC. Each point is the mean value of two independent measurements.

Concerning aseptic batch bioreactor cultures, biomass production seemed to be insignificantly enhanced (especially by means of X_{max}) (Table 3.7.) compared to the respective shake-flask trial, while the strain reached its kinetics plateau earlier in shake-flasks (~96 h) than in bioreactor cultures. X_{max} quantities up to 4.7 g L^{-1}, corresponding to conversion yield of biomass produced per glucose consumed ($Y_{X/Glc}$, g g^{-1}) up to 0.19 g g^{-1} and L_{max} quantities up to 1.1 g L^{-1}, corresponding to lipid in dry weight ($Y_{L/X}$) up to 0.27 g g^{-1} were obtained (Table 3.7.). A representative kinetics is shown in Fig. 3.14. (a) and (b) (aseptic batch bioreactor culture in nitrogen-limited OMW-based media with initial phenolic compounds at 2.90±0.25 g L^{-1} and Glc_0~35 g L^{-1}).

(a)



(b)

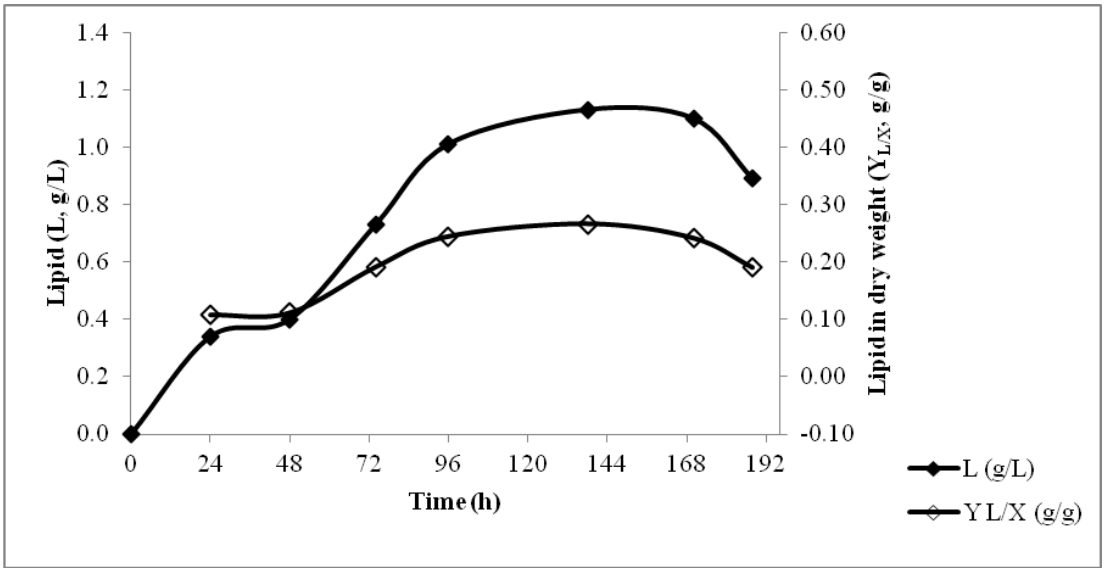


Figure 3. 14. (a) Biomass (X, g L^{-1}), total citric acid (Cit, g L^{-1}) and glucose (Glc, g L^{-1}); (b) cellular lipids (L, g L^{-1}) and total cellular lipid in dry weight ($Y_{L/X}$, g g^{-1}) evolution during growth of *Yarrowia lipolytica* strain ACA-YC 5033 on OMW-based media (initial phenolic compounds concentration 2.90±0.25 g L^{-1}) enriched with commercial glucose in nitrogen-limited media. Culture conditions: aseptic batch bioreactor cultures agitated at 300 rpm, Glc_0~35.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% *v/v*, incubation temperature *T*=28 ºC, and sparge of air 1.0 vvm. Each point is the mean value of two independent measurements.

In both trials, Glc~10.0 g L^{-1} remained unconsumed at the end of the fermentation, while the assimilation rate of glucose was almost linear for both shake-flasks and bioreactor experiments, with glucose consumption rate (r'''_{Glc}, in g L^{-1} h^{-1}) being higher in shake-flasks cultures than in the bioreactor cultures (r'''_{Glc}~0.17 g L^{-1} h^{-1}against 0.13 g L^{-1} h^{-1}, respectively) (Fig. 3.15.).

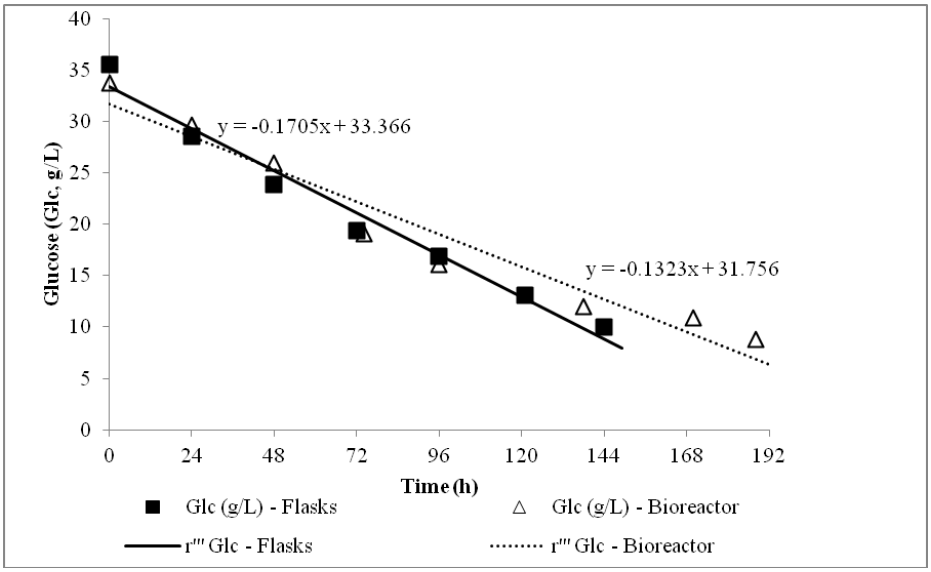
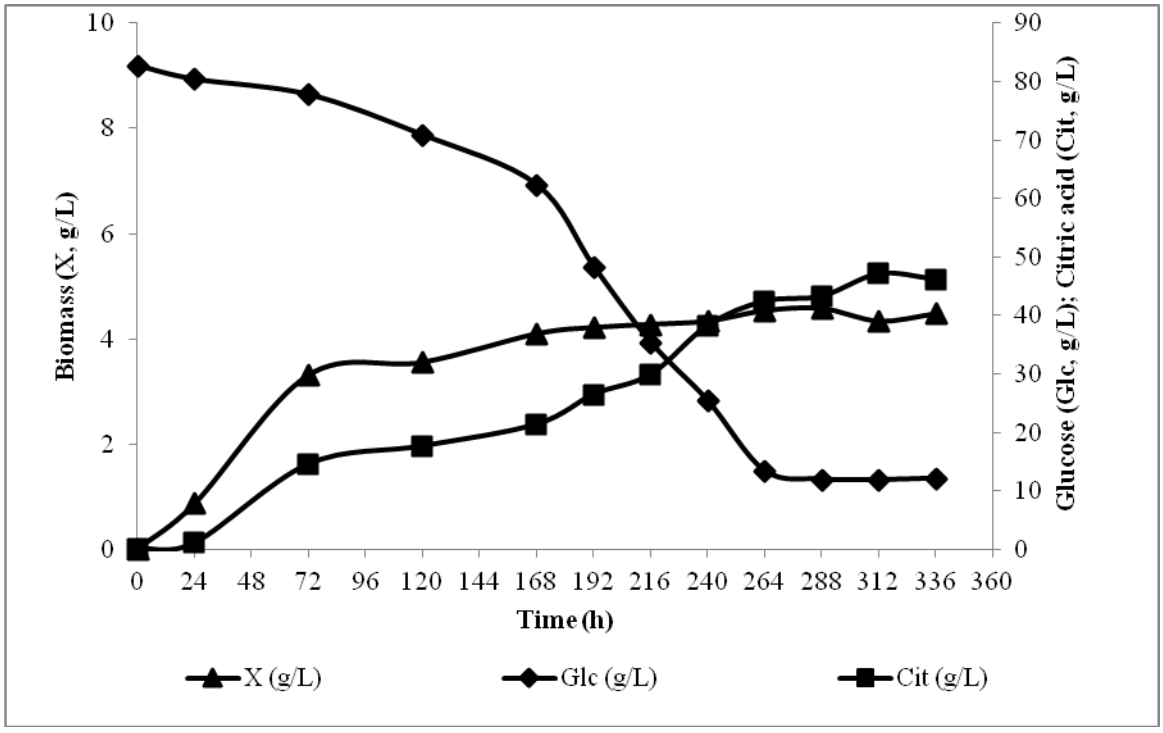


Figure 3. 15. Comparison of *Yarrowia lipolytica* strain ACA-YC 5033 kinetics between aseptic shake-flask and aseptic batch-bioreactor cultures regarding glucose (Glc, g L^{-1}) evolution on OMW-based media enriched with commercial glucose with initial phenolic compounds concentration 2.90±0.25 g L^{-1}. Culture conditions: growth on aseptic shake-flask 250-mL cultures agitated at 180 ± 5 rpm, Glc_0~35.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% *v/v*, incubation temperature *T*=28 ºC; aseptic batch bioreactor cultures agitated at 300 rpm, Glc_0~35.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% *v/v*, incubation temperature *T*=28 ºC, and sparge of air 1.0 vvm. Each point is the mean value of two independent measurements.

As mentioned above, in order to demonstrate the feasibility and the potential of the pasteurized fermentation process, pasteurized batch bioreactor trials (Glc_0~35.0 g L^{-1}; initial phenolic compounds concentration in the medium of 2.90±0.25 g L^{-1}) were performed and compared with the respective aseptic experiments in which axenic cultures were used (see Table 3.7.). Indeed, between aseptic and pasteurized cultures no significant differences were observed for biomass and total cellular lipids production.

Finally, a trial with significantly higher initial glucose concentration as compared with the previous experiments (Glc_0~80.0 g L^{-1}) was performed in shake-flask aseptic cultures. In that trial, OMWs were added into the medium in order to yield in an initial phenolic compounds concentration 2.90±0.25 g L^{-1}. The rationale of such glucose concentration selected was to give initial concentration of reducing sugars corresponded to glucose quantity that can usually be found in OMWs derived from press extraction systems (Crognale *et al.*, 2006; Papanikolaou *et al.*, 2008a). Concerning this fermentation, X_{max} quantities up to 4.6 g L^{-1}, corresponding to $Y_{X/Glc}$ values up to 0.07 g g^{-1} were obtained [Table 3.7.; Fig. 3.16. (a)]. Comparing with the respective trial in which lower initial glucose concentration was employed into the medium (culture with initial phenolic compounds concentration 2.90±0.25 g L^{-1} and Glc_0~35.0 g L^{-1}), only in terms of biomass yield on glucose consumed, the addition of glucose into the medium led to lower $Y_{X/Glc}$ values (Table 3.7.). L_{max} quantities up to 1.4 g L^{-1} (overall the highest value throughout *Y. lipolytica* ACA-YC 5033 experiments), corresponding to lipid in dry weight ($Y_{L/X}$) values up to 0.33 g g^{-1} were obtained when OMW was added into the medium [Table 3.7.; Fig. 3.16. (b)], suggesting, as previously, stimulation of reserve lipid accumulation process, due to increased presence of OMWs into the culture medium.

(a)



(b)

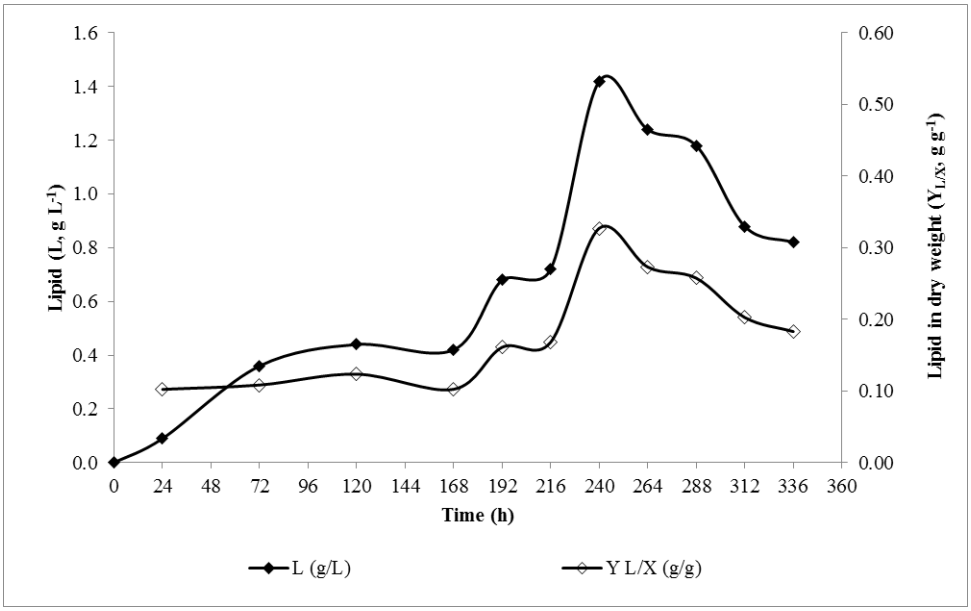


Figure 3. 16. (a) Biomass (X, g L^{-1}), total citric acid (Cit, g L^{-1}) and glucose (Glc, g L^{-1}); (b) cellular lipids (L, g L^{-1}) and total cellular lipid in dry weight ($Y_{L/X}$, g g^{-1}) evolution during growth of *Yarrowia lipolytica* strain ACA-YC 5033 on OMW-based media (initial phenolic compounds concentration 2.90±0.25 g L^{-1}) enriched with commercial glucose in nitrogen-limited conditions. Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, Glc_0~80.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% *v/v*, incubation temperature *T*=28 ºC. Each point is the mean value of two independent measurements.

3.2.3. Citric acid production

In nitrogen-limited experiments, *Y. lipolytica* strain ACA-YC 5033 produced Cit; the total citric acid concentration achieved was 19.0 g L^{-1} (the highest overall Cit_{max} value throughout all experiments of this study for the biotechnological production of citric acid from OMW-based media with initial glucose into the medium at ~35 g L^{-1}) with simultaneous conversion yield of citrate produced per glucose consumed $Y_{Cit/Glc}$=0.74 g g^{-1}, in the sterile shake-flask experiment with initial phenolic compounds concentration 2.90±0.25 g L^{-1}. Cit_{max} concentration was insignificantly reduced with the addition of OMW into the medium for the trial with initial phenolic compounds concentration 2.00±0.20 g L^{-1}. Concerning the strain ACA-YC 5033, it should be highlighted that citric acid production remained almost unaffected (by means of Cit_{max} values) with the addition of OMWs into the culture medium compared to blank experiments (Tables 3.1. and 3.7.), while comparison between the aseptic trial with the one performed into the pasteurized medium revealed some reduction of citric acid production in the latter by both means of Cit_{max} and $Y_{Cit/Glc}$ values (Table 3.7.).

Comparing aseptic shake-flask and aseptic batch bioreactor fermentations of OMW-based media that presented almost equal initial Glc concentrations (~35.0 g L^{-1}) and phenolic compounds (2.90±0.25 g L^{-1}), one could conclude that citric acid production seems to decrease in the bioreactor cultures [by both means of Cit_{max} and $Y_{Cit/Glc}$ values; Table 3.7.; Fig. 3.17.]. Finally, it should be noted that comparing aseptic with pasteurized batch bioreactor fermentations, citric acid production decreased insignificantly in pasteurized trials (Table 3.7.).

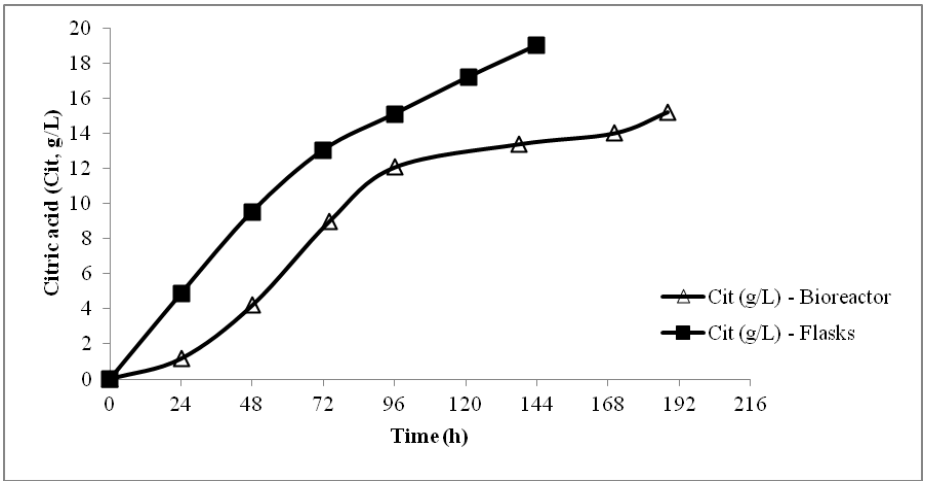


Figure 3. 17. Comparison of *Yarrowia lipolytica* strain ACA-YC 5033 kinetics between aseptic shake-flask and aseptic batch-bioreactor cultures regarding citric acid (Cit, g L^{-1}) evolution on OMW-based media enriched with commercial glucose with initial phenolic compounds concentration 2.90±0.25 g L^{-1}. Culture conditions: growth on aseptic shake-flask 250-mL cultures agitated at 180 ± 5 rpm, Glc_0~35.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% *v/v*, incubation temperature *T*=28 ºC; aseptic batch bioreactor cultures agitated at 300 rpm, Glc_0~35.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% *v/v*, incubation temperature *T*=28 ºC, and sparge of air 1.0 vvm. Each point is the mean value of two independent measurements.

Regarding aseptic shake-flask fermentations with initial phenolic compounds concentration 2.90±0.25 g L^{-1} and Glc_0~80.0 g L^{-1}, Cit_{max} quantities up to 47.1 g L^{-1} (overall highest value throughout nitrogen-limited experiments of all strains), corresponding to $Y_{Cit/Glc}$ values up to 0.67 g g^{-1} were obtained [Table 3.7.; Fig. 3.16. (a)]. Comparing with the respective trial (initial phenolic compounds concentration 2.90±0.25 g L^{-1} and Glc_0~35.0 g L^{-1}), only in terms of citric acid yield on glucose consumed, the addition of glucose into the medium led to insignificantly lower $Y_{Cit/Glc}$ values (Table 3.7.). This is quite suprising as result, since the production of citric acid is a secondary anabolic action and, theoretically, with a constant initial nitrogen availability into the medium, the more glucose concentration increases the more citric acid quantity (in both absolute and relative values) should increase (Papanikolaou and Aggelis, 2009; Papanikolau et al., 2002b; 2013). Potentially, when Glc_0~80.0 g L^{-1}, the carbon flow was directed more efficiently towards

lipid and biomass formation ($Y_{L/X}$=0.33 g g^{-1} against 0.27 g g^{-1}, L_{max}=1.4 g L^{-1} against 1.0 g L^{-1}, X_{max}=4.6 g L^{-1} against 3.7 g L^{-1}).

In the sterile shake-flask trials with initial phenolic compounds concentration 4.50±0.35 g L^{-1} and 5.50±0.40 g L^{-1}, the high phenol content, as expected, seriously inhibited the growth of the microorganism. The production of citric acid (by means of Cit_{max}) reached in both trials the values of ~4.0 g L^{-1} and of ~2.0 g L^{-1} respectively. Finally, in the carbon-limited fermentations, as expected, insignificant citric acid quantities were produced (Cit<1.5 g L^{-1}) as the kinetics of ammonium nitrogen uptake and citric acid accumulation into the medium, showed that the onset of citric acid production occurred only after complete NH_4^+ exhaustion from the medium (see also: Anastassiadis *et al.* 2002; Papanikolaou *et al.* 2008a; Papanikolaou and Aggelis, 2009), that was not reported in the carbon-limited trials (at the end of growth and after complete glucose removal of the medium, a significant quantity of NH_4^+ ions remained into the medium - data not presented).

3.2.4. Decolorization - removal of phenolic compounds

Significant color removal was performed in both nitrogen and carbon-limited fermentations. The overall maximum decolorization achieved was within the range 36-58%. The decolorization process seemed to insignificantly decrease by the addition of waste excluding the sterile batch bioreactor trial and the experiments with initial phenolic compounds 4.50±0.35 g L^{-1} and 5.50±0.40 g L^{-1}, where was clearly decreased (Table 3.8.). On the other hand, the overall maximum reduction of phenolic compounds ranged between 5 and 51% *w/w*. In nitrogen-limited experiments, the maximum decolorization achieved was 55.9% (with initial phenolic compounds 2.00±0.20 g L^{-1}) and the maximum reduction in phenol compounds obtained was, surprisingly enough (as serious inhibited growth was observed), 50.9% *w/w* and 29.1% *w/w* (at initial phenolic compounds 5.50±0.40 g L^{-1} and 4.50±0.35 g L^{-1}, respectively). Thus, the removal of phenolic compounds increased by the addition of the waste into the medium, especially in the trials with initial phenolic compounds 4.50±0.35 g L^{-1} and 5.50±0.40 g L^{-1} (Table 3.8.). Besides the total phenol values reduction of the medium due to their possible adsorption into yeast cell surface, such a surprising result (as serious growth inhibition and therefore low biomass values observed) could strongly suggest the partial utilization of phenolic compounds as carbon source by the microorganism. In fact, such high removal of phenolic compounds from the growth medium due to yeast fermentation is a rather surprising and original result, since it is known that natural yeast strains (like *Y. lipolytica*) lack in the synthesis of phenol-degrading enzymes (like laccases, lignin peroxidases etc.). Only in a very limited number of reports, significant reduction of phenolic compounds has been observed in cultures performed by yeast species (Chtourou *et al.*, 2004; D'Annibale *et al.*,

2006), with phenolic compounds removal of ~51% *w/w*, being one of the best values reported in the international literature.

In carbon-limited fermentations, the maximum decolorization achieved was 58.1% whereas the maximum reduction in phenolic compounds was 13.8% *w/w* (at initial phenolic compounds ~2.90 g L^{-1}), lower compared to nitrogen-limited trials. The kinetics of color and phenolic compounds removal from the culture medium in one case (pasteurized shake-flask fermentation with initial phenolic compounds concentration 2.90±0.25 g L^{-1}) is shown in Fig. 3.18..

Table 3. 8. Data of *Yarrowia lipolytica* strains ACA-YC 5033 concerning removal of phenol compounds and color obtained from kinetics in media (sterile and pasteurized shake-flask cultures and sterile batch bioreactor cultures) containing commercial glucose and various initial O.M.W. concentrations. Representation of initial and final phenol compounds concentration in the culture medium, phenol compounds removal (% *w/w*) and color removal (%) from the medium in nitrogen-limited (a) and carbon-limited (b) experiments. Each point is the mean value of two independent measurements.

	Initial Phenolics (g L^{-1})	Final Phenolics (g L^{-1})	Phenolic Compounds Reduction (% *w/w*)	Color Removal (%)
a	2.10±0.20	1.83±0.20	12.9±1.0	55.9±4.0
	2.82±0.25	2.31±0.20	17.9±1.0	55.7±4.0
	2.83±0.25 Pasteurised	2.36±0.20	16.7±1.0	54.9±4.0
	3.05±0.25 Glc_0~80.0 g L^{-1}	2.53±0.20	17.1±1.0	56.7±4.0
	2.96±0.25 Bioreactor	2.50±0.20	15.4±1.0	35.6±3.5
	3.04±0.25 Bioreactor Pasteurized	2.51±0.20	17.4±1.0	37.8±3.5
	4.51±0.35	3.20±0.30	29.0±1.5	46.5±4.0
	5.47±0.40	2.68±0.25	50.9±2.5	45.3±4.0
b	2.10±0.20	2.00±0.20	4.8±0.5	58.1±4.5
	2.90±0.25	2.50±0.20	13.8±1.0	55.3±4.0

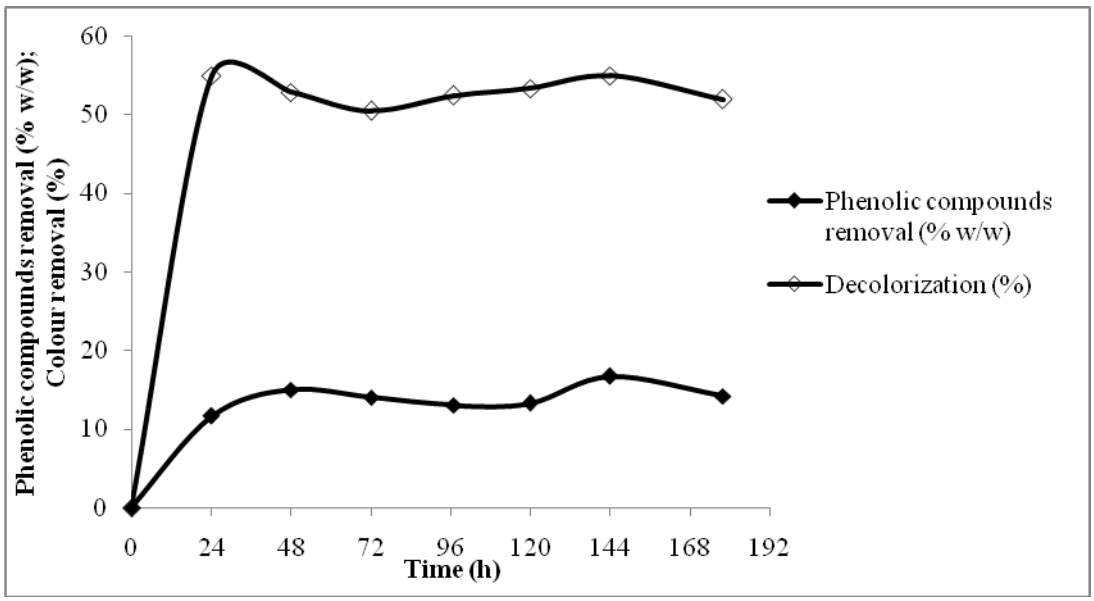


Figure 3. 18. Phenolic compounds removal (% *w/w*) and color removal (%) during growth of *Yarrowia lipolytica* strain ACA-YC 5033 on pasteurized [medium in 80 ºC for 5 min, inoculated with 3mL (6% inoculum)] OMW-based media enriched with commercial glucose, with initial phenolic compounds concentration 2.90±0.25 g L^{-1} in nitrogen limited media. Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, Glc_0~35.0 g L^{-1}, $(NH_4)_2SO_4$=0.5 g L^{-1}, yeast extract=0.5 g L^{-1}, initial pH=6.0 ± 0.1, pH ranging between 5.0 and 6.0, DOT>20% *v/v*, incubation temperature *T*=28 ºC. Each point is the mean value of two independent measurements.

3.2.5. Fatty acid composition analysis

Fatty acid composition of intra-cellular lipids was analyzed in all trials at various growth phases (data not presented). The fatty acid composition for the various strains presented similarities irrespective of the presence or absence of nitrogen from the culture medium. The principal FAs detected were these with C16 and C18 aliphatic chains. The fatty acid composition presented not significant changes with fermentation time and the addition of OMWs into the medium.

3.3. Ethanol, biomass and cellular lipid production by *Saccharomyces cerevisiae* strain MAK-1 cultivated on olive mill wastewater-based media

3.3.1. General presentation

In the third and fourth part of the manuscript, it was desirable to study aspects related with the production of bioethanol by microbial fermentations of OMW-based media. The microorganism used in the conversions, *Saccharomyces cerevisiae* strain MAK-1, has been previously studied upon simultaneous remediation-detoxification and bio-ethanol production bioprocess. In fact, the strain was cultivated on pasteurized grape must and the fungicide quinoxyfen was added in various concentrations; significant quantities of biomass were produced regardless of the addition of fungicide to the medium, ethanol was synthesized in very high quantities and the fungicide concentration was remarkably reduced (Sarris *et al.*, 2009).

In the present part, fermentations were carried out under aseptic and non-aseptic conditions in 250-mL Erlenmeyer flasks as also in laboratory scale bioreactor (total volume 3.5 L; working volume 3.0 L) by *Saccharomyces cerevisiae* strain MAK-1. Concerning shake-flasks experiments, to investigate the biochemical response of *S. cerevisiae* strain MAK-1 grown on OMW-based media, fermentations in aseptic (axenic) and non-aseptic shake-flask cultures were carried out. OMWs and glucose were added, and media presenting various initial concentrations of phenolic compounds and Glc were created (see Table 3.9.). Initial phenolic compounds concentration of 0.00 g L^{-1} corresponded to the control experiment (without OMW addition), whereas two non-sterilized trials were also performed (Glc_0~40.0 g L^{-1} with initial phenolic compounds concentration at 0.00 g L^{-1} and Glc_0~75.0 g L^{-1} with initial phenolic compounds concentration at 2.90±0.25 g L^{-1}). Maximum Glc concentration selected (~75.0 g L^{-1}), as previously stressed, corresponds to initial glucose quantity that can usually be found in OMWs that derive from press extraction systems (Crognale *et al.*, 2006; Papanikolaou *et al.*, 2008a). Equally, the initial concentrations of phenolic compounds correspond to quantities that can be found in typical OMWs (for state-of-the-art review see: Crognale *et al.* 2006). Dry cell weight (X, g L^{-1}) and ethanol (EtOH, g L^{-1}) produced and assimilated sugars (Glc_{cons}, g L^{-1}) as well as the yields of ethanol produced per sugar consumed ($Y_{EtOH/Glc}$, g g^{-1}) and total lipid in dry cell weight ($Y_{L/X}$, g g^{-1}) were quantified for all trials and are presented in Table 3.9.. Regarding bioreactor experiments, fermentations were carried out under aseptic and non-aseptic conditions. Initially, no phenolic compounds were added into the growth medium (control experiment without OMW addition) and the influence of sterilization or non-sterilization on the bioprocess was quantified (Table 3.10.). Thereafter, the impact of the aeration rate on the fermentation efficiency was studied, and finally OMWs were added to the medium to

yield initial phenolic compounds concentration of 2.80±0.25 g L^{-1} in order to study the impact of OMWs addition on the conversion (see Table 3.10.). Glucose was added into the media giving an initial glucose concentration of ~75.0 g L^{-1}.

Table 3. 9. Experimental data of *Saccharomyces cerevisiae* strain MAK-1 originated from kinetics on media presenting various initial concentrations of glucose blended with OMWs added in different concentrations. Representations of total biomass (X, g L^{-1}), ethanol (EtOH, g L^{-1}), total cellular lipid (L, g L^{-1}) and consumed substrate (Glc_{cons}, g L^{-1}) concentrations at different fermentation points of each trial:

Initial Phenolics (g L^{-1})	Glc_0 (g L^{-1})	Fermentation time (h)	X (g L^{-1})	EtOH (g L^{-1})	L (g L^{-1})	Glc_{cons} (g L^{-1})	$Y_{EtOH/Glc}$ (g g^{-1})	$Y_{L/X}$ (g g^{-1})
0.00	40.0	24[b]	3.5±0.4	13.4±1.0	0.2±0.1	40.0±3.5	0.34	0.06
		132[a]	7.2±0.6	0.0	0.6±0.1	43.2±3.5	–	0.09
	55.0	20[b]	3.5±0.4	15.7±1.5	0.2±0.1	52.8±4.0	0.30	0.05
		132[a]	10.7±0.8	4.1±0.5	0.2±0.1	55.2±4.0	0.07	0.02
	75.0	36[b]	6.3±0.5	19.5±1.5	0.2±0.1	67.4±4.5	0.29	0.03
		136[a]	11.2±0.8	9.3±1.0	0.1±0.1	67.4±4.5	0.14	0.01
	40.0 Non-aseptic	28[b]	4.2±0.4	12.9±1.0	–	43.2±3.5	0.30	ND
		132[a]	7.2±0.6	0.0	–	43.2±3.5	–	ND
1.20±0.10	40.0	16[b]	5.3±0.4	15.6±1.5	0.2±0.1	37.4±3.5	0.42	0.04
		132[a]	12.0±0.8	0.0	0.2±0.1	39.6±3.5	–	0.02
	55.0	16[b]	6.8±0.5	19.3±1.5	0.2±0.1	52.7±4.0	0.37	0.03
		120[a]	15.8±0.9	0.0	0.2±0.1	55.4±4.0	–	0.01
	75.0	16[b]	7.8±0.6	21.7±1.5	0.2±0.1	66.8±4.5	0.32	0.03
		132[a]	16.0±0.9	1.3±0.5	0.2±0.1	68.5±4.5	0.02	0.01
2.00±0.20	40.0	16[b]	5.3±0.4	17.2±1.5	0.2±0.1	36.4±3.5	0.47	0.04
		108[a]	13.2±0.8	0.0	0.2±0.1	39.5±3.5	–	0.02
	55.0	16[b]	6.3±0.5	19.8±1.5	0.2±0.1	48.4±3.5	0.41	0.03
		132[a]	17.3±0.9	0.0	0.2±0.1	51.3±4.0	–	0.01
	75.0	20[b]	7.9±0.6	22.8±1.5	0.2±0.1	69.3±4.5	0.33	0.03
		132[a]	17.7±0.9	3.9±0.5	0.2±0.1	69.3±4.5	0.06	0.01
2.90±0.25	40.0	16[b]	6.8±0.5	15.2±1.5	0.4±0.1	38.3±3.5	0.40	0.06
		132[a]	15.0±0.8	2.8±0.5	0.2±0.1	43.9±3.5	0.06	0.02
	55.0	16[b]	8.1±0.6	17.8±1.5	0.3±0.1	50.1±4.0	0.36	0.04
		132[a]	18.2±0.9	0.0	0.2±0.1	51.9±4.0	–	0.01
	75.0	16[b]	8.2±0.6	26.1±1.5	0.4±0.1	73.1±4.5	0.36	0.05
		132[a]	18.7±1.0	0.0	0.2±0.1	76.8±4.5	–	0.01
	75.0 Non-aseptic	16[b]	8.8±0.6	25.8±1.5	–	73.8±4.5	0.35	ND
		132[a]	18.9±1.0	3.9±0.5	–	76.8±4.5	0.06	ND

[a]when X_{max} concentration was achieved; [b]when $EtOH_{max}$ concentration was achieved; ND: Not determined; Fermentation time, conversion yield of ethanol produced per glucose consumed ($Y_{EtOH/Glc}$, g g^{-1}) and total lipid in dry biomass ($Y_{L/X}$, g g^{-1}) are presented for all points of the trials (exception for the representation of lipids in the non-aseptic trials). Culture conditions: growth on 250-mL aseptic and non-aseptic shake flasks at 180 ± 5 rpm, Glc_0 (in g L^{-1}) ~40.0, ~55.0 and ~75.0 g L^{-1}, initial pH=3.5 ± 0.1, incubation temperature *T*=28 °C. Each point is the mean value of two independent measurements.

Table 3. 10. Experimental data of *Saccharomyces cerevisiae* strain MAK-1 originated from kinetics during growth on glucose-based media (blank experiments in various aeration conditions) and glucose enriched OMW-based media under aseptic and non-aseptic batch bioreactor fermentations.

Representations of biomass concentration (X, g L^{-1}) when maximum ethanol ($EtOH_{max}$, g L^{-1}) concentrations were achieved at the different trials:

	Initial phenolics (g L^{-1})	Glc_0 (g L^{-1})	Aeration (vvm)	X (g L^{-1})	$EtOH_{max}$ (g L^{-1})	$Y_{EtOH/Glc}$ (g g^{-1})
Aseptic	0.00	~75.0	1.5	4.9±0.4	33.6±2.0	0.44
Non-aseptic	0.00	~75.0	1.5	5.2±0.4	33.8±2.0	0.45
			1.0	5.1±0.4	33.7±2.0	0.42
			0.5	4.8±0.4	34.2±2.0	0.45
			0.0	4.8±0.4	33.9±2.0	0.45
	2.80±0.25	~75.0	0.0	8.3±0.6	33.1±2.0	0.45
		~115.0		8.9±0.6	52.0±3.0	0.46

Conversion yields of biomass produced per glucose consumed ($Y_{X/Glc}$, g g^{-1}) and ethanol produced per glucose consumed ($Y_{EtOH/Glc}$, g g^{-1}) are also presented when $EtOH_{max}$ concentration was achieved. Culture conditions: growth in batch bioreactor experiments, 300 rpm, initial pH=3.50 ± 0.02 and incubation temperature *T*=28 °C. Each point is the mean value of two independent measurements.

3.3.2. Kinetics of *Saccharomyces cerevisiae* strain MAK-1 grown in shake-flask cultures

Regarding aseptic shake-flask trials, although OMWs contained compounds that could provoke biomass inhibition (e.g. phenolic compounds), dry cell weight production achieved, surprisingly, clearly increased with OMWs addition into the medium (Fig. 3.19.); for all of the Glc_0 concentrations tested, while ANOVA test indicated that X_{max} concentrations increased significantly with the rise of phenolic compounds quantities, in the range of 2.00±0.20–2.90±0.25 g L^{-1} (Table 3.11.). For the aseptic trials, the highest X_{max} value achieved was in media with initial phenolic compounds concentration 2.90±2.50 g L^{-1} and Glc_0~75.0 g L^{-1}, being 18.7 g L^{-1}, while the X_{max} value for the control experiment (Glc_0~75.0 g L^{-1} without OMW addition) was only 11.3 g L^{-1} (Table 3.9.). Moreover, as it was expected, for cultures with varying initial phenolic compounds concentration, statistical analysis revealed significant biomass increase in the higher range of Glc_0 concentration (55-75 g L^{-1}) (Table 3.11.). In all trials, glucose was totally and very rapidly consumed (~20 h after inoculation), since the cellular metabolism was shifted towards the synthesis of ethanol despite the remarkable presence of oxygen in the medium (due to the Crabtree effect) (Ratledge, 1991; Αγγελής, 2007).

After glucose assimilation, *S. cerevisiae* consumed previously accumulated ethanol, and new biomass formation was observed ("diauxic growth") (Fig. 3.20.). In several cases, complete

ethanol re-consumption was observed; this is evident in Table 3.9., as when X_{max} concentration was recorded, the respective ethanol value was 0.0 g L^{-1} (it is evident that in a potential process scale-up, fermentation should stop ~25-30 h after inoculation, in order not to lose the valuable ethanol). A representative kinetics is shown in Fig. 3.20. (strain grown on OMW-based media with initial phenol content at 2.00±2.0 g L^{-1} and Glc_0~40.0 g L^{-1}).

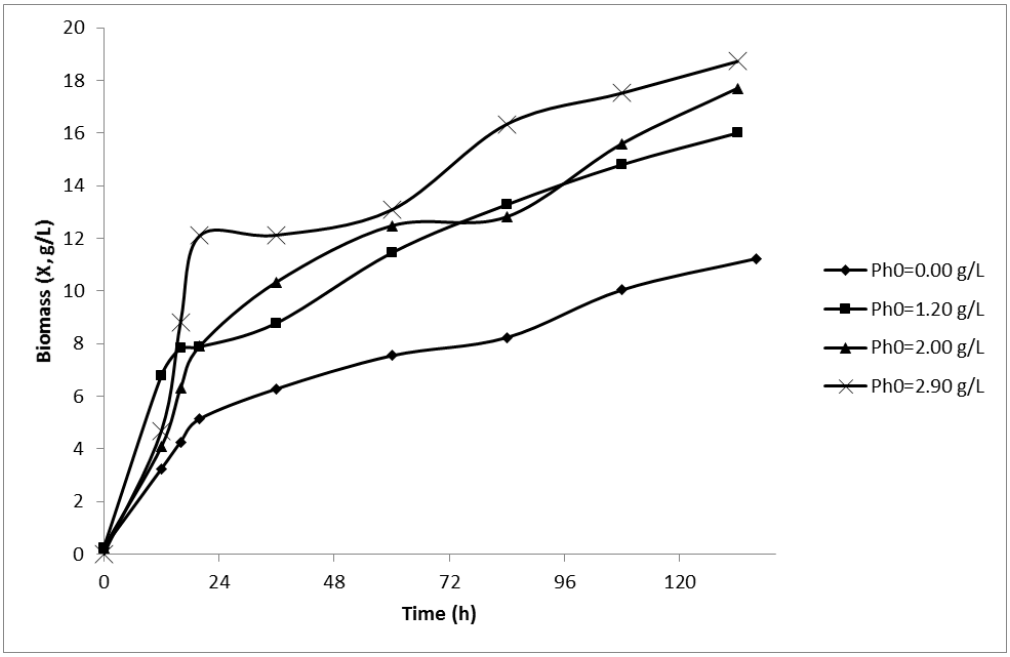


Figure 3. 19. Biomass (X, g L^{-1}) evolution during growth of *Saccharomyces cerevisiae* strain MAK-1 on OMW-based media (initial phenolic compounds concentration 0.00 g L^{-1} (no OMW addition), 1.20±0.10 g L^{-1}, 2.00±0.20 g L^{-1} and 2.90±0.25 g L^{-1}) enriched with commercial glucose. Culture conditions: growth on 250-mL aseptic flasks at 180 ± 5 rpm, Glc_0~75.0 g L^{-1}, initial pH=3.5 ± 0.1, incubation temperature *T*=28 °C. Each point is the mean value of two independent measurements.

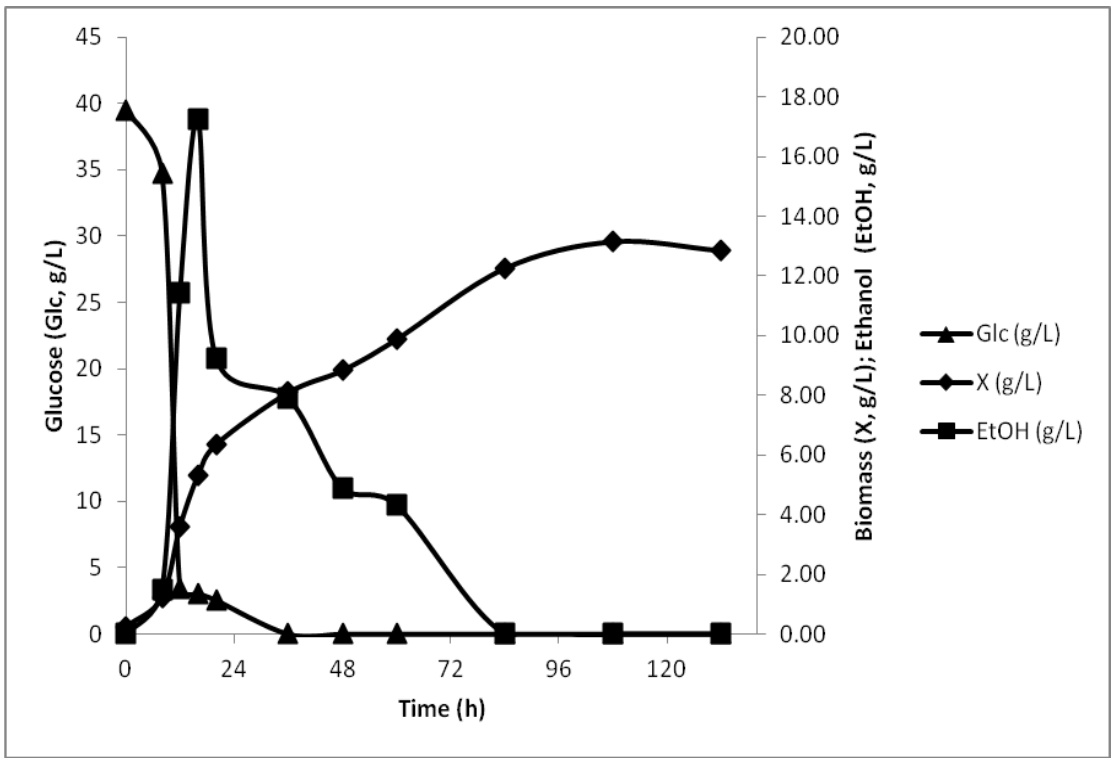


Figure 3. 20. Biomass (X, g L^{-1}), ethanol (EtOH, g L^{-1}) and glucose (Glc, g L^{-1}) evolution during growth of *Saccharomyces cerevisiae* strain MAK-1 on OMW-based media (initial phenolic compounds concentration 2.00±0.20 g L^{-1}) enriched with commercial glucose. Culture conditions: growth on 250-mL aseptic flasks at 180 ± 5 rpm, Glc_0~40.0 g L^{-1}, initial pH=3.5 ± 0.1, incubation temperature *T*=28 °C. Each point is the mean value of two independent measurements.

In order to enhance ethanol production so as to valorize OMWs as process water in such fermentations, commercial glucose was added into the medium in various amounts. The $EtOH_{max}$ concentration achieved for axenic flask cultures was 26.1 g L^{-1} (yield of ethanol produced per glucose consumed – $Y_{EtOH/Glc}$ at 0.36 g g^{-1}), during growth of the microorganism on the trial with initial phenolic compounds concentration 2.90±0.25 g L^{-1} and Glc_0~75.0 g L^{-1}. The maximum $Y_{EtOH/Glc}$ value achieved was 0.47 g g^{-1} ($EtOH_{max}$=17.2 g L^{-1}) and was obtained in the fermentation with initial phenolic compounds concentration 2.00±0.20 g L^{-1} and Glc_0 at ~40.0 g L^{-1} (Table 3.9.). Taking into consideration the yield $Y_{EtOH/Glc}$ for all Glc_0 concentrations tested, statistically significantly higher ethanol yield occurred in the media with initial phenolic compounds adjusted at 2.00±0.20 g L^{-1} (Table 3.11.). Further addition of OMWs into the culture medium, which resulted in the presence of initial phenolics at 2.90±0.25 g L^{-1}, somehow lowered the maximum value of yield $Y_{EtOH/Glc}$. In any case, it must be stressed that despite the presence of inhibitors (e.g. phenolic compounds) in the medium, the addition of OMWs up to a specific level seemed to enhance the production of ethanol (Tables 3.9. and 3.11.; Fig. 3.21.).

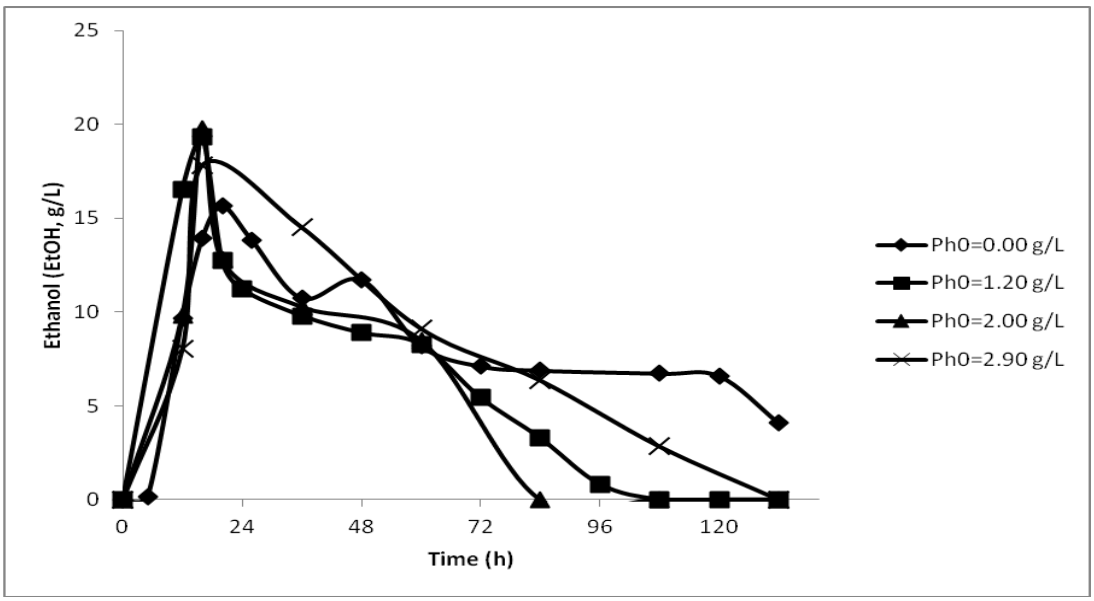


Figure 3. 21. Ethanol (X, g L^{-1}) evolution during growth of *Saccharomyces cerevisiae* strain MAK-1 on OMW-based media (initial phenolic compounds concentration 0.00 g L^{-1} (no OMW addition), 1.20±0.10 g L^{-1}, 2.00±0.20 g L^{-1} and 2.90±0.20 g L^{-1}) enriched with commercial glucose. Culture conditions: growth on 250-mL aseptic flasks at 180 ± 5 rpm, Glc_0~55.0 g L^{-1}, initial pH=3.5 ± 0.1, incubation temperature *T*=28 °C. Each point is the mean value of two independent measurements.

In all aseptic flask trials, as it was expected, maximum ethanol production in absolute values ($EtOH_{max}$=13.4-26.1 g L^{-1}) increased with the addition of glucose into the synthetic medium (Fig. 3.22.). However, ANOVA tests revealed that maximum yield $Y_{EtOH/Glc}$ values (ranging between 0.29-0.47 g g^{-1}, at the point when $EtOH_{max}$ was noted) were statistically significantly reduced when cultures were performed at increasing Glc_0 concentrations, regardless of the addition of OMW into the medium (Table 3.11.), and this was probably due to the fact that when increased Glc_0 concentrations were employed, media were not equally supplemented with nitrogen and cultures were performed at higher initial C/N values in the medium (in several cases, trial were performed under sufficiently nitrogen-limited conditions), which were potentially not adequate for the elaboration of alcoholic fermentation. Finally, the addition of OMW in the medium reduced the time at which $EtOH_{max}$ values were noted, compared with the control experiment. The impact of the different initial glucose and phenolic compounds concentrations upon the maximum values of biomass concentration (X_{max}, g L^{-1}) and ethanol yield per Glc consumed ($Y_{EtOH/Glc}$, g g^{-1}) for all trials are summarized in Fig. 3.23..

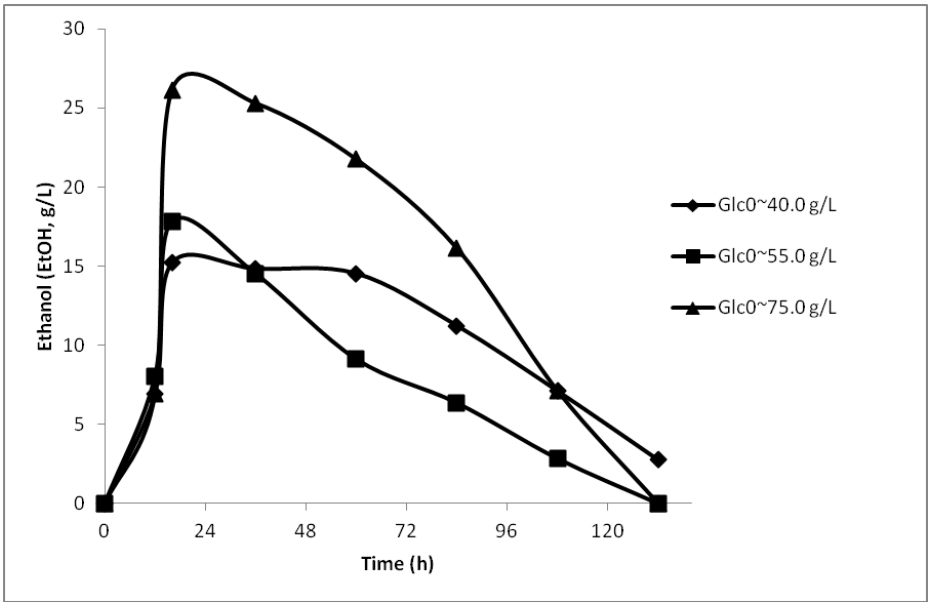


Figure 3. 22. Ethanol (EtOH, g L^{-1}) evolution during growth of *Saccharomyces cerevisiae* strain MAK-1 on OMW-based media enriched with commercial glucose (initial glucose concentration ~40.0 g L^{-1}, ~55.0 g L^{-1} and ~75.0 g L^{-1}). Culture conditions: growth on 250-mL aseptic flasks at 180 ± 5 rpm, initial phenolic compounds concentration 2.90±0.25 g L^{-1}, initial pH=3.5 ± 0.1, incubation temperature *T*=28 °C. Each point is the mean value of two independent measurements.

Table 3. 11. Two-way ANOVA test results for the factors initial phenolic compounds concentration (g L^{-1}) (a) and initial Glc concentration (g L^{-1}) (b) during the growth of *Saccharomyces cerevisiae* strain MAK-1 on various concentrations of glucose-based media (Glc_0 levels: ~40.0, ~55.0 and ~75.0 g L^{-1}) with OMWs addition (levels: 0.00, 1.20±0.10, 2.00±0.20, 2.90±0.25 g L^{-1}). Culture conditions: growth on 250-mL aseptic and non-aseptic shake flasks at 180 ± 5 rpm, Glc_0 ~40.0, ~55.0 and ~75.0 g L^{-1}, initial pH=3.5 ± 0.1, incubation temperature *T*=28 °C. Least Significant Difference (LSD) test at 5% level of probability was used to compare mean values. Mean values of each parameter and factor within the same column not sharing common letters are significantly different (P<0.05).

	X_{max} (g L^{-1})		$Y_{EtOH/Glc}$ (g g^{-1})		$Y_{L/X}$ (g g^{-1})	
Initial phenolics (g L^{-1})	P=0.0000*	LSD	P=0.0007	LSD	P=0.0000	LSD
0.0	9.68	a	0.31	a	0.071	b
1.2	14.60	b	0.37	b	0.029	a
2.0	16.07	c	0.40	c	0.038	a
2.9	17.30	c	0.37	b	0.038	a
Glc_0 (g L^{-1})	P=0.0000	LSD	P=0.0002	LSD	P=0.0006	LSD
40.0	11.85	a	0.41	c	0.053	b
55.0	15.49	b	0.36	b	0.047	b
75.0	15.90	b	0.33	a	0.030	a

*P-values less than 0.05 indicate a statistically significant effect on tested parameters at the 95.0% confidence level

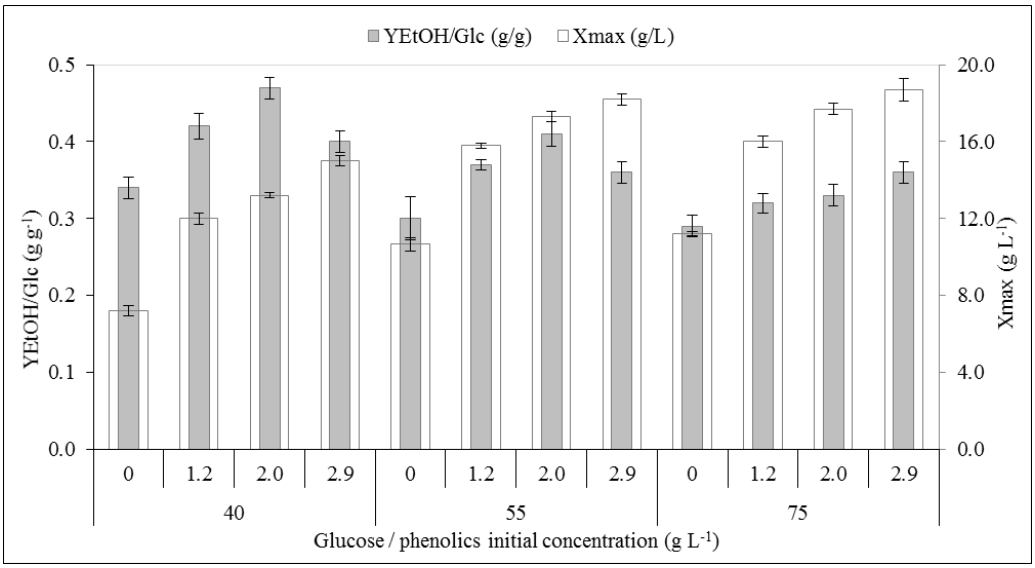


Figure 3. 23. Impact of the initial concentration of glucose (Glc) and phenolic compounds upon the maximum biomass concentration (X_{max}, g L^{-1}) and maximum ethanol yield per glucose consumed ($Y_{EtOH/Glc}$, g g^{-1}) for all shake-flask cultures realized, on media composed of different mixtures of olive-mill wastewaters and glucose. Culture conditions: growth on 250-mL aseptic and non-aseptic shake flasks at 180 ± 5 rpm, Glc_0 (in g L^{-1}) ~40.0, ~55.0 and ~75.0 g L^{-1}, initial pH=3.5±0.1, incubation temperature *T*=28 °C. Each point is the mean value of two independent measurements.

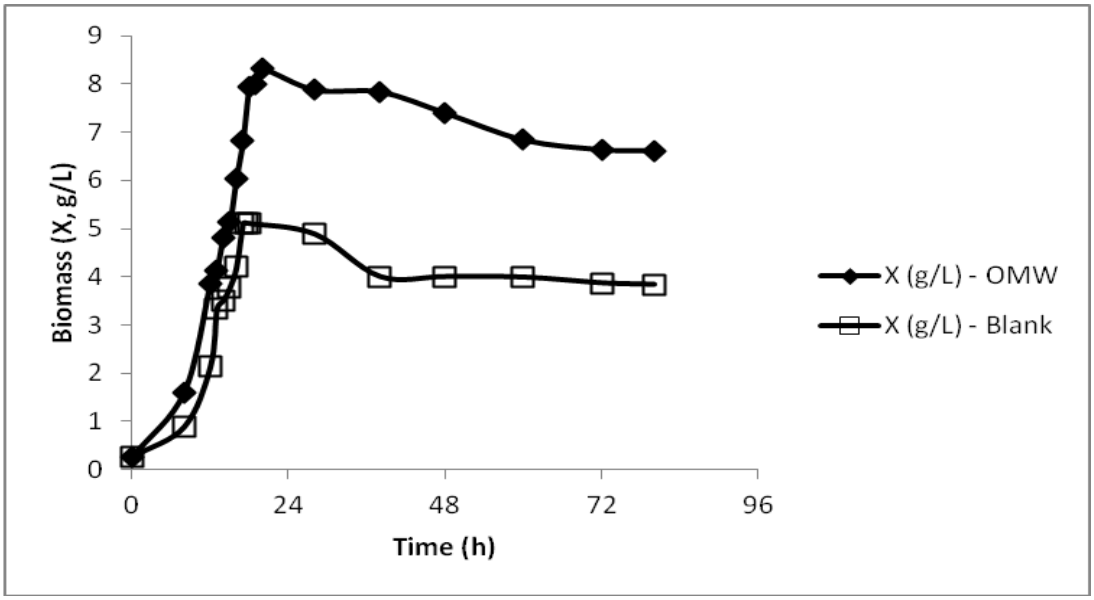
In order to demonstrate the feasibility and the potential of the non-aseptic fermentation process, non-aseptic trials (Glc_0~40.0 g L^{-1} when no OMWs were added into the medium and Glc_0~75.0 g L^{-1} when OMWs were added to yield an initial phenolic compounds concentration in the medium of 2.90±0.25 g L^{-1}) were performed and compared with the respective aseptic experiments in which axenic cultures were used (see Table 3.9.). Indeed, between aseptic and non-aseptic cultures no statistically significant differences were observed for both biomass (by means of X_{max}) and ethanol production (by means of $Y_{EtOH/Glc}$, g g^{-1}) (statistical analysis not shown). During non-aseptic fermentations, samples were checked under the microscope after Gram coloration had been done in order to ensure the purity of the culture. These microscopic observations revealed that only cells of the microorganism *S. cerevisiae* were found in the fermentation medium. In fact, a slight presence of bacteria was found during the initial stages of the culture (0-10 or 15 h after inoculation), but these microorganisms rapidly disappeared due to the secretion of ethanol and lack of nutrients. However, this slight fermentation diversity observed in comparison with the aseptic (axenic) culture, was potentially caused by this (very) little presence of bacteria found in the medium during the early growth steps due to the unsterilized conditions.

3.3.3. Kinetics of *Saccharomyces cerevisiae* strain MAK-1 grown in bioreactor cultures

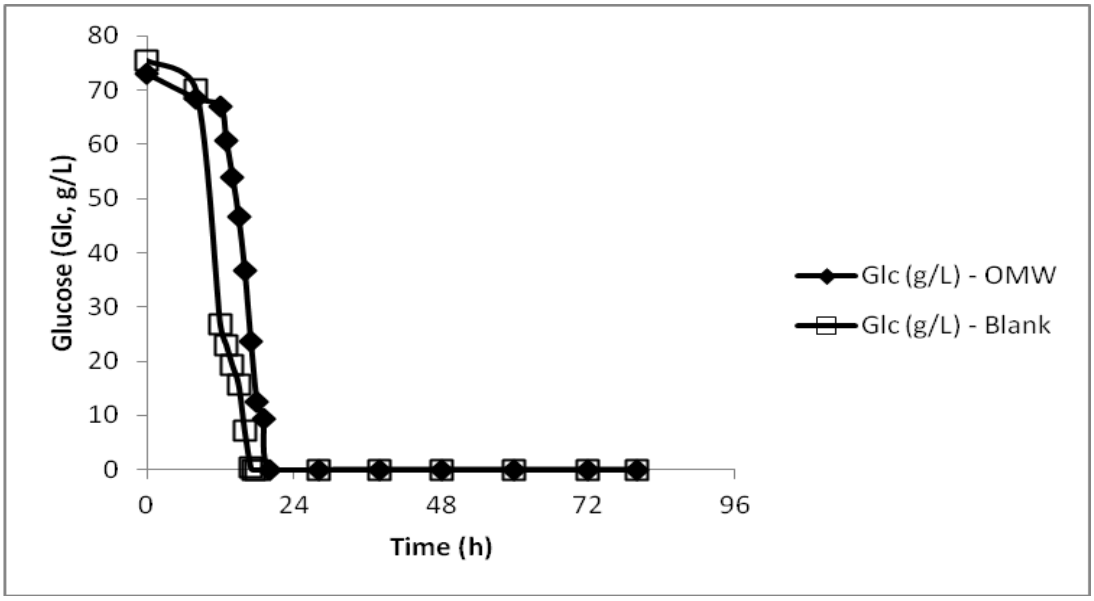
In bioreactor trials with no OMW addition, ANOVA tests demonstrated no significant differences in biomass and ethanol production (by means of maximum concentration and yield of product synthesized per unit of glucose consumed) when comparing aseptic and non-aseptic fermentations (statistical analysis not shown). Likewise, comparing the various aeration regimes (aeration rate imposed at 1.5, 1.0, 0.5 and 0.0 vvm) in the non-aseptic experiments, no remarkable differences were found concerning maximum concentrations of ethanol and biomass achieved. Thus, it may be assumed that glucose-based bioreactor fermentations performed by *S. cerevisiae* MAK-1 under non-aseptic conditions in which no aeration was imposed were ideal concerning bioethanol production by this strain (all these findings are advantageous for a potential scale-up of the process). In non-aseptic bioreactor batch cultures, the addition of OMWs to the synthetic medium seemed, according to the ANOVA test, to significantly enhance the production biomass ($X=8.3\ g\ L^{-1}$) compared with the equivalent experiment in which no OMW addition was performed ($X=4.9\ g\ L^{-1}$, Table 3.10.; statistical analysis not shown).

On the contrary, in non-aseptic bioreactor batch cultures, the addition of OMW into the synthetic medium did not seem to have a statistically significant impact on the conversion of glucose into ethanol compared with the equivalent experiment in which no OMW addition was made (Table 3.10.). As in the shake-flask experiments, in the non-sterilized fermentations, samples were checked under the microscope after Gram coloration and it was revealed that only the microorganism *S. cerevisiae* was found in the fermentation medium. The kinetics of *S. cerevisiae* MAK-1 on bioreactor experiments with either OMW addition or not (“control” experiment) under non-aseptic conditions and no aeration imposed (0 vvm), is presented in Fig. 3.24. (a); (b); (c). Clearly higher dry cell mass values were obtained for the experiment with OMW added to the medium [Fig. 3.24. (a)], while the kinetics of Glc assimilation and ethanol biosynthesis presented similar trends [Fig. 3.24. (b) and (c)].

(a)


Biomass (X, g/L)
0
1
2
3
4
5
6
7
8
9
0
24
48
72
96
Time (h)
X (g/L) - OMW
X (g/L) - Blank

(b)


Glucose (Glc, g/L)
0
10
20
30
40
50
60
70
80
0
24
48
72
96
Time (h)
Glc (g/L) - OMW
Glc (g/L) - Blank

(c)

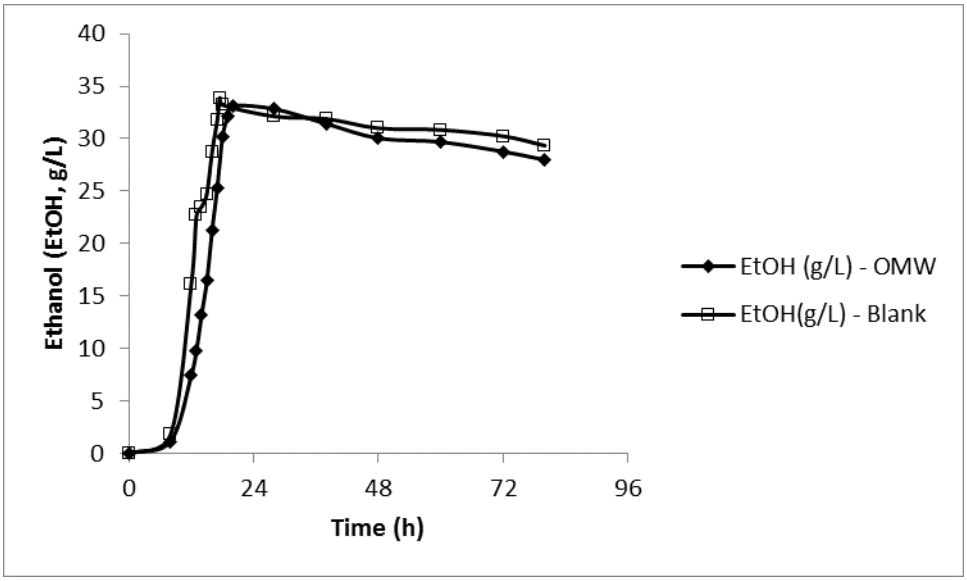
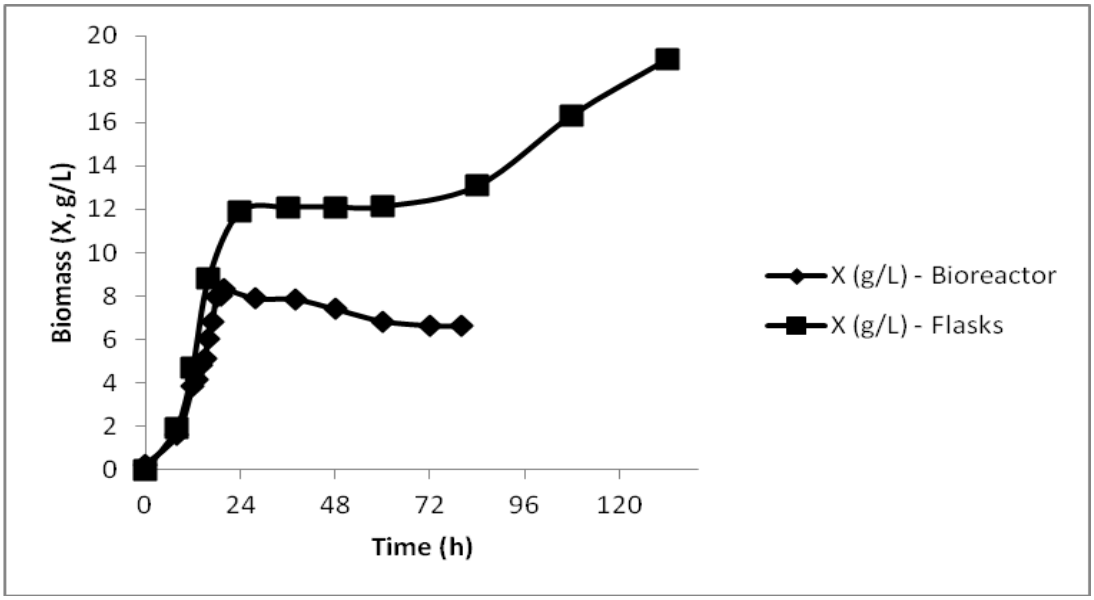


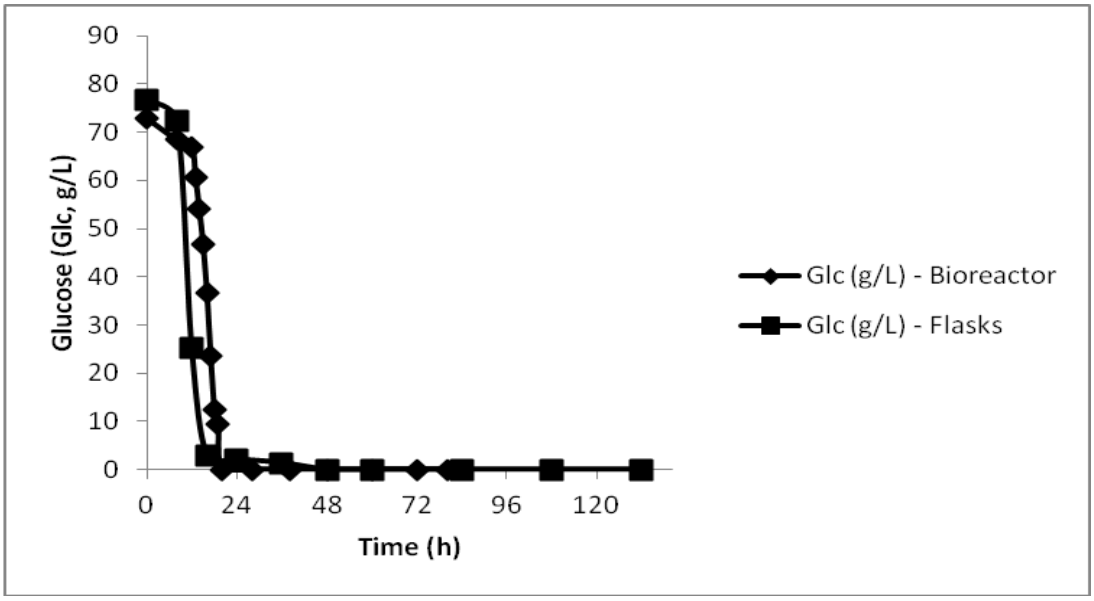
Figure 3. 24. (a) Biomass (X, g L^{-1}), (b) glucose (Glc, g L^{-1}) and (c) ethanol (EtOH, g L^{-1}) evolution during growth of *Saccharomyces cerevisiae* strain MAK-1 on blank (no OMW addition; Glc_0~75.0 g L^{-1}) and OMW-based (initial phenolic compounds concentration 2.80±0.25 g L^{-1}; Glc_0~75.0 g L^{-1}) non-aseptic batch bioreactor cultures enriched with commercial glucose. Culture conditions: agitation at 300 rpm, initial pH=3.50 ± 0.02, incubation temperature *T*=28°C, no aeration imposed. Each point is the mean value of two independent measurements.

As mentioned above, in order to perform a process scale-up for several microbial conversions, an important aspect that is taken into consideration and studied is the comparison and the potential differences in the physiological and kinetic features between experiments performed in shake-flask and batch bioreactor cultures, given that agitation and aeration conditions may be different in these fermentation configurations (Aggelis *et al.*, 2003; Mantzavinos and Kalogerakis, 2005; Crognale *et al.*, 2006). Comparison was performed between the (non-aseptic) batch bioreactor and shake-flask fermentations of OMW-based media that presented almost equal initial Glc concentrations (~75.0 g L^{-1}) and phenolic compounds (2.80±0.25 g L^{-1}) [Fig. 3.25. (a), (b) and (c)].

(a)


Biomass (X, g/L)
20
18
16
14
12
10
8
6
4
2
0
0
24
48
72
96
120
Time (h)
X (g/L) - Bioreactor
X (g/L) - Flasks

(b)


Glucose (Glc, g/L)
90
80
70
60
50
40
30
20
10
0
0
24
48
72
96
120
Time (h)
Glc (g/L) - Bioreactor
Glc (g/L) - Flasks

(c)

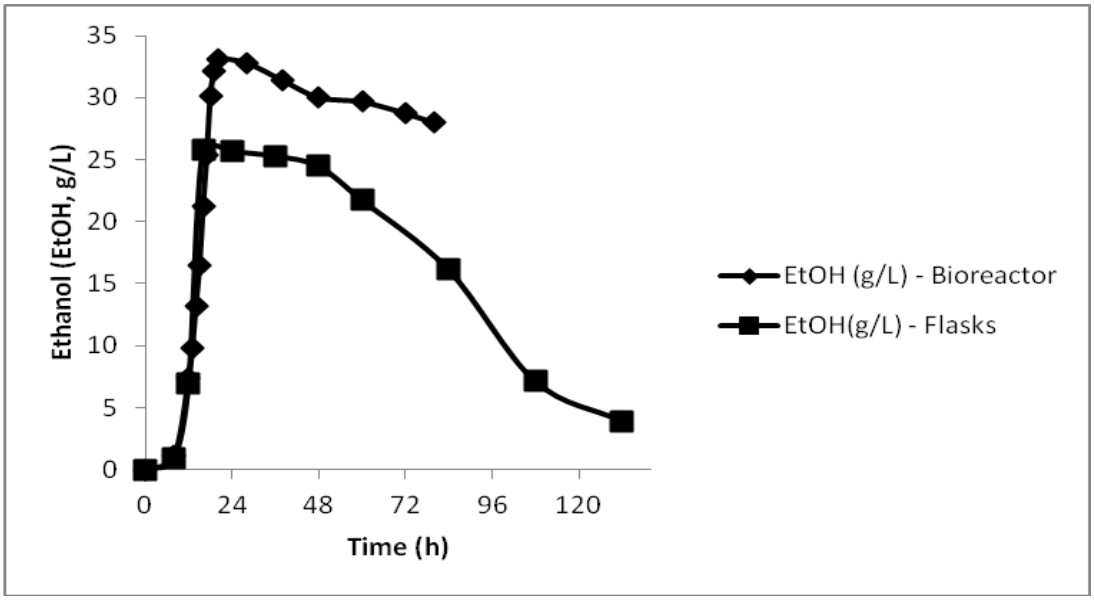


Figure 3. 25. Comparison of *Saccharomyces cerevisiae* strain MAK-1 kinetics between non-aseptic shake-flask and non-aseptic batch-bioreactor cultures regarding (a) biomass (X, g L^{-1}), (b) glucose (Glc, g L^{-1}) and (c) ethanol (EtOH, g L^{-1}) evolution on OMW-based media enriched with commercial glucose. Culture conditions: non-aseptic shake-flask 250-mL cultures agitated at 180 ± 5 rpm, initial phenolic compounds concentration 2.90±0.25 g L^{-1}, Glc_0 at ~75.0 g L^{-1}, initial pH=3.5 ± 0.1, incubation temperature *T*=28 ºC; non-aseptic batch bioreactor cultures agitated at 300 rpm, initial phenolic compounds concentration 2.80±0.25 g L^{-1}, Glc_0 at ~75.0 g L^{-1}, initial pH=3.50 ± 0.02, incubation temperature *T*=28 ºC, no air sparging. Each point is the mean value of two independent measurements.

Dry cell mass production was indeed lower in the bioreactor trial compared with the shake-flasks, while the strain reached its kinetics plateau earlier in bioreactor trial (~18–20 h) than in the shake-flask culture [Fig. 3.25. (a)]. In both trials, virtually no glucose remained unconsumed at the end of the fermentation, while the assimilation rate of glucose was similar in both the shake-flask and the bioreactor experiment [Fig. 3.25. (b)]. Ethanol production was clearly enhanced in the bioreactor cultures, while after total depletion of glucose in the culture media, the microorganism used the previously produced ethanol as carbon source for further biomass proliferation, leading to non-negligible ethanol concentration reduction [this was seen specifically for the shake-flask experiment in which significant oxygen quantities were found into the medium - Fig. 3.25. (c)]. In order to further increase the maximum ethanol level achieved, a supplementary batch bioreactor non-sterilized trial was performed, in which OMWs were added in a (relatively concentrated) glucose-based medium, and the initial Glc and phenolic compounds concentrations of the fermentation medium were ~115.0 and 2.90±0.25 g L^{-1}, respectively. The kinetics of biomass and ethanol production and Glc consumption is illustrated in Fig. 3.26..

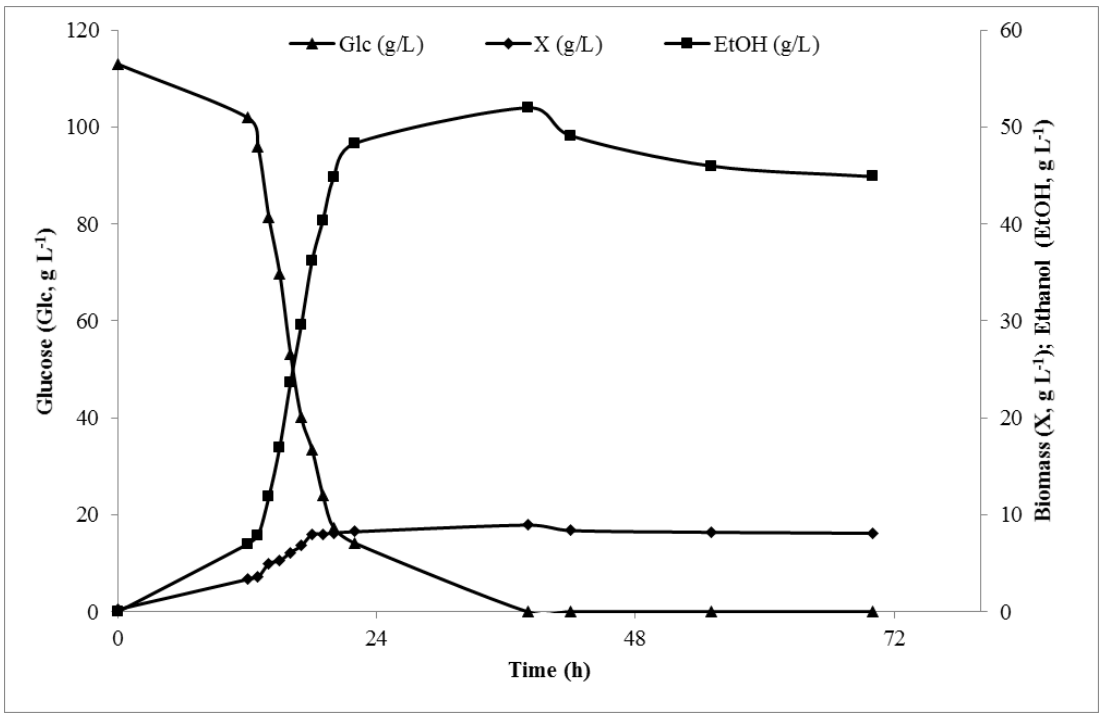


Figure 3. 26. Biomass (X, g L^{-1}), ethanol (EtOH, g L^{-1}) and glucose (Glc, g L^{-1}) evolution during growth of *Saccharomyces cerevisiae* strain MAK-1 on OMW-based media enriched with commercial glucose. Non-aseptic batch bioreactor cultures agitated at 300 rpm, initial phenolic compounds concentration 2.90±0.25 g L^{-1}, Glc_0 at ~115.0 g L^{-1}, initial pH=3.50 ± 0.02, incubation temperature *T*=28 ºC, no air sparging. Each point is the mean value of two independent measurements.

As in all previous fermentations, glucose was rapidly consumed (within ~48 h), although the fermentation was accomplished later compared with the previous trials (Fig. 3.24.) potentially due to the higher initial glucose quantity employed. In any case, an $EtOH_{max}$ concentration of 52.0 g L^{-1} was reported 38 h after inoculation (concomitant $Y_{EtOH/Glc}$=0.46 g g^{-1}), while ethanol concentration presented a slight decrease after sugar consumption from the growth medium. As in the previous bioreactor experiments, ethanol concentration decrease was not followed by biomass concentration rise, the value of which remained almost constant (X~8.5 g L^{-1}).

3.3.4. Decolorization - removal of phenolic compounds

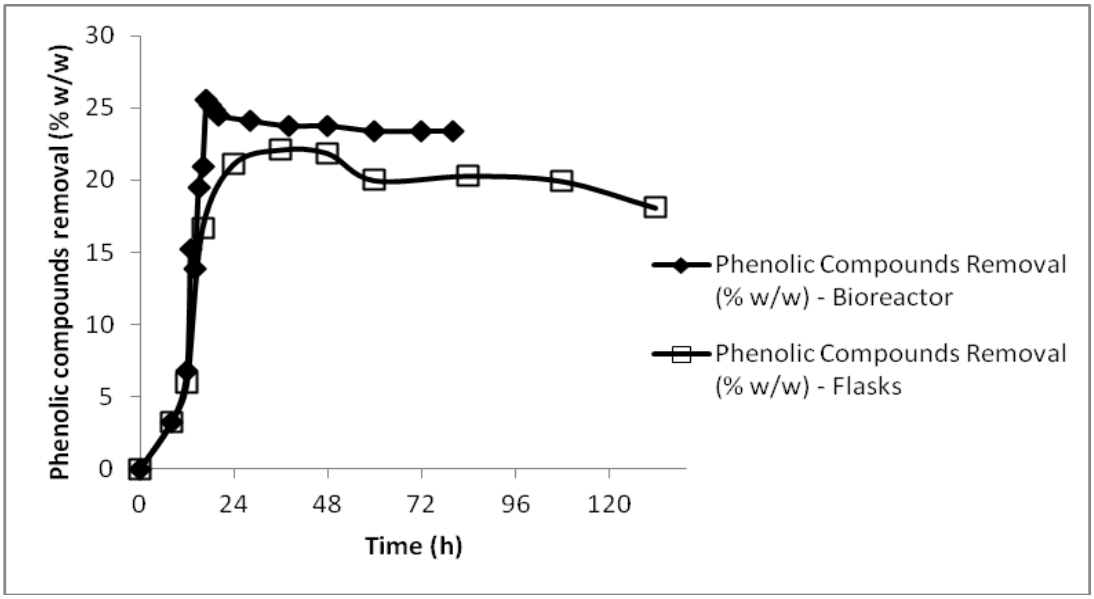
In order to identify whether reduction of phenolic compounds concentration and/or color reduction from the culture medium occurred due to agitation (and, thus, aeration) alone, experiments were carried out in which OMWs and glucose were added to the growth medium, as previously, giving initial phenolic compounds concentration (in g L^{-1}) 1.20±0.10, 2.00±0.20 and 2.90±0.25 and Glc_0~40.0 g L^{-1} but without inoculating the medium (therefore there was no presence of *S. cerevisiae* cells). It was found that the phenolic content of the media showed no reduction at all, whereas the color intensity increased to approximately 5-7%, potentially due to

auto-oxidation of phenolic compounds (data not presented). Therefore neither phenolic compounds nor color were removed from the medium due to the agitation performed (in contrast, as stated, the coloration of the residue slightly increased due to the agitation). Remarkable color removal was performed in both aseptic and unsterilized shake-flask fermentations (Table 3.12.). It appears that for a given Glc_0 concentration, decolorization rate seemed to increase with increased initial phenolic content media, while no correlation can be established between the removal of phenolic compounds from the medium and the initial concentration of phenolics or glucose in the medium. In non-aseptic batch bioreactor fermentations, the maximum decolorization and reduction of phenolic compounds concentration achieved was 59.6% and 27.4% (*w/w*) respectively. Comparing non-aseptic shake-flask and non-aseptic bioreactor cultures that presented similar initial concentrations of sugar (~75.0 g L^{-1}) and phenolic compounds (2.80±0.25 g L^{-1}), one can conclude that decolorization and reduction of phenolic compounds values were similar. Moreover, comparison of bioreactor experiments presenting different initial sugar concentrations (~75.0 and ~115.0 g L^{-1}) and almost equal initial phenolic compounds quantities, demonstrated similar values of decolorization and removal of phenolic compounds. The kinetics of color and phenolic compounds removal from the culture medium (comparison of flask and bioreactor trials) is shown in Fig. 3.27. (a) and (b). From the trends of the kinetics obtained that were similar to all other shake-flask or bioreactor experiments, it can be readily assumed that rapid color and phenolic compounds removal occurred (maximum phenolic compounds removal and decolorization within the first 24-30 h) which coincided with the maximum values of ethanol achieved.

Table 3. 12. Data of *Saccharomyces cerevisiae* strain MAK-1 concerning removal of phenol compounds and color, obtained from kinetics in media (in aseptic and non-aseptic shake-flask cultures and non-aseptic batch bioreactor cultures) containing commercial glucose and various initial OMW concentrations. Representation of initial and final phenol compounds concentration in the culture medium, phenol compounds removal (% *w/w*) and color removal (%) from the medium. Each point is the mean value of two independent measurements.

Glc_0 (g L^{-1})	Initial phenolics (g L^{-1})	Final phenolics (g L^{-1})	Phenol removal (%, *w/w*)	Color removal (%)
~40.0	1.11±0.10	0.86±0.10	22.8±2.0	49.7±4.0
~55.0	1.25±0.10	0.82±0.10	34.4±2.0	55.0±4.0
~75.0	1.13±0.10	0.92±0.10	18.5±1.5	44.5±4.0
~40.0	2.06±0.20	1.57±0.15	23.6±2.0	56.6±4.0
~55.0	1.95±0.20	1.51±0.15	22.9±2.0	61.5±4.5
~75.0	2.01±0.20	1.46±0.10	27.4±2.0	61.5±4.5
~40.0	3.01±0.25	2.34±0.20	22.3±1.5	62.9±4.5
~55.0	2.88±0.25	2.29±0.20	20.5±1.5	60.3±4.5
~75.0	2.97±0.25	2.35±0.20	20.8±1.5	59.9±4.5
~75.0, non-aseptic shake-flask	2.95±0.25	2.30±0.20	22.1±1.5	58.5±4.0
~75.0, non-aseptic bioreactor	2.82±0.25	2.10±0.20	25.5±2.0	59.6±4.5
~115.0, non-aseptic bioreactor	2.89±0.25	2.10±0.20	27.3±2.0	59.5±4.5

(a)



(b)

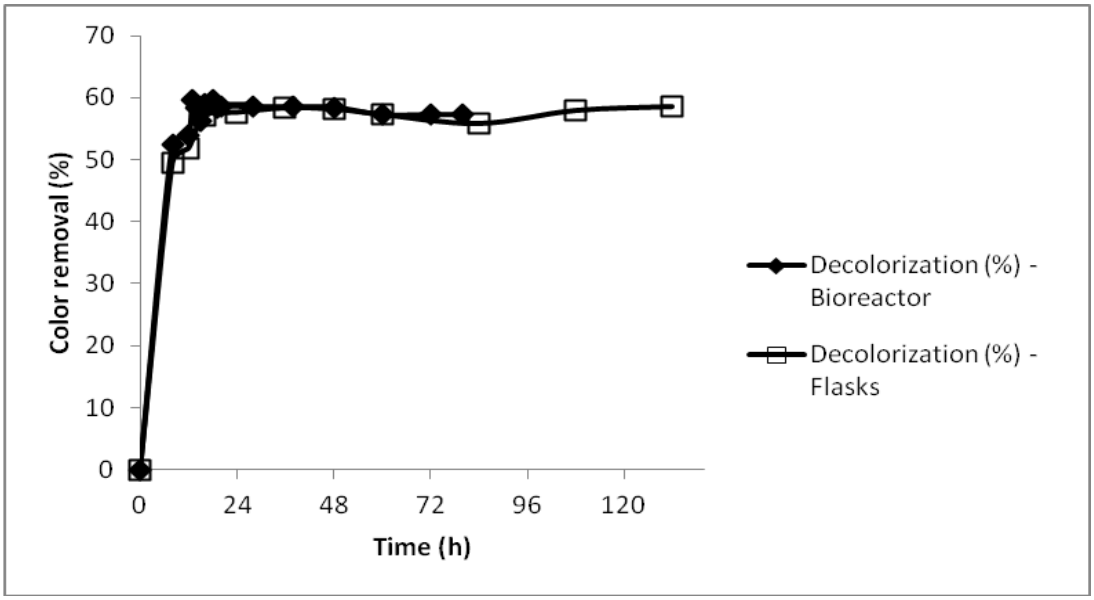


Figure 3. 27. (a) Phenolic compounds removal (% *w/w*) and (b) color removal (%) during growth of *Saccharomyces cerevisiae* strain MAK-1 on OMW-based media enriched with commercial glucose in 250-mL non-aseptic shake-flask (initial phenolic compounds concentration 2.90±0.25 g L^{-1}; Glc_0 at ~75.0 g L^{-1}, 180 ± 5 rpm, initial pH=3.5 ± 0.1, incubation temperature *T*=28 ºC) and non-aseptic batch bioreactor (initial phenolic compounds concentration 2.80±0.25 g L^{-1}; Glc_0 at ~75.0 g L^{-1}, 300 rpm, initial pH=3.50 ± 0.02, incubation temperature *T*=28 ºC, no air sparging) cultures. Each point is the mean value of two independent measurements.

3.3.5. Fatty acid composition analysis

Total cellular lipids were quantified in all growth phases, and quantities ≤10% (*w/w*) in dry matter were found (Table 3.9.), indicating, in full accordance with the literature (Fakas *et al.*, 2009), that no lipid accumulation occurs in *S. cerevisiae* strains, in spite of the fact that in several cases of the present investigation, the increase of Glc_0 concentration rendered the media nitrogen-limited [nitrogen limitation is prerequisite in order for lipid accumulation to occur (Fakas *et al.*, 2009; Papanikolaou and Aggelis, 2011a)]. ANOVA tests showed that significantly lower quantities of lipids per g of dry weight ($Y_{L/X}$) were observed in the media with Glc_0~75.0 g L^{-1}, compared with the trials at Glc_0~55.0 g L^{-1} and Glc_0~40.0 g L^{-1} (Table 3.11.). Likewise, monitoring of lipid produced by the strain suggested that the presence of OMWs in the medium seemed to have effects on the biogenesis of lipids; two-way ANOVA tests revealed that maximum lipid in dry weight $Y_{L/X}$ values were significantly higher in the control experiment (initial phenolic compounds at 0.00 g L^{-1}) compared with the trials in which OMW quantities were added to the medium. On the other hand, maximum $Y_{L/X}$ values (ranging between 0.029 and 0.038 g g^{-1}) were not statistically significantly different, irrespective of the (low or high) initial quantity of phenolic compounds added to the medium (Table 3.11.). The impact of different initial Glc and initial phenolic

compounds concentrations on the maximum values of lipid synthesized per g of dry yeast mass ($Y_{L/X}$, in g g^{-1}) for all trials is summarized in Fig. 3.28.. Indeed, the presence of phenolic compounds in the medium, even in small concentrations, as well as the increasing initial glucose quantities negatively affected the concentration of lipids produced by the microorganism. FA composition of intracellular lipids was analyzed in all trials at various growth phases (Table 3.13.). The principal FAs detected belonged to the C16 and C18 aliphatic chains. The FA composition changed with fermentation time and the addition of OMWs into the medium. Specifically, in the absence of OMWs in the medium, the concentration of stearic acid (C18:0) and oleic acid ($^{\Delta 9}$C18:1) clearly decreased with time since the concentration of palmitoleic ($^{\Delta 9}$C16:1) increased (Table 3.10.).

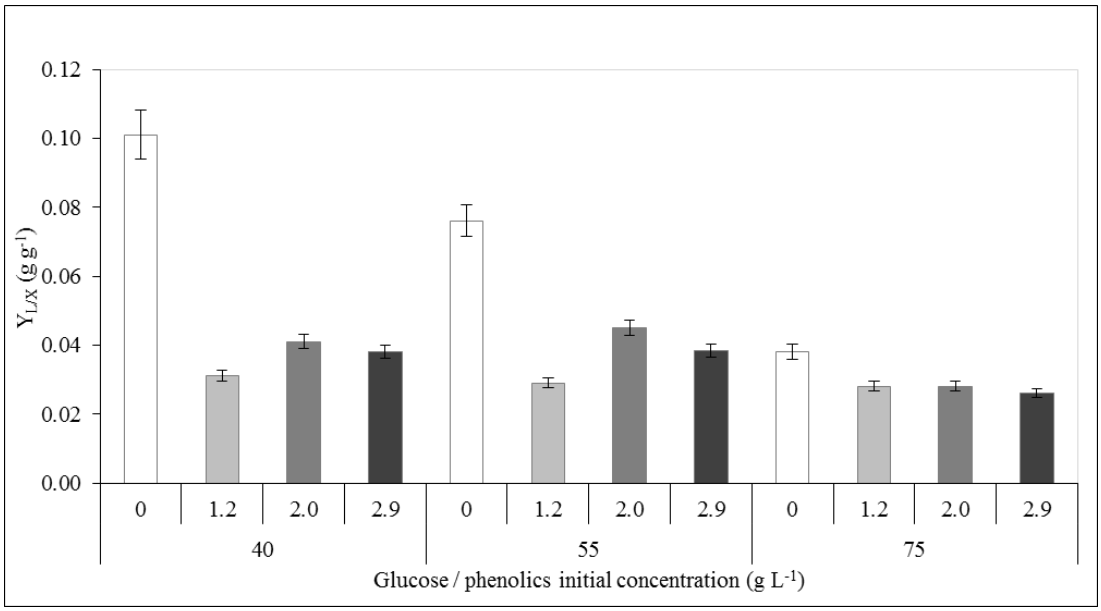


Figure 3. 28. Impact of the initial concentration of glucose (Glc, g L^{-1}) and phenolic compounds upon the maximum quantity of lipids produced per unit of dry yeast mass ($Y_{L/X}$, g g^{-1}) for all shake-flask cultures realized, on media composed of different mixtures of olive-mill wastewaters and glucose. Culture conditions: growth on 250-mL aseptic and non-aseptic shake flasks at 180 ± 5 rpm, Glc_0 (in g L^{-1}) ~40.0, ~55.0 and ~75.0 g L^{-1}, initial pH=3.5 ± 0.1, incubation temperature *T*=28 °C. Each point is the mean value of two independent measurements.

Moreover, in the cultures that were not supplemented with OMWs, the addition of glucose to the medium also had a strong impact on the total FA composition of the lipids since the concentration of $^{\Delta 9}$C18:1 and $^{\Delta 9}$C16:1 decreased with Glc_0 rise in the medium, while the respective concentration of FA $^{\Delta 9,12}$C18:2 increased. The addition of OMW to the culture medium even in quantities that resulted in minimal elevated initial phenolic compounds concentration (e.g. 1.20±0.10 g L^{-1}) resulted in differences in the FA composition of cellular lipids produced. In almost all cases, the addition of phenolic compounds resulted in a remarkable rise in the concentration of the cellular FA $^{\Delta 9}$C18:1 (and to lesser extent of the FA $^{\Delta 9,12}$C18:2) whereas the respective quantities

of the FAs C18:0 and $^{\Delta 9}$C16:1 drastically decreased (Table 3.13.). Finally, the addition of OMWs did not result in drastic FA composition changes as a function of the fermentation time, in contrast with the control experiments in which no OMW addition was performed (Table 3.13.).

Table 3. 13. Fatty acid composition (% *w/w*) of cellular lipids by *Saccharomyces cerevisiae* MAK-1

Initial phenolics (g L^{-1})	Glc_0 (g L^{-1})	Time (h)	C16:0	$^{\Delta 9}$C16:1	C18:0	$^{\Delta 9}$C18:1	$^{\Delta 9,12}$C18:2
0.00	~40.0	12	14.3	14.1	7.2	60.3	4.1
		24	14.7	32.0	7.1	40.7	3.8
		60	14.1	35.3	6.4	38.8	4.0
		132	16.9	29.1	3.9	42.7	7.3
	~55.0	12	16.7	9.9	16.6	54.2	2.6
		24	17.5	10.9	15.5	41.3	14.7
		60	16.9	24.4	7.4	37.4	13.8
		132	17.6	22.5	3.2	40.3	16.4
	~75.0	12	23.6	12.1	5.5	47.6	3.5
		24	23.2	15.4	5.5	45.9	4.0
		60	20.3	22.1	T.	44.6	12.7
		132	17.8	24.0	T.	41.1	16.5
~1.20	~40.0	12	4.5	T.	8.0	58.0	18.9
		24	7.6	T.	6.0	57.4	14.0
		60	16.6	7.1	T.	59.7	15.7
		132	17.6	8.0	T.	57.1	14.8
	~55.0	12	21.0	T.	T.	69.9	7.7
		24	15.2	T.	T.	65.4	12.4
		60	15.4	T.	T.	65.2	17.7
		132	13.4	7.9	T.	67.6	11.0
	~75.0	12	15.7	10.5	T.	59.6	14.2
		24	22.7	T.	T.	57.3	12.8
		60	15.1	T.	T.	68.0	12.9
		132	15.5	T.	T.	68.5	15.5
~2.00	~40.0	12	13.1	8.5	T.	61.4	16.3
		24	13.8	T.	T.	68.4	15.6
		60	15.3	T.	T.	70.1	13.6
		132	16.4	2.0	T.	63.5	15.1
	~55.0	12	14.5	9.9	T.	61.0	14.6
		24	16.5	T.	T.	68.4	15.1
		60	16.7	T.	T.	65.3	12.8
		132	17.2	T.	T.	67.7	11.5
	~75.0	12	16.2	T.	T.	70.2	13.5
		24	17.5	T.	T.	68.8	11.6
		60	15.4	3.7	T.	67.8	7.7
		132	15.3	1.5	T.	68.9	12.8
~2.90	~40.0	12	4.8	T.	T.	69.4	15.7
		24	7.0	T.	T.	66.8	16.2
		60	4.5	T.	T.	68.3	14.1
		132	15.9	T.	T.	69.0	14.3
	~55.0	12	16.9	2.7	T.	65.0	13.5
		24	15.8	T.	T.	67.3	16.5
		60	14.3	1.3	T.	66.9	14.6
		132	19.2	T.	T.	68.8	12.0
	~75.00	12	17.3	T.	T.	67.8	14.8
		24	17.1	T.	T.	65.4	16.8
		60	17.3	T.	T.	66.3	16.0
		132	14.3	T.	T.	68.0	13.5

T.<0.5%, *w/w*

The results of the aforementioned chapter have been published in a peer-reviewed journal as follows:

Sarris, D.; Giannakis, M.; Philippoussis, A.; Komaitis, M.; Koutinas, A. A.; Papanikolaou, S., Conversions of olive mill wastewater-based media by *Saccharomyces cerevisiae* through sterile and non-sterile bioprocesses. *Journal of Chemical Technology and Biotechnology* **2013,** 88, 958-969.

3.4. Ethanol and biomass production by *Saccharomyces cerevisiae* strain MAK-1 cultivated on blends of molasses and olive mill wastewaters

3.4.1. General presentation

In the previous part of this study (paragraph 3.3.), it was desirable to study aspects related with the production of bioethanol by microbial fermentations of OMW-based media under non-aseptic shake-flasks (with the addition of various amounts of OMWs) and non-aseptic batch bioreactor cultures (with use of different aeration regimes). Goal of the fourth part of this study was to investigate the potential of *S. cerevisiae* strain MAK-1 to produce bioethanol and biomass under completely non-aseptic aerated and non-aerated conditions when blends of molasses and OMWs were used as substrates. The rationale of the utilization of these blends was to study the effect of the utilization of these mixtures of residues upon the physiological and kinetic behavior of the strain, since in a scale-up of the process in large-scale operations, OMWs could be used as tap water substitute for molasses dilution. Moreover, molasses could simultaneously substitute external supplementation of sugars for OMW-based media for the production of higher amounts of added-value products through microbial conversions.

The fermentations were carried out under sterile and non-sterile conditions in 250-mL Erlenmeyer flasks as also in non-sterile aerated and non-aerated conditions in laboratory scale bioreactor (total volume 3.5 L; working volume 3.0 L) by *Saccharomyces cerevisiae* MAK-1. To investigate the biochemical response of *S. cerevisiae* strain MAK-1 grown on media composed of mixtures of molasses and OMWs, fermentations in sterile and non-sterile flask cultures and non-sterile batch bioreactor cultures were carried out. In the first part of the present study, sterile control (without OMW addition; initial total sugars into the medium TS_0, ~100.0 g L^{-1}) flask cultures with salts added compared to cultures without salts addition. In the second part, sterile flask control cultures without salts added compared to non-sterile ones (TS_0~100.0 g L^{-1}). In the third experimental part, OMW was added into the non-sterile molasses flask cultures in various ratios (% *v/v*): 0 (control experiment, no OMW addition), 10, 20, 30, 40 and 50 giving respectively initial concentration of phenolic compounds (in g L^{-1}): 2.6±0.2, 3.9±0.3, 4.5±0.4, 5.2±0.4, 5.5±0.5 and 6.3±0.5. The initial total sugars concentration was ~100.0 g L^{-1}. Finally, non-sterile batch

fermentations were conducted in a laboratory scale bioreactor under aerated (1.2 vvm) and non-aerated conditions (0.0 vvm). In fermentations carried out under non-aerated conditions, OMW was added into the medium in ratio 20% *v/v* giving initial phenolic compounds concentration 4.7±0.4 g L^{-1}. Molasses added in various amounts giving initial total sugars concentration (in g L^{-1}): ~100.0, ~135.0, ~150.0 and ~200.0. A non-sterile aerated bioreactor trial with aeration imposed 1.2 vvm (initial phenolic compounds concentration 4.7±0.4 g L^{-1}; TS_0~100.0 g L^{-1}) was also performed. [Note: All values of the photometric analysis of phenols include also the reaction of melanoidins (of molasses) with Folin-Chiocalteau reagent]

3.4.2. Effect of salts addition and sterilization on the kinetic behavior of *Saccharomyces cerevisiae* strain MAK-1 grown on molasses and OMWs blends

Initially the kinetic behavior of *S. cerevisiae* was evaluated on media composed of molasses (without OMW addition – "blank" experiments; in these cultures TS_0 was adjusted to ~100.0 g L^{-1}) in which addition of mineral compounds (see paragraph 2.2.2.) had been done, and comparisons were performed with trial that was carried out without supplementary salts addition (it is common knowledge that molasses contain already non-negligible amounts of mineral elements). Shake-flask experiments were performed, and before inoculation, sterilization of the culture media had been performed. The addition of salts into the medium affected negatively dry cell weight (X_{max}=6.2 against 7.2 g L^{-1}, yield of dry weight produced per total sugars consumed $Y_{X/TS}$ =0.06 against 0.07 g g^{-1}) and ethanol ($EtOH_{max}$=33.5 against 35.7 g L^{-1}, yield of ethanol produced per total sugars consumed $Y_{ETOH/TS}$ =0.36 against 0.36 g g^{-1}) production (Table 3.14. and Fig. 3.29.). Therefore no necessity of salts addition existed and, thus, these elements were not added at the trials that followed.

Table 3. 14. Experimental data of *Saccharomyces cerevisiae* strain MAK-1 originated from kinetics on molasses-based media (blank experiments; no OMW addition) under addition and no addition of salts, in aseptic flask fermentations.

Representations of total biomass (X, g L^{-1}), ethanol (EtOH, g L^{-1}) and consumed substrate (TS_{cons}, g L^{-1}) concentrations at different fermentation points of each trial:

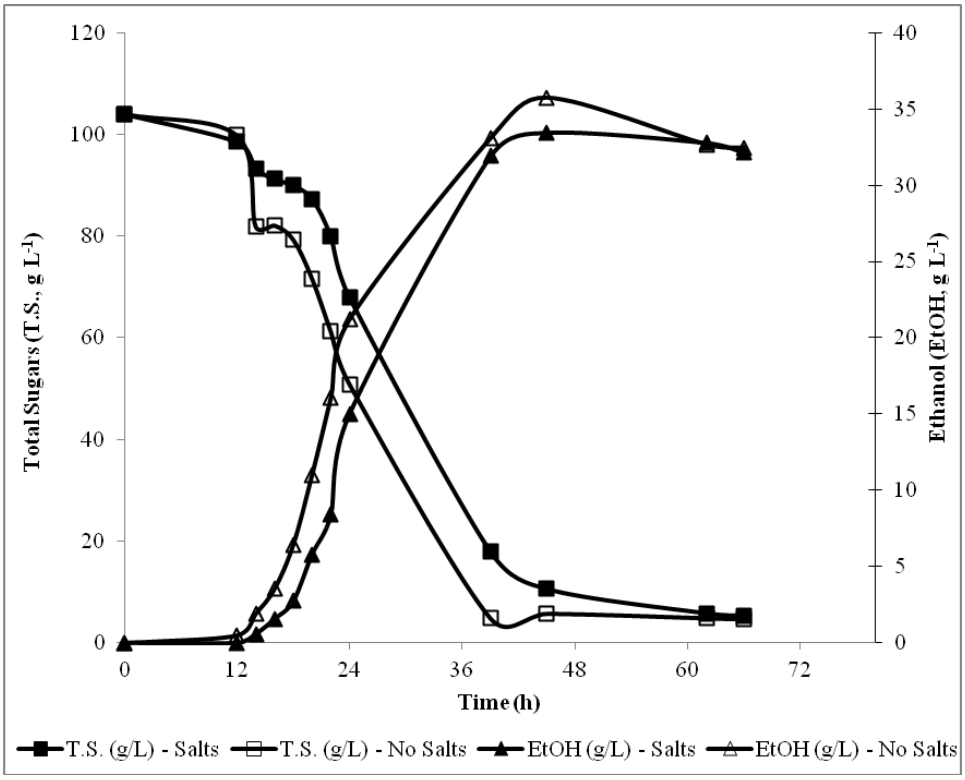
	Fermentation Time (h)	X (g L^{-1})	EtOH (g L^{-1})	TS_{Cons} (g L^{-1})	$Y_{X/TS}$ (g g^{-1})	$Y_{EtOH/TS}$ (g g^{-1})
Salts	45[a]	5.3±0.4	33.5±2.5	93.4±7.5	0.06	0.36
	66[b]	6.2±0.5	32.2±2.5	98.7±7.5	0.06	0.33
No Salts	45[a]	6.1±0.5	35.7±3.0	98.3±7.5	0.06	0.36
	62[b]	7.2±0.6	32.1±2.5	99.3±7.5	0.07	0.33

[a] when $EtOH_{max}$ concentration was achieved;

[b] when X_{max} concentration was achieved.

Fermentation time, conversion yield of biomass produced per total sugars consumed ($Y_{X/TS}$, g g^{-1}) and conversion yield of ethanol produced per total sugars consumed ($Y_{EtOH/TS}$, g g^{-1}) are presented for all points of the trials. Culture conditions: growth on 250-mL sterile flasks at 180 ± 5 rpm, TS_0~100.0 g L^{-1}, initial pH=3.5 ± 0.1, incubation temperature *T*=28 °C. Each point is the mean value of two independent measurements.

(a)



(b)

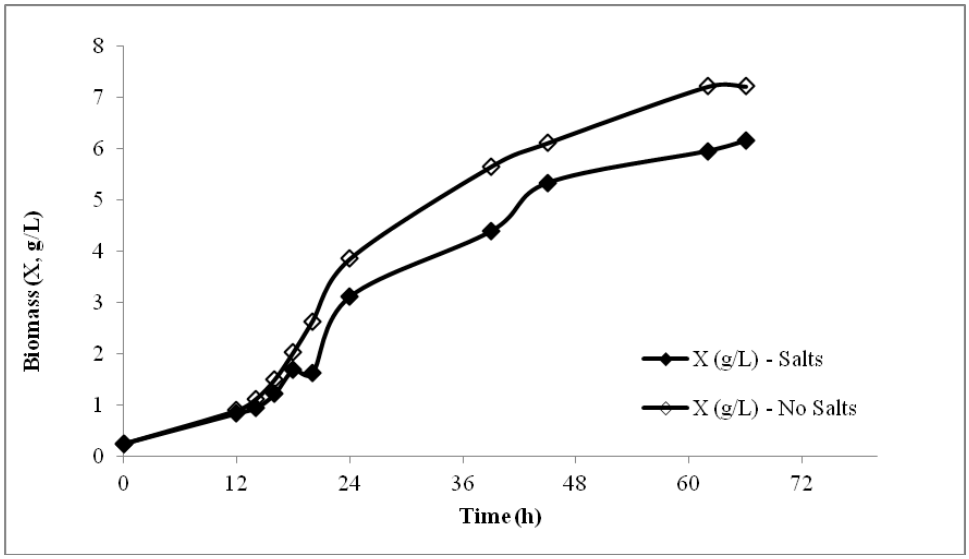
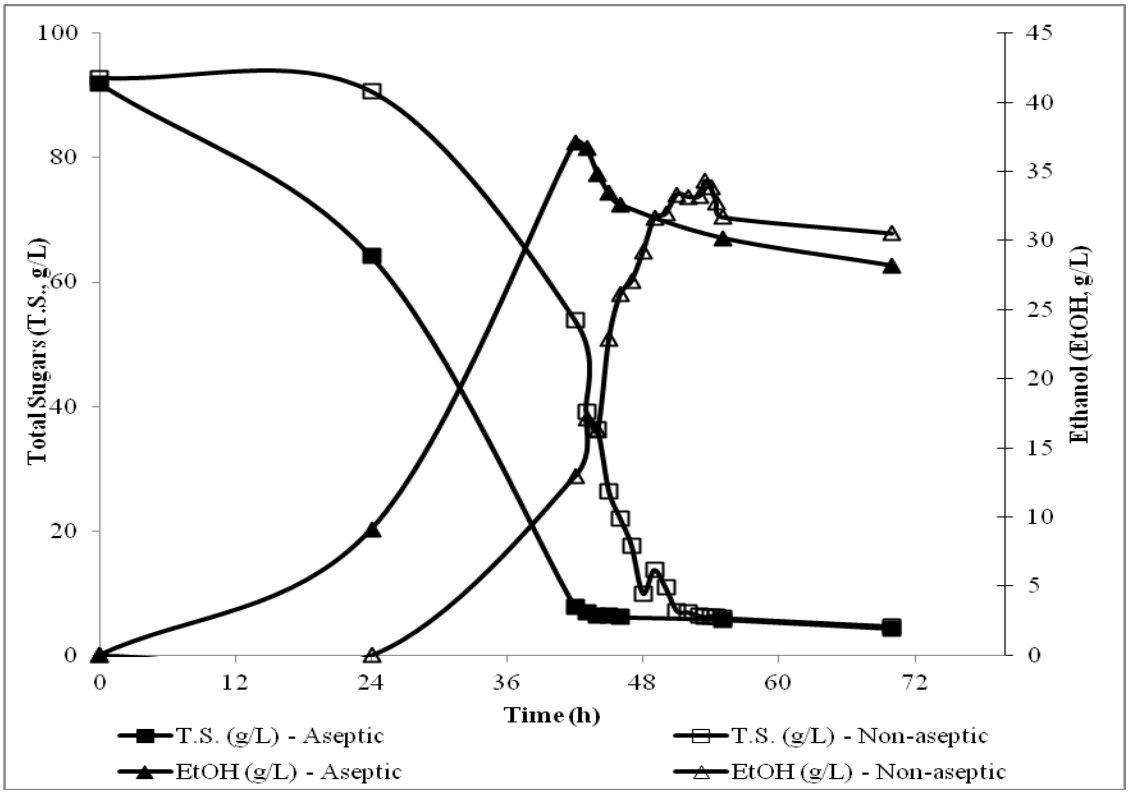


Figure 3. 29. (a) Total sugars (TS, g L^{-1}) ethanol (EtOH, g L^{-1}) and (b) biomass (X, g L^{-1}) evolution during growth of *Saccharomyces cerevisiae* strain MAK-1 on molasses based media (blank experiments; no OMW addition) under addition and no addition of salts in sterile flask fermentations. Culture conditions: growth on 250-mL sterile flasks at 180 ± 5 rpm, Glc_0~100.0 g L^{-1}, initial pH=3.5 ± 0.1, incubation temperature *T*=28 °C. Each point is the mean value of two independent measurements.

Previously sterilized (without salts addition) blends of molasses and OMWs (10% *v/v*; initial total phenolics at 3.9±0.3 g L^{-1}; TS_0~100.0 g L^{-1}) were subjected to shake-flask fermentations and were compared with non-aseptic shake-flask cultures containing the same initial quantities of phenolic compounds and TS [Fig. 3.30. (a) and (b)]. At the trials performed under aseptic conditions, ethanol values were slightly higher compared to non-aseptic trials ($EtOH_{max}$=37.1 against 34.3 g L^{-1}, $Y_{ETOH/TS}$=0.44 against 0.40 g g^{-1}; Table 3.15.), while also the substrate was consumed earlier in the former case. This most probably was attributed to the presence of bacteria (rods) grown together with the yeast strain at the early fermentation stages. The presence of bacteria was almost completely eliminated with the subsequent rise in the concentration of ethanol, as the fermentation proceeded. On the other hand, biomass production (by means of X_{max} and $Y_{X/TS}$ values) was enhanced at the non-previously sterilized fermentations compared with the aseptic trials (X_{max}=7.3 against 5.8 g L^{-1}, $Y_{X/TS}$=0.08 against 0.07 g g^{-1}; Table 3.15.). The results with the trial under non-aseptic conditions were considered as satisfactory, and, therefore, it was decided to proceed with the following trials with no previous sterilization of the medium.

(a)



(b)

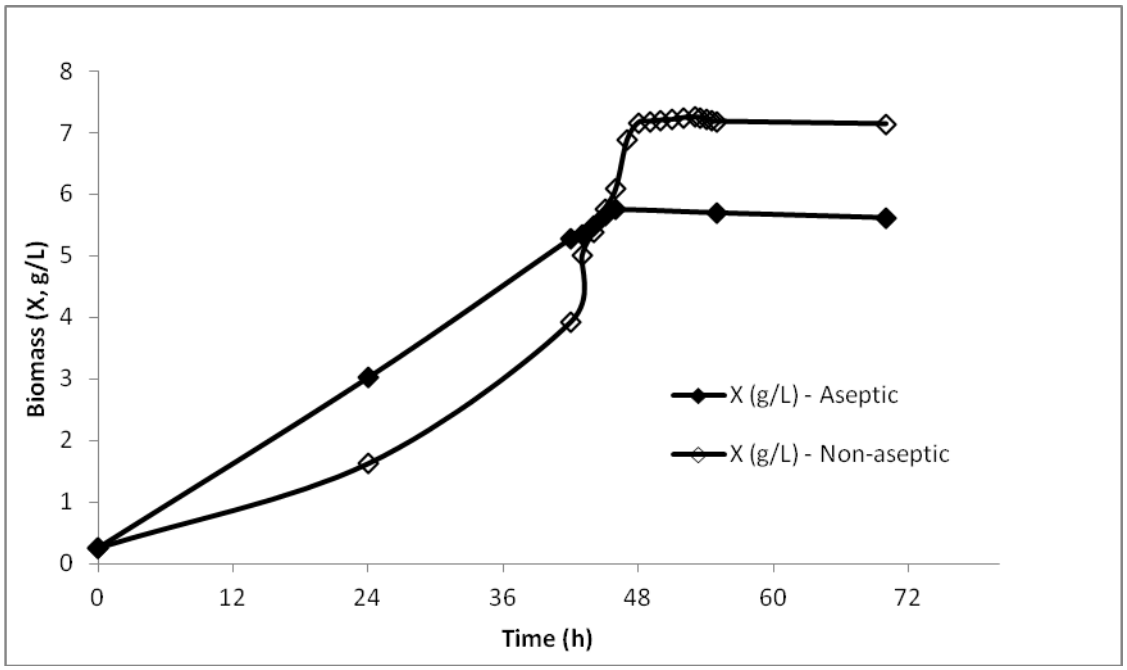


Figure 3. 30. (a) Total sugars (TS, g L^{-1}), ethanol (EtOH, g L^{-1}) and (b) biomass (X, g L^{-1}) evolution during growth of *Saccharomyces cerevisiae* strain MAK-1 on molasses and OMWs (10% *v/v*; initial phenolic compounds concentration 3.9±0.3 g L^{-1}) blends. Culture conditions: growth on 250-mL aseptic and non-aseptic flasks at 180 ± 5 rpm, TS_0~100.0 g L^{-1}, initial pH=3.5 ± 0.1, incubation temperature *T*=28 °C. Each point is the mean value of two independent measurements.

Table 3. 15. Experimental data of *Saccharomyces cerevisiae* strain MAK-1 grown on mixtures of molasses and OMWs (10% *v/v*), under aseptic and non-aseptic flask fermentations.

Representations of total biomass (X, g L^{-1}), ethanol (EtOH, g L^{-1}) and consumed substrate (TS_{cons}, g L^{-1}) concentrations at different fermentation points of each trial:

	Fermentation time (h)	X (g L^{-1})	EtOH (g L^{-1})	TS_{Cons} (g L^{-1})	$Y_{X/TS}$ (g g^{-1})	$Y_{EtOH/TS}$ (g g^{-1})
Aseptic	42[a]	5.3±0.4	37.1±4.0	84.1±6.0	0.06	0.44
	46[b]	5.8±0.4	32.6±2.5	85.8±6.5	0.07	0.38
Non-aseptic	53[a,b]	7.3±0.6	34.3±3.0	86.7±7.0	0.08	0.40

[a] when $EtOH_{max}$ concentration was achieved;

[b] when X_{max} concentration was achieved.

Fermentation time, conversion yield of biomass produced per total sugars consumed ($Y_{X/TS}$, g g^{-1}) and conversion yield of ethanol produced per total sugars consumed ($Y_{EtOH/TS}$, g g^{-1}) are presented for all points of the trials. Culture conditions: growth on 250-mL sterile and non-sterile flasks at 180 ± 5 rpm, initial phenolic compounds concentration 3.9±0.3 g L^{-1}, TS_0~100.0 g L^{-1}, initial pH=3.5 ± 0.1, incubation temperature *T*=28 °C. Each point is the mean value of two independent measurements.

3.4.3. Biomass production by *Saccharomyces cerevisiae* strain MAK-1 grown on non-aseptic molasses and OMWs blends

In the shake-flask trials (TS_0~100.0 g L^{-1}; OMWs added in various amounts) performed under non-aseptic conditions, biomass production (expressed as X_{max} and $Y_{X/TS}$) was not significantly reduced by the addition of phenolic compounds (OMW) into the diluted molasses-based media (X_{max}=6.3-8.0 g L^{-1}; $Y_{X/TS}$~0.07-0.09 g g^{-1}) (see Table 3.16.). The highest X_{max} and $Y_{X/TS}$ values were presented at the control (without OMW addition) experiment (Table 3.16.). The values of total sugars concentration that remained unconsumed (TS_f=5.1-18.3 g L^{-1}; ~70 hours after inoculation) rose proportionally to the addition of OMWs indicating that the increasing presence of inhibitors of the effluent in the diluted molasses-based media negatively affected the metabolism of the strain. A representative kinetics is shown in Fig. 3.31. (a); (b) (strain grown non-aseptically on blends of molasses and OMWs with initial phenol content at 6.3±0.5 g L^{-1} and TS_0~100.0 g L^{-1}). Addition of OMWs at a ratio of 20%, *v/v*, gave satisfactory results as regards the production of both yeast biomass and ethanol in shake-flask cultures of *S. cerevisiae*.

Table 3. 16. Experimental data of *Saccharomyces cerevisiae* strain MAK-1 grown on molasses and OMWs blends, with OMWs added in various amounts.

Representations of total biomass (X, g L^{-1}), ethanol (EtOH, g L^{-1}) and consumed substrate (TS_{cons}, g L^{-1}) concentrations at different fermentation points of each trial:

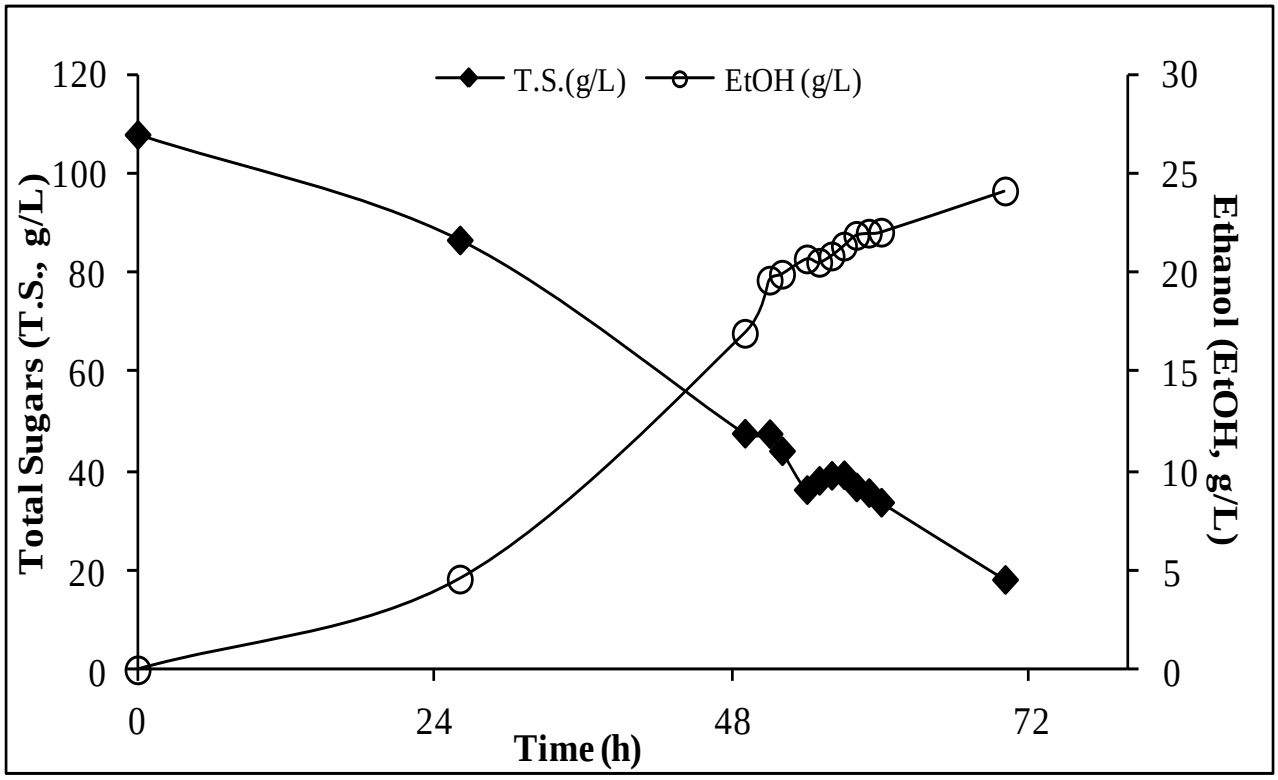
OMWs (% *v/v*)	Initial phenolics (g L^{-1})	Fermentation time (h)	X (g L^{-1})	EtOH (g L^{-1})	TS_{cons} (g L^{-1})	$Y_{X/TS}$ (g g^{-1})	$Y_{EtOH/TS}$ (g g^{-1})
0	2.6±0.2	58[a]	6.7±0.5	37.3±4.0	84.9±6.5	0.08	0.44
		73[b]	8.0±0.6	33.7±3.0	88.3±7.0	0.09	0.38
10	3.9±0.3	53[a,b]	7.3±0.6	34.3±3.0	86.7±7.0	0.08	0.40
20	4.5±0.4	51[a]	6.4±0.5	33.9±3.0	82.5±6.0	0.08	0.41
		55[b]	6.7±0.6	31.4±2.5	84.7±6.5	0.08	0.37
30	5.2±0.4	55[b]	6.3±0.5	30.2±2.5	87.5±7.0	0.07	0.35
		56[a]	6.2±0.5	31.0±2.5	87.6±7.0	0.07	0.35
40	5.5±0.5	54[b]	6.4±0.5	26.8±2.0	76.3±5.0	0.08	0.35
		58[a]	6.0±0.4	28.4±2.5	80.9±5.5	0.07	0.35
50	6.3±0.5	70[a,b]	6.5±0.5	24.2±2.0	89.8±7.0	0.07	0.27

[a] when $EtOH_{max}$ concentration was achieved;

[b] when X_{max} concentration was achieved.

Fermentation time, conversion yield of biomass produced per total sugars consumed ($Y_{X/TS}$, g g^{-1}) and conversion yield of ethanol produced per total sugars consumed ($Y_{EtOH/TS}$, g g^{-1}) are presented for all points of the trials. Culture conditions: growth on 250-mL not previously sterilized shake-flasks at 180 ± 5 rpm, TS_0~100.0 g L^{-1}, initial pH=3.5 ± 0.1, incubation temperature *T*=28 °C. Each point is the mean value of two independent measurements.

(a)



(b)

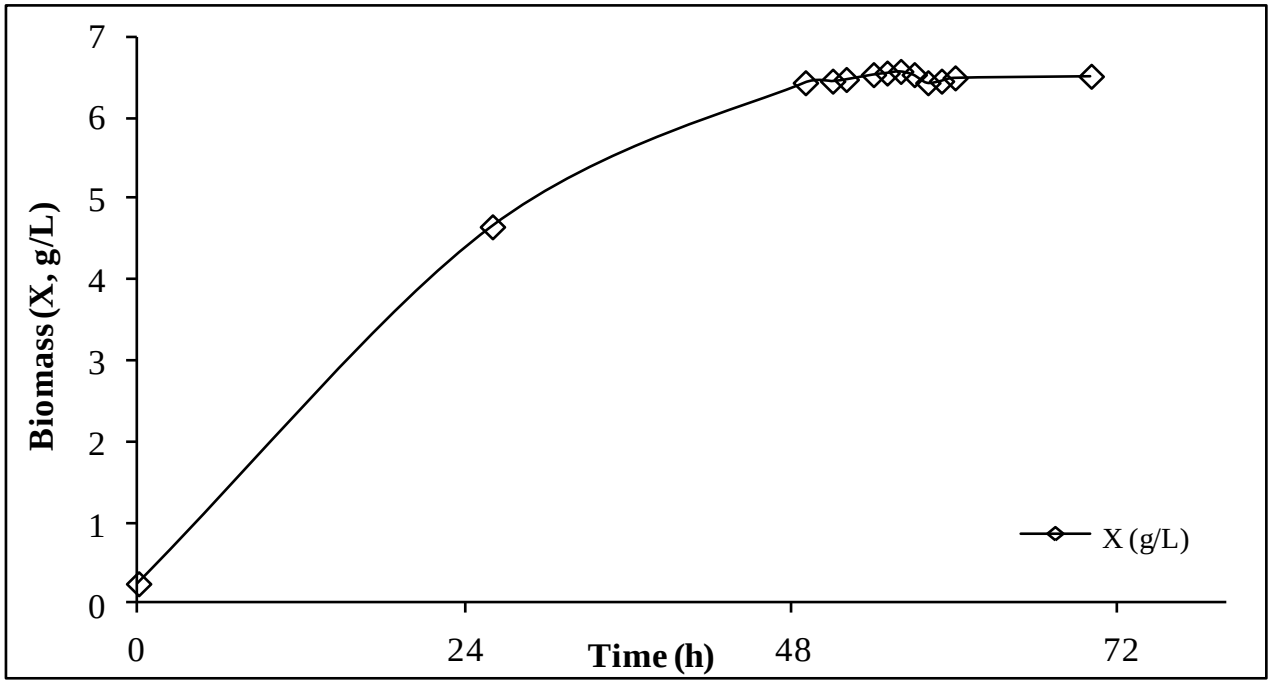
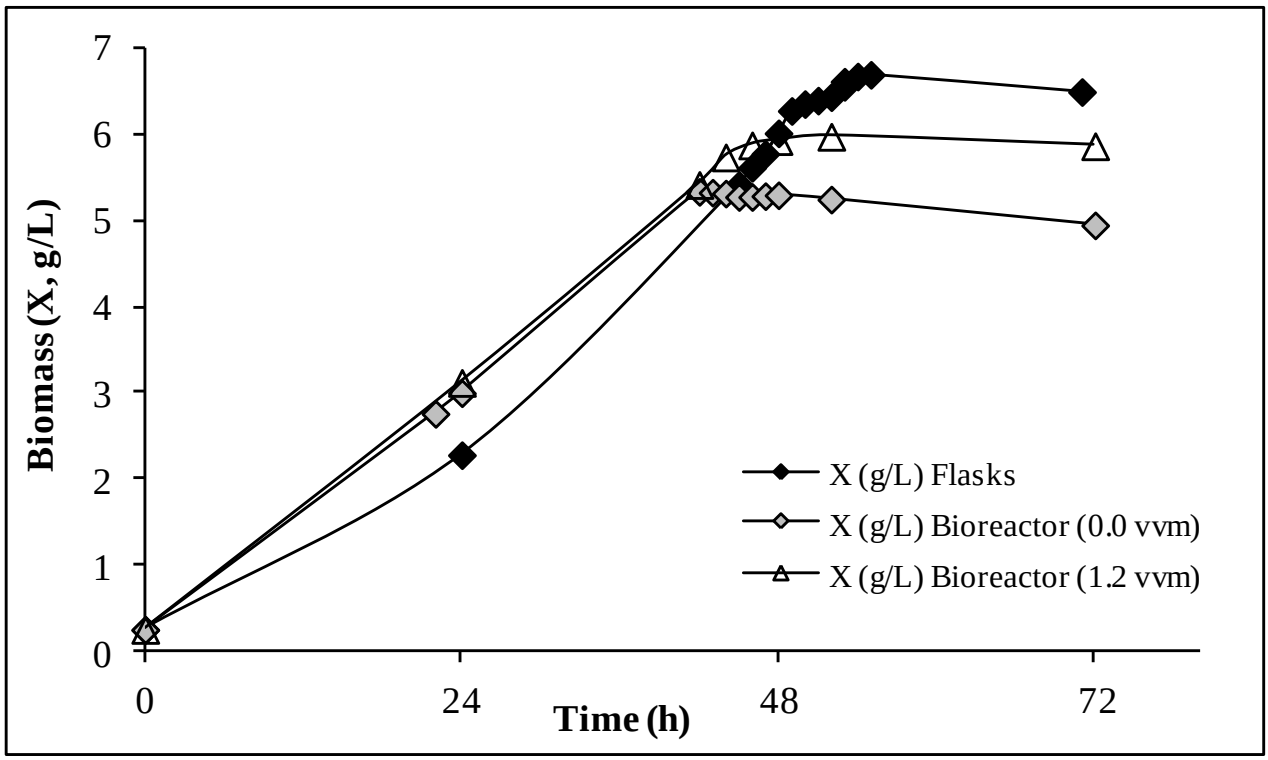


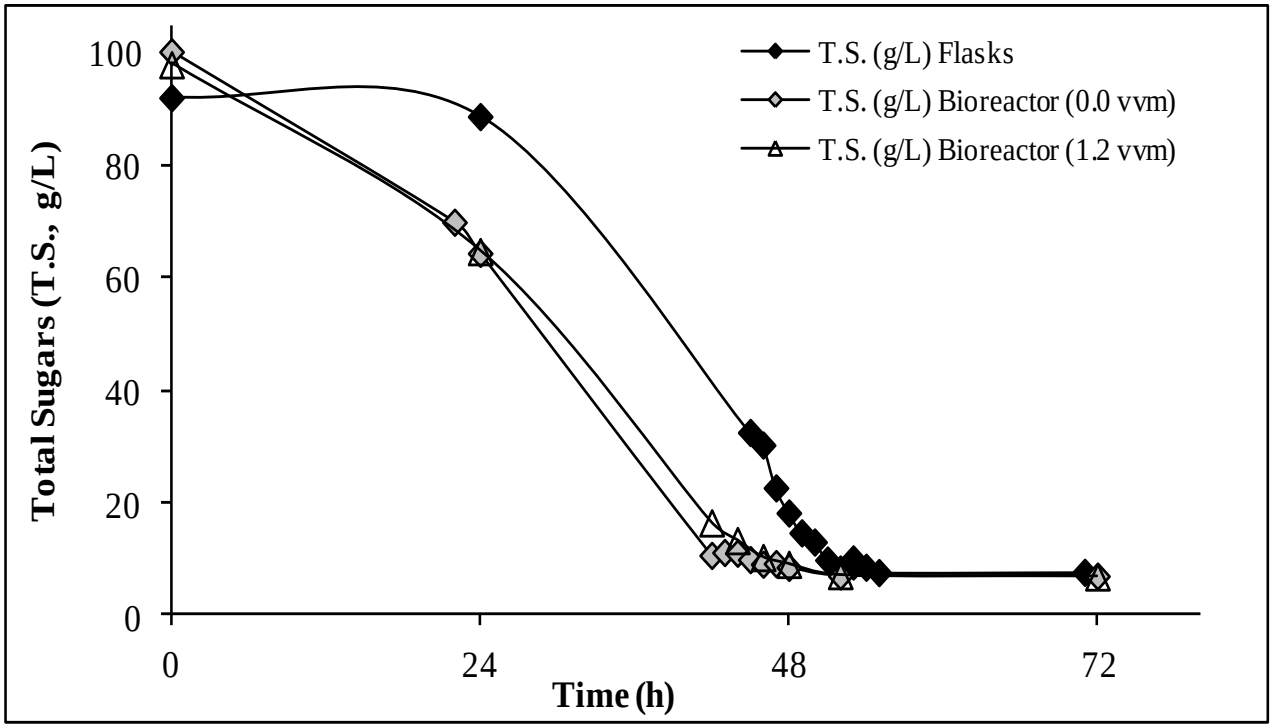
Figure 3. 31. (a) Total sugars (TS, g L^{-1}), ethanol (EtOH, g L^{-1}) and (b) biomass (X, g L^{-1}) evolution during growth of *Saccharomyces cerevisiae* strain MAK-1 on molasses and OMWs (50% *v/v*; initial phenolic compounds concentration 6.3±0.5 g L^{-1}) blends. Culture conditions: growth on 250-mL non-sterile flasks at 180 ± 5 rpm, TS_0~100.0 g L^{-1}, initial pH=3.5 ± 0.1, incubation temperature *T*=28 °C. Each point is the mean value of two independent measurements.

In the next step, it was desirable to perform batch bioreactor trials and compare the results with the respective ones performed in shake-flask cultures (comparative trials on media with TS_0=100.0 g L^{-1} with OMWs added to a ratio of 20%, *v/v*). In this step the effect of aeration or no-aeration on the bioreactor experiments upon the microbial metabolism was also identified. Thus, in the bioreactor experiments, either constant aeration (1.2 vvm) or no aeration (0.0 vvm) throughout the fermentation was imposed. The obtained results are depicted in Figs 3.32. (a); (b); (c). When non-aseptic bioreactor experiments under no aeration conditions (0.0 vvm) with aeration imposed (1.2 vvm) were compared, biomass production showed some slight increase in the latter case. In any case though, as regards both bioreactor trials, biomass production was clearly reduced by means of X_{max} value achieved when compared with the respective shake-flask experiment [Fig. 3.32 (a)]. Moreover, biomass evolution reached at its plateau earlier in bioreactor (~42 hours) than in flask cultures (~52 hours). In the shake-flask (and in less extent in the bioreactor under aeration), after glucose was exhausted, some "diauxic growth" of *S. cerevisiae* was observed (Pyun *et al.*, 1989; Zhang *et al.*, 1994; Piškur *et al.*, 2006) and the strain consumed the previously accumulated into the growth medium ethanol towards the formation of new biomass (in accordance with several of the trials performed in the paragraph 3.3.). On the other hand, in all trials, total sugars were consumed within 50 hours after inoculation with relatively comparable consumption rates [Fig. 3.32. (b)], since virtually the microbial metabolism was directed towards the accumulation of ethanol into the culture medium spite of the fact that oxygen (concerning shake-flask and aerated batch-bioreactor trials) existed into the medium (as previously, the Crabtree effect was very clear in our trials). Moreover, comparing non-aerated and aerated bioreactor trials, it was seen that ethanol production slightly increased by means of $EtOH_{max}$ values in the trial under no aeration imposed [Fig. 3.32. (c)]. Comparing both bioreactor trials (aeration 1.2 vvm and 0.0 vvm) with the respective non-sterile shake-flask experiment, it was seen that ethanol biosynthesis was clearly favored in the reactor trials by means of $EtOH_{max}$ values rather than in the shake-flask experiment [Fig. 3.32. (c)], suggesting a clear positive effect of the scale-up of the process towards ethanol production. The $EtOH_{max}$ value was noted earlier in bioreactor (~44-46 hours) than in flask cultures (~52 hours).

(a)


Biomass (X, g/L)
0
1
2
3
4
5
6
7
0
24
48
72
Time (h)
X (g/L) Flasks
X (g/L) Bioreactor (0.0 vvm)
X (g/L) Bioreactor (1.2 vvm)

(b)


Total Sugars (T.S., g/L)
0
20
40
60
80
100
0
24
48
72
Time (h)
T.S. (g/L) Flasks
T.S. (g/L) Bioreactor (0.0 vvm)
T.S. (g/L) Bioreactor (1.2 vvm)

(c)

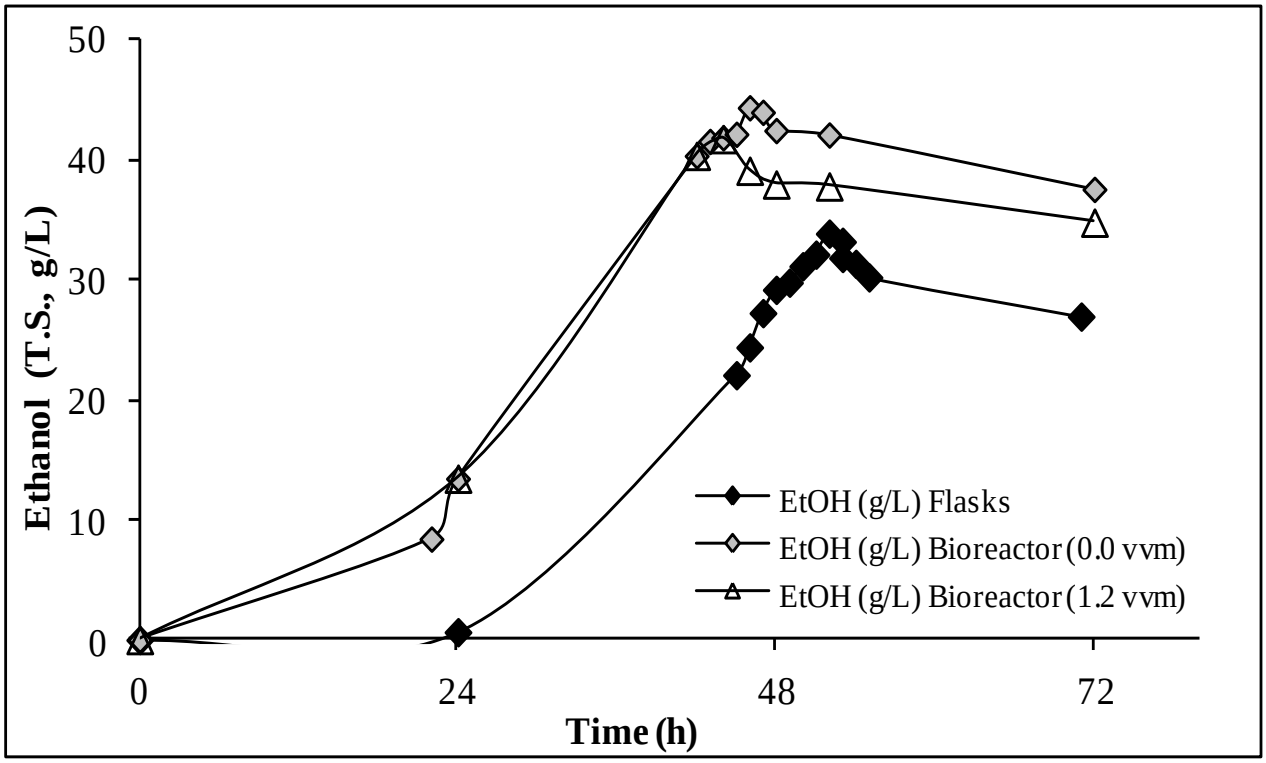


Figure 3. 32. (a) Biomass (X, g L^{-1}), (b) total sugars (TS, g L^{-1}) and (c) ethanol (EtOH, g L^{-1}) evolution during growth of *Saccharomyces cerevisiae* MAK-1 under non-aseptic conditions on molasses and OMWs (20% *v/v*) blends. Culture conditions: 250-mL non-sterile flasks at 180 ± 5 rpm, initial phenolic compounds concentration 4.50±0.4 g L^{-1}, TS_0~100.0 g L^{-1}, initial pH=3.5 ± 0.1, incubation temperature *T*=28 °C; non-sterile batch bioreactor at 300 rpm, initial phenolic compounds concentration 4.7±0.4 g L^{-1}, TS_0~100.0 g L^{-1}, initial pH=3.50 ± 0.02, incubation temperature *T*=28 °C, under aeration (1.2 vvm) and no aeration (0.0 vvm) conditions. Each point is the mean value of two independent measurements.

By taking into consideration that in the non-aseptic bioreactor culture with no aeration imposed (non-aerated trial) slightly higher ethanol quantities were achieved, in the next series of experiments it was desirable to study the effect on increasing addition of molasses upon the growth of the strain under no aeration conditions (0.0 vvm) in media with OMWs added to a ratio of 20%, *v/v*, therefore, molasses were added in increasing amounts in OMW-based media resulting in increasing TS_0 concentrations as follows (in g L^{-1}): ~100.0; ~135.0, ~150.0 and ~200.0 g L^{-1}. The increase of addition of molasses quantity into the medium negatively affected biomass production in terms of absolute values (g L^{-1}) achieved (Table 3.17.). Moreover, increment of molasses addition into the medium, negatively affected sugar assimilation by the strain, since the final total sugars concentration was (~ in g L^{-1}) 7.0, 13.0, 25.0 and 190.0 respectively, indicating that high phenolic (principally melanoidin) content affected negatively the strain metabolism. *S. cerevisiae* strain MAK-1 did not grow sufficiently at the trial with TS_0~200.0 g L^{-1}.

Table 3. 17. Experimental data of *Saccharomyces cerevisiae* strain MAK-1 grown on molasses and OMWs (20% *v/v*) blends, with molasses added in various quantities. Representations of biomass (X, g L^{-1}), ethanol (EtOH, g L^{-1}) and consumed substrate (TS_{cons}, g L^{-1}) concentrations. Fermentation time, conversion yield of biomass produced per total sugars consumed ($Y_{X/TS}$, g g^{-1}) and conversion yield of ethanol produced per total sugars consumed ($Y_{EtOH/TS}$, g g^{-1}) are presented. Representations are given when maximum ethanol concentrations ($EtOH_{max}$, g L^{-1}) were achieved. Culture conditions: growth on not previously sterilized batch bioreactor, agitation at 300 rpm, OMWs added at 20% *v/v*, initial pH=3.50 ± 0.02, incubation temperature *T*=28 °C, no aeration imposed (except the trial presented in the last line). Each point is the mean value of two independent measurements ($EtOH_{max}$ and X_{max} concentration achieved simultaneously at the trials below).

TS_0 (g L^{-1})	Initial Phenolics (g L^{-1})	Fermentation time (h)	X (g L^{-1})	EtOH (g L^{-1})	TS_{cons} (g L^{-1})	$Y_{X/TS}$ (g g^{-1})	$Y_{EtOH/TS}$ (g g^{-1})
~100	4.7±0.4	46	5.3±0.3	44.4±3.5	91.4±7.0	0.06	0.49
~135	5.6±0.5	98	4.6±0.3	52.4±4.0	110.3±8.0	0.04	0.48
~150	6.1±0.5	72	4.5±0.3	50.6±4.0	112.5±8.0	0.04	0.45
~200	7.8±0.6	70	2.8±0.2	n.d.	7.8±1.5	0.36	0.00
~100 (1.2 vvm)	4.7±0.4	44	5.7±0.4	41.8±3.5	84.9±6.5	0.07	0.49

n.d.: Not detected

3.4.4. Ethanol production by *Saccharomyces cerevisiae* strain MAK-1 grown on non-aseptic blends of molasses and OMWs

As far as ethanol biosynthesis is concerned, in non-sterile flask cultures (TS_0~100.0 g L^{-1}), $EtOH_{max}$ and maximum $Y_{EtOH/TS}$ values achieved were 37.3 g L^{-1} and 0.44 g g^{-1}, during growth of the microorganism on the control experiment (without OMW addition; Table 3.16.). In all non-aseptic flask trials, the addition of OMWs into the molasses medium reduced maximum ethanol production (EtOHmax=24.2-37.3 g L^{-1}; $Y_{EtOH/TS}$~0.29-0.44 g g^{-1}) comparing with the control experiment (Fig. 3.33.). Moreover, $EtOH_{max}$ values were noted earlier at the fermentations of the media containing mixtures of molasses and OMWs compared to the control experiment [excluding trials with initial phenolic compounds concentration (in g L^{-1}): 5.5±0.5 and 6.3±0.5] (Table 3.16.). Comparing non-aerated and aerated bioreactor trials, it was seen that ethanol production slightly increased by means of $EtOH_{max}$ values in the trial under no aeration imposed [Fig. 3.32. (c)]. In non-sterile non-aerated batch bioreactor cultures (OMWs added at 20% *v/v*) the increase of total sugars concentration raised ethanol production up to the trial with TS_0~135.0 g L^{-1} ($EtOH_{max}$=52.4 g L^{-1}; $Y_{EtOH/TS}$=0.48 g g^{-1}). The (overall) maximum $Y_{EtOH/TS}$=0.49 g g^{-1} ($EtOH_{max}$=44.4 g L^{-1}) was presented at the fermentation with TS_0~100.0 g L^{-1}. The fermentation with TS_0~200.0 g L^{-1} was accompanied by no ethanol detected into the medium (detection threshold of the HPLC analysis of ~0.2 g L^{-1}) suggesting potentially that the high melanoidin content might negatively affected the metabolism of the strain (Table 3.17.). Comparing both bioreactor trials (aeration 1.2 vvm and 0.0 vvm; initial phenolic compounds concentration 4.7±0.4 g L^{-1}; TS_0~100.0 g L^{-1}) with the respective non-sterile shake-flask (initial phenolic compounds concentration 4.5±0.4 g L^{-1}; TS_0~100.0 g L^{-1}) fermentations, ethanol production was clearly enhanced in reactor cultures by means of $EtOH_{max}$ values rather than in the shake-flask experiment [Fig. 3.32. (c)]. The $EtOH_{max}$ value was noted earlier in bioreactor (~44-46 hours) than in flask cultures (~52 hours).

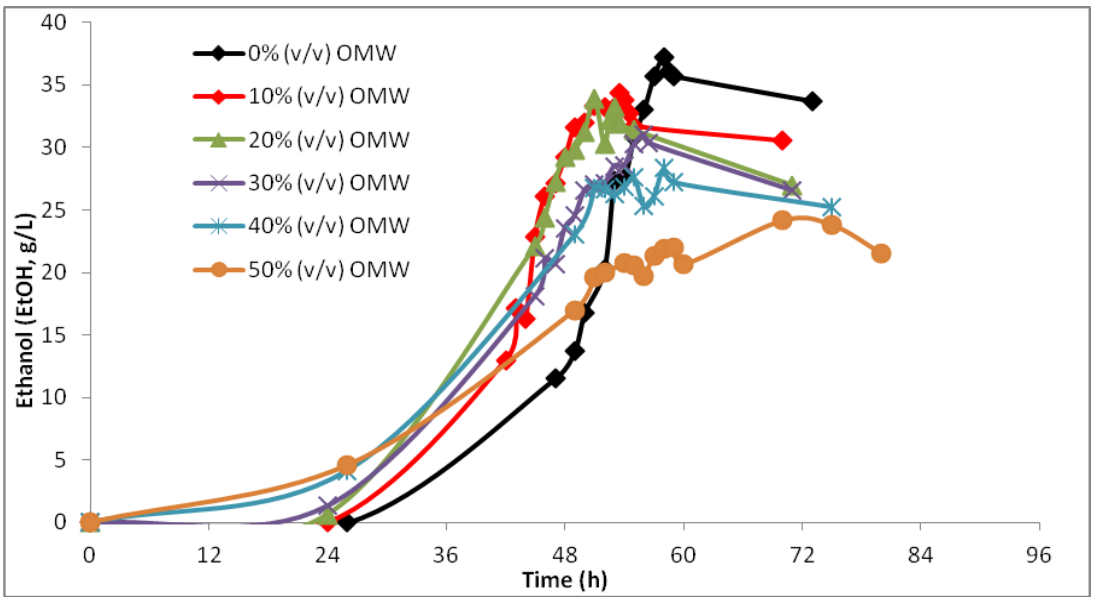


Figure 3. 33. Ethanol (EtOH, g L^{-1}) evolution during growth of *Saccharomyces cerevisiae* strain MAK-1 on blends of molasses and OMWs (in % *v/v*): 0 (control experiment; without OMWs addition), 10, 20, 30, 40 and 50. Culture conditions: 250-mL non-aseptic shake flasks agitated at 180 ± 5 rpm, initial phenolic compounds concentration (in g L^{-1}): 2.6±0.2, 3.90±0.3, 4.50±0.4, 5.20±0.4, 5.50±0.5 and 6.30±0.5, TS_0~100.0 g L^{-1}, initial pH=3.5 ± 0.1, incubation temperature *T*=28 °C. Each point is the mean value of two independent measurements.

3.4.5. Decolorization – removal of phenolic compounds

Remarkable color removal was performed in all non-sterile flask fermentations (TS_0~100 g L^{-1}). The overall maximum decolorization achieved was 28-60%. The overall maximum reduction of phenolic compounds ranged between 12 and 26% (*w/w*) (Table 3.18.). The overall maximum decolorization and the overall maximum reduction of phenolic compounds achieved at the trial with initial phenolic compounds 6.3±0.5 g L^{-1} (Table 3.18.). In non-sterile batch bioreactor fermentations, the maximum decolorization and reduction of phenolic compounds concentration achieved was 54.4% (at trial with TS_0~135.0 g L^{-1}) and 27.5% *w/w* (at trial with TS_0~100.0 g L^{-1}) respectively. No color and phenol reduction noted at reactor cultures with TS_0~200.0 g L^{-1} (Table 3.19.). Comparing non-sterile flask and non-sterile bioreactor cultures, one can conclude that decolorization and reduction of phenolic compounds values are almost equal. The kinetics of color and phenolic compounds removal from the culture medium (comparison of flask and bioreactor trials) is shown in Fig. 3.34. (a) and (b).

Table 3. 18. Experimental data of *Saccharomyces cerevisiae* strain MAK-1 concerning removal of phenol compounds and color performed in not previously sterilized shake-flask cultures, containing blends of molasses and OMWs added at various initial concentrations, in which total sugars were at ~100.0 g L^{-1}.

Representation of initial and final phenol compounds concentration in the culture medium, phenol compounds removal (% *w/w*) and color removal (%) from the medium. Each point is the mean value of two independent measurements.

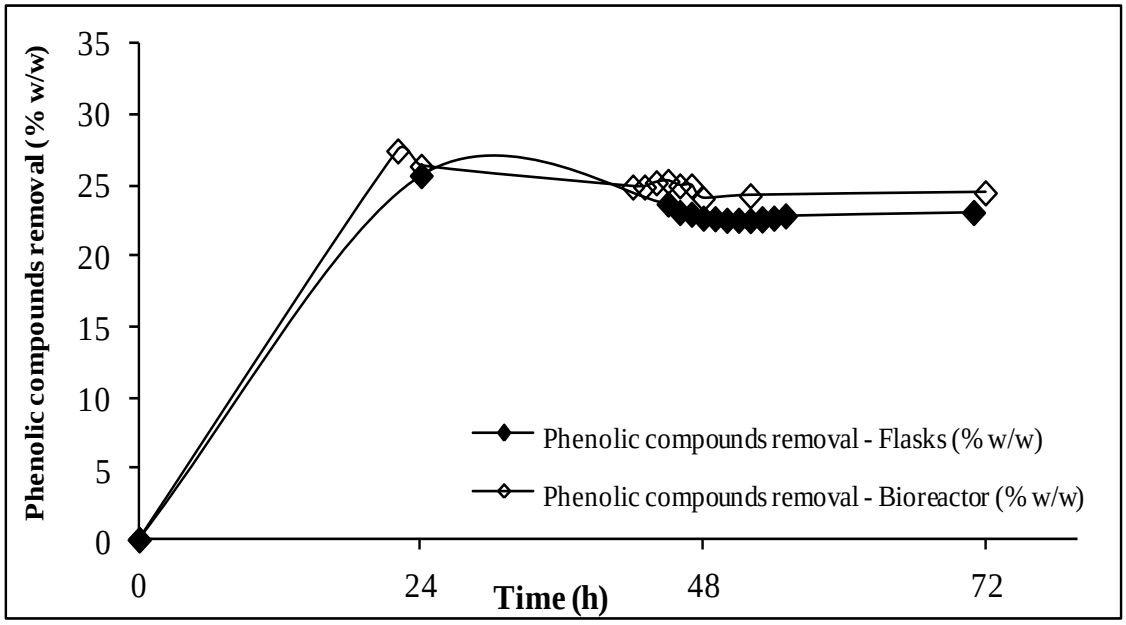
OMWs (% *v/v*)	Initial phenolics (g L^{-1})	Final phenolics (g L^{-1})	Phenol removal (% *w/w*)	Color removal (%)
0	2.60±0.3	1.70±0.2	35.1±3.0	35.9±3.0
10	3.90±0.4	3.20±0.3	19.3±1.5	52.7±4.0
20	4.50±0.4	3.30±0.3	25.7±2.0	49.6±4.0
30	5.20±0.4	4.40±0.3	15.0±1.0	28.4±2.5
40	5.50±0.5	4.90±0.4	12.1±1.0	46.9±3.5
50	6.30±0.5	4.60±0.4	26.2±2.0	59.9±4.5

Table 3. 19. Experimental data of *Saccharomyces cerevisiae* strain MAK-1 concerning removal of phenol compounds and color performed in not previously sterilized batch bioreactor cultures, containing mixtures of molasses and OMWs (added at ratio of 20% *v/v*), with molasses added in various amounts.

Representation of initial and final phenol compounds concentration in the culture medium, phenol compounds removal (% *w/w*) and color removal (%) from the medium. Each point is the mean value of two independent measurements.

TS_0 (g L^{-1})	Initial Phenolics (g L^{-1})	Final Phenolics (g L^{-1})	Phenol Removal (% *w/w*)	Color Removal (%)
~100	4.70±0.4	3.40±0.3	27.6±2.0	48.7±4.0
~135	5.60±0.5	4.70±0.4	16.4±1.0	54.4±4.0
~150	6.10±0.5	5.20±0.4	15.2±1.0	51.6±4.0
~200	7.80±1.0	7.80±1.0	0.0	0.0
~100 (1.2 vvm)	4.70±0.4	3.50±0.3	26.5±2.0	51.1±4.0

(a)



(b)

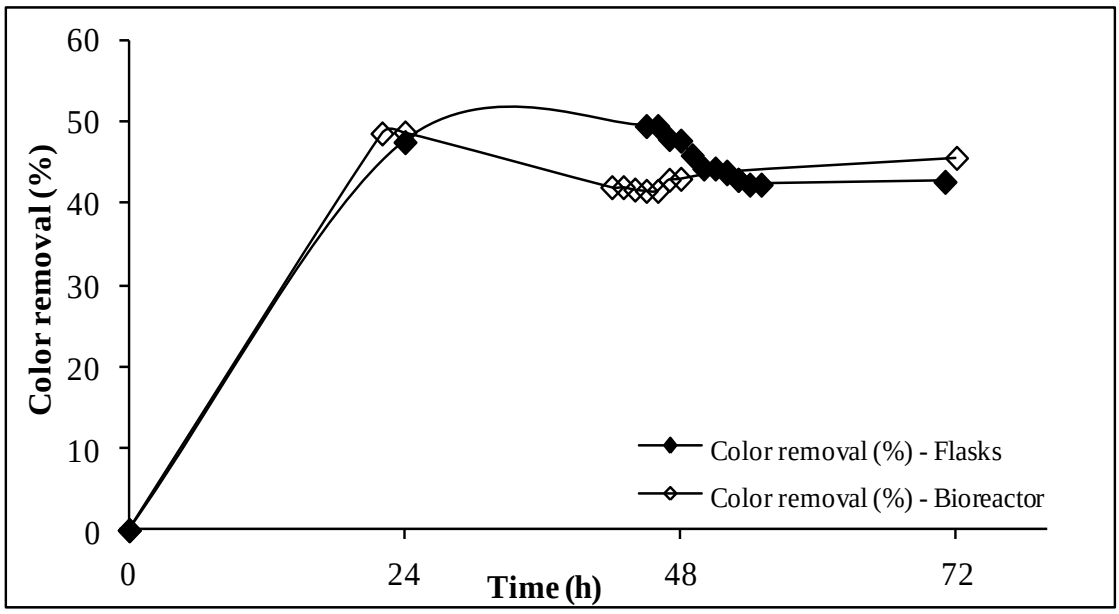


Figure 3. 34. (a) Phenolic compounds removal (% *w/w*) and (b) color removal (%) during growth of *Saccharomyces cerevisiae* strain MAK-1 on blends of molasses and OMWs (20% *v/v*) under non-aseptic 250-mL shake-flask (initial phenolic compounds concentration 4.5±0.4 g L^{-1}; TS_0~100.0 g L^{-1}) and non-aseptic batch bioreactor (initial phenolic compounds concentration 4.7±0.4 g L^{-1}; TS_0~100.0 g L^{-1}) cultures. Culture conditions: 250-mL non-aseptic flasks at 180 ± 5 rpm, initial pH=3.5 ±0.1, incubation temperature *T*=28 °C; non-aseptic batch bioreactor at 300 rpm, initial pH=3.50 ± 0.02, incubation temperature *T*=28 °C, sparge of air 0.0 vvm. Each point is the mean value of two independent measurements.

The results of the current chapter have been published in a peer-reviewed journal as follows:

Sarris, D.; Matsakas, L.; Aggelis, G.; Koutinas, A. A.; Papanikolaou, S., Aerated vs non-aerated conversions of molasses and olive mill wastewaters blends into bioethanol by *Saccharomyces cerevisiae* under non-aseptic conditions. *Ind Crops Prod* **2014,** 56, 83-93.

4. DISCUSSION

The ability of strains of two yeast species, namely *Yarrowia lipolytica* [strains ACA-YC 5028, ACA-YC 5033 and W29 (ATCC 20460)] and *Saccharomyces cerevisiae* (strain MAK-1; Sarris *et al.* 2009), to simultaneous bioremediate (reduce phenolic content and color) OMW-based media and produce added value products (biomass, citric acid, ethanol, cellular lipids) was assessed at the present study, which was divided into four main parts: At the first part, the ability of three *Y. lipolytica* strains [ACA-YC 5028, ACA-YC 5033 and W29 (ATCC 20460)] to grow on and convert glucose-enriched OMWs into biomass, cellular lipids and citric acid in carbon- and nitrogen-limited aseptic shake-flask cultures was assessed. Furthermore, at the second part of this study, fermentations by *Y. lipolytica* strain ACA-YC 5033 were carried out under aseptic and pasteurized conditions in 250-mL Erlenmeyer flasks as also in aseptic and pasteurized laboratory scale batch bioreactor (total volume 3.5 L; working volume 3.0 L) with the addition of higher amounts of OMWs into the medium. The FAMEs profile of total cellular lipids produced, the removal of phenolic compounds from the medium and the decolorization of the medium were also tested. The three *Y. lipolytica* strains tested presented non-negligible biomass production in both nitrogen- and carbon-limited shake-flask cultures, when various amounts of OMWs were added. In nitrogen-limited trials, satisfactory biomass and citric acid quantities were obtained, while a non-negligible phenolic compounds concentration reduction (up to ~34% *w/w*; excluding strain ACA-YC 5033 at trial with initial phenolic compounds concentration 5.50±0.5 g L^{-1} where surprisingly significant phenolic compounds concentration reduction reached the value of ~51% *w/w* occurred) and a remarkable decolorization (up to ~63%) in the media supplemented with OMWs was seen.

It is well documented (Anastassiadis *et al.*, 2002; Papanikolaou *et al.*, 2008a; 2008b; 2009; 2013; André *et al.*, 2009; Papanikolaou and Aggelis, 2009; Makri *et al.*, 2010) that in various yeast strains including *Y. lipolytica*, by increasing nitrogen limitation, yeast metabolism gradually turns from cell growth to citric acid and/or storage lipid formation. In carbon-limited conditions, significant biomass formation (X_{max}=10.0–13.3 g L^{-1}, maximum biomass yield on sugar consumed ~0.52 g g^{-1}) was observed while small quantities of citric acid were produced. Interestingly, in all carbon-limited trials, OMW addition enhanced biomass production compared with the control experiment (no waste addition into the medium). Moreover, some reduction of biomass concentration occurred during the stationary growth phase, suggesting possible cell autolysis, while waste addition did not affect glucose consumption in all strains throughout these trials; trials performed in carbon-limited conditions showed a higher sugar uptake than the nitrogen-limited trials. Phenolic compounds removal was higher in the nitrogen-limited trials than in the carbon-limited trials although in the former case notably less biomass was synthesized due to the imposed nitrogen limitation.

In the current study, in order for citric acid production to be carried out, nitrogen was the limiting substrate, since citric acid biosynthesis is a secondary anabolic activity performed only under carbon-excess conditions (Papanikolaou and Aggelis, 2009). *C. oleophila* or *Y. lipolytica* strains have been shown capable of producing noticeable citric acid quantities after nitrogen exhaustion during growth on glucose or similarly metabolized substrates (e.g. glycerol) as secondary anabolic activity in several fermentation configurations (Rane and Sims, 1993; 1996; Antonucci *et al.*, 2001; Anastassiadis *et al.*, 2002; Anastassiadis and Rehm, 2005; Moeller *et al.*, 2007; Papanikolaou *et al.*, 2008a). The culture conditions (e.g. initial C/N molar ratio, carbon source, pH evolution etc.) have serious impact upon the process of citric acid production (Anastassiadis *et al.*, 2002; Anastassiadis and Rehm, 2005; Papanikolaou *et al.*, 2002b; 2008a; 2008b). For instance, in one case, a *Y. lipolytica* strain growing on media with glycerol as sole carbon source and under nitrogen limitation, produced high concentrations of citric acid in shake-flasks fermentations whereas when grown in continuous bioreactor cultures under high agitation and aeration it accumulated high amounts of cellular lipids (Papanikolaou *et al.*, 2002b; Papanikolaou and Aggelis, 2002). Likewise, this process, as also demonstrated in the current investigation, is indeed significantly influenced by the strain used (Antonucci *et al.*, 2001; Kamzolova *et al.*, 2005; André *et al.*, 2009). In our study, Cit_{max} concentration achieved in the control experiment was 18.9 g L^{-1} during growth of strain ACA-YC 5033. Cit_{max} concentration of this strain was insignificantly reduced in fermentations where OMW was added. On the other hand, citric acid production by the strain W29 was strongly affected by the addition of OMW into the medium. In the current investigation, overall Cit_{max} concentration achieved (~47.0 g L^{-1} that was obtained during growth of the strain ACA-YC 5033 in the experiment with initial phenolic compounds concentration ~2.90 g L^{-1} and glucose ~80.0 g L^{-1}), although satisfactory, is somehow lower in comparison with the highest literature values reported so far (i.e. ranging between 50 and 160 g L^{-1}) when other *Y. lipolytica* and *C. oleophila* strains were cultivated on various substrates such as glucose, fats, glycerol and ethanol (Rane and Sims, 1993; 1996; Antonucci *et al.*, 2001; Anastassiadis *et al.*, 2002; Kamzolova *et al.*, 2003; 2005; Anastassiadis and Rehm, 2005; Moeller *et al.*, 2007; Papanikolaou *et al.*, 2008b). Moreover, the values of yield of citric acid produced per substrate consumed reported in the literature by strains growing on sugars (or similarly metabolized compounds such as glycerol) ranges between 0.40 and 0.80 g g^{-1} (Rane and Sims, 1993; 1996; Antonucci *et al.*, 2001; Anastassiadis *et al.*, 2002; Morgunov *et al.*, 2004; Anastassiadis and Rehm, 2005; Moeller *et al.*, 2007; Papanikolaou *et al.*, 2008a; André *et al.*, 2009; Makri *et al.*, 2010). Only in a very limited number of reports this conversion yield was >0.90 g g^{-1} (case of mutant *Y. lipolytica* strain growing on waste biodiesel-derived glycerol – see: Morgunov *et al.* 2013;

Papanikolaou *et al.* 2013). An experimental results overview of citric acid-producing yeasts when grown under various fermentation configurations including the current investigation is presented in Table 4.1..

Table 4. 1. Experimental results of citric acid-producing yeasts during their growth under various fermentation configurations.

Microorganism	Substrate	Culture mode	Cit ($g\ L^{-1}$)	$Y_{Cit/S}$ ($g\ g^{-1}$)	Reference
Saccharomycopsis lipolytica D 1805	Glucose	Batch culture, bioreactor	95.0	0.75	Briffaud and Engasser (1979)
Saccharomycopsis lipolytica NRRL Y7576	Glucose	Batch culture, bioreactor	51.5	0.71	Klasson *et al.* (1989)
Candida guillermondii IMK 1	Galactose	Batch culture, flasks	13.5	0.38	Gutierrez *et al.* (1993)
Candida lipolytica Y1095	Glucose	Fed–batch culture bioreactor	13.6-78.5	0.79	Rane and Sims (1993)
Yarrowia lipolytica ATCC 20346	Glucose	Fed-batch culture, bioreactor	50.0-69.0	0.52	Moresi (1994)
Candida lipolytica Y 1095	Glucose	Continuous culture recycling, bioreactor	40.0-50.0	0.72	Rane and Sims (1995)
Candida oleophila ATCC 20177	Glucose	Batch culture, flasks, bioreactor	50.1-79.1	0.55	Anastassiadis *et al.* (2002)
Yarrowia lipolytica ACA-DC 50109	Raw glycerol	Batch culture, flasks	35.1	0.44	Papanikolaou *et al.* (2002b)
Yarrowia lipolytica N1	Ethanol	Fed-batch culture, bioreactor	120.0	0.85	Kamzolova *et al.* (2003)
Candida lipolytica NRRL-Y-1095	n-Paraffins	Fed-batch culture bioreactor	30.0-40.0	0.99	Crolla and Kennedy (2004)
Candida oleophila ATCC 20177	Glucose	Continuous culture bioreactor	37.6-57.8	0.40	Anastassiadis and Rehm (2005)
Yarrowia lipolytica 187/1	Rapeseed oil	Fed-batch culture, bioreactor	135.1	1.55	Kamzolova *et al.* (2005b)
Yarrowia lipolytica ACA-DC 50109	Raw glucose	Batch culture, flasks	42.9	0.56	Papanikolaou *et al.* (2006)
Yarrowia lipolytica AWG-7	Glucose syrup	Batch culture, flasks	36.7	0.31	Rymowicz and Cibis (2006)

Microorganism	Substrate	Culture mode	Cit ($g\ L^{-1}$)	$Y_{Cit/S}$ ($g\ g^{-1}$)	Reference
Yarrowia lipolytica Wratislavia 1.31	Raw glycerol	Batch culture bioreactor	124.5	0.62	Rymowicz *et al.* (2006)
Yarrowia lipolytica Wratislavia AWG7			88.1	0.46	
Yarrowia lipolytica Wratislavia K1			75.7	0.40	
Strain	Substrate	Culture mode	Cit ($g\ L^{-1}$)	$Y_{Cit/S}$ ($g\ g^{-1}$)	Reference
Yarrowia lipolytica NRRL YB-423	Pure glycerol	Batch culture, flasks	21.6	0.55	Levinson *et al.* (2007)
Yarrowia lipolytica NCIM 3589	Raw glycerol	Batch culture, flasks	77.4	-	Imandi *et al.* (2007)
Yarrowia lipolytica ACA-DC 50109	Raw glycerol	Batch culture, flasks	62.5	0.56	Papanikolaou *et al.* (2008b)
Yarrowia lipolytica A-101-1.22	Raw glycerol	Batch culture bioreactor	112.0	0.60	Rymowicz *et al.* (2010)
Yarrowia lipolytica ACA-YC 5033	Raw glycerol	Batch culture, flasks	50.1	0.44	André *et al.* (2009)
Yarrowia lipolytica A-101	Pure glycerol	Batch culture bioreactor	0.44	66.5	Rywińska *et al.* (2010a)
	Raw glycerol		0.43	66.8	
Yarrowia lipolytica Wratislavia K1	Pure glycerol		53.3	0.34	
	Raw glycerol		36.8	0.25	
Yarrowia lipolytica Wratislavia 1.31	Raw glycerol	Fed-batch culture bioreactor	126.0	0.63	Rywińska *et al.* (2010b)
Yarrowia lipolytica Wratislavia AWG7			157.5	0.58	
Yarrowia lipolytica Wratislavia 1.31			155.2	0.55	
Yarrowia lipolytica Wratislavia AWG7	Raw glycerol	Repeated batch culture bioreactor	154.0	0.78	Rywińska and Rymowicz (2010)
Yarrowia lipolytica N15	Pure glycerol	Batch culture, flasks	19.08	0.55	Kamzolova *et al.* (2011)
		Fed-batch culture bioreactor	98.0	0.70	

Microorganism	Substrate	Culture mode	Cit ($g\ L^{-1}$)	$Y_{Cit/S}$ ($g\ g^{-1}$)	Reference
Yarrowia lipolytica Wratislavia AWG7	Pure glycerol	Continuous culture bioreactor	86.5	0.59	Rywińska *et al.* (2011)
			63.3	0.67	
Yarrowia lipolytica NG40/UV7	Pure glycerol	Fed-batch culture bioreactor	115.0	0.64	Morgunov *et al.* (2013)
	Raw glycerol		112.0	0.90	
Yarrowia lipolytica JMY1203	Raw glycerol	Batch culture, flasks	57.7	0.92	Papanikolaou *et al.* (2014)
***Yarrowia lipolytica* ACA-YC 5028**	**OMW-based media**	**Batch culture, flasks**	**8.4**	**0.25**	**Present study**
***Yarrowia lipolytica* ACA-YC W29**			**15.8**	**0.46**	
***Yarrowia lipolytica* ACA-YC 5033**	**OMW-based media**	**Batch culture, flasks**	**47.1**	**0.67**	
		Batch culture, flasks pasteurized	**15.5**	**0.68**	
		Batch culture bioreactor	**15.2**	**0.61**	
		Batch culture bioreactor pasteurized	**13.9**	**0.58**	

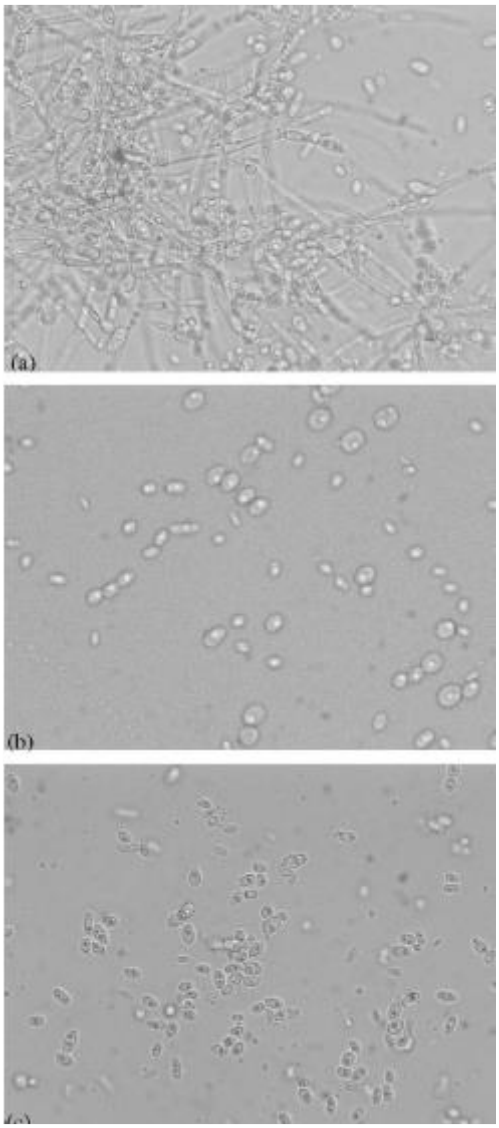


Figure 4. 1. The morphology of the yeast *Y. lipolytica* grown on glycerol is growth phase-dependent.

(a) Short true mycelia and pseudo-mycelia were predominant during biomass production phase; (b) obese yeast cells with large lipid globules appeared on lipogenic phase; and (c) small yeasts cells appeared on CA production phase which occurred simultaneously with the lipid turnover phase (Makri *et al.*, 2010)

Remarkable quantities of intra-cellular lipids were accumulated by *Y. lipolytica* strain W29 (up to 34% *w/w* in dry matter) and strain ACA-YC 5033 (up to 27% *w/w* in dry matter) in nitrogen-limited media supplemented with OMWs, although in the control experiment small lipid quantities (up to 11% *w/w*) were produced (see Tables 3.1. and 3.7.). The presence of OMWs in the medium favored this process. This is an interesting result since only in a few reports does the addition of natural compounds (e.g. *Teucrium polium* L. aqueous extracts) enhance the process of lipid accumulation inside yeast cells (Aggelis and Komaitis, 1999). It should be noted that in the cases in which non-negligible lipid accumulation was observed (i.e. cellular lipids >25% *w/w* of dry biomass – strains W29 and ACA-YC 5033 on OMW-based nitrogen-limited cultures), some reduction of lipids concentration in the early stationary growth phase was observed before significant amounts of citric acid began to be secreted into the medium, in full agreement with the literature [strain ACA-DC 50109 growing in nitrogen-limited glycerol-based bioreactor cultures (Makri *et al.*, 2010)].

Specifically, according to Makri *et al.* (2010), three distinct phases (namely biomass production phase, lipogenic phase and citric acid production phase) with specific morphological (Fig. 4.1.) and biochemical features characterizing yeast cells, were identified. During the exponential growth phase [biomass production phase; when high activity of NAD^+ dependent iso-citric dehydrogenase (NAD^+–ICDH) was detected] short true mycelia and pseudo-mycelia were predominant, while a few yeast-like cells were observed and high respiration activity was observed. Following, as growth proceeded, only yeast cells appeared in the culture, while the different percentage of lipid accumulated in the various growth phases was notably reflected on the cell size. Actually, large obese cells with discernible lipid globules appeared in the early stationary phase (lipogenic phase;

decreased activity of NAD^+-ICDH). Finally, lipid globule sizes were diminished (storage lipid turnover) during the late stationary phase (citric acid production phase; further drop in NAD^+–ICDH activity to minimal levels and a decrease in glycerol kinase activity) and significant quantities of citric acid were secreted in the growth environment. It is also of great interest to note that, microbial lipid turnover was a dominant event even for genetically modified strains that presented decreased expression of acyl-CoA oxidases used by Papanikolaou *et al.* (2009). The aforementioned strains under-express, or do not express at all, various acyl-CoA oxidases implicated in the catabolism (β-oxidation) of intracellular fatty acid. Thus the lipid in dry matter should, theoretically, have remained more or less constant at the stationary phase; a fact that, finally, did not happen suggesting that microbial lipid turnover is a universal phenomenon in *Y. lipolytica* strains (Papanikolaou *et al.*, 2009). The lipids synthesis of different strains of *Y. lipolytica* used in various configurations is presented in Table 4.2..

In the present study, the main cellular fatty acid of *Y. lipolytica* lipid produced during all trials was the $^{\Delta 9}$C18:1. Amounts of this fatty acid increased with the presence of waste into the growth medium. In several cases, high quantities of this fatty acid (>60% *w/w* of total lipids) were found into the cellular lipids (see Tables 3.4. and 3.5.). In general, the distribution of intra-cellular fatty acids in *Y. lipolytica* strains growing on glucose or other hydrophilic compounds (glycerol or ethanol) seems to be strain-dependent, while other parameters influencing the fatty acid composition of *Y. lipolytica* yeasts are the initial (for batch cultures) or the inlet (for continuous cultures) substrate concentration, the fermentation time and the dilution rate (Aggelis and Komaitis, 1999; Finogenova *et al.*, 2002; Papanikolaou *et al.*, 2008b; 2009; 2013; André *et al.*, 2009; Makri *et al.*, 2010). Similarly with the current study, the concentrations of cellular $^{\Delta 9}$C16:1 and $^{\Delta 9}$C18:1 fatty acids for another *Y. lipolytica* strain increased when the culture medium was enriched with OMWs. This was attributed to microbial adaptation on the high-phenol concentration media (Papanikolaou *et al.*, 2008a). Finally, Finogenova *et al.* (2002) reported significant decrease of cellular $^{\Delta 9}$C16:1 and $^{\Delta 9,12}$C18:2 and concomitant rise of cellular $^{\Delta 9}$C18:1 concentrations during growth of *Y. lipolytica* strain N1 in continuous cultures on ethanol used as substrate at constant dilution rate imposed and increased concentrations of inlet ethanol. It should be noted that the majority of *Y. lipolytica* strains lack the ability of *de novo* lipid accumulation (in contrast with the usual phenomenon of *ex novo* lipid synthesis in huge amounts), since the metabolism is shifted towards citric acid production (Papanikolaou *et al.*, 2009). It is possible that shift from lipid accumulation to citric acid synthesis is attributed to the extremely low concentration of nitrogen into the medium, since it is possible that some nitrogen quantities are requested in order for SCO to be produced

through *de novo* pathway regarding this yeast (Beopoulos et al., 2009; Makri *et al.*, 2010; Fontanille *et al.*, 2012; Papanikolaou *et al.*, 2013).

Table 4. 2. Lipid content of different strains of *Yarrowia lipolytica* used in various configurations.

Strain	Substrate	Culture mode	L ($g\ L^{-1}$)	$Y_{L/X}$ ($g\ g^{-1}$)	Reference
LGAM S(7)1	Glucose	Continuous culture, bioreactor	3.1	0.33	Aggelis and Komaitis (1999)
LGAM S(7)1	Mixtures of industrial fats	Batch culture, flasks	0.6-3.8	0.08-0.44	Papanikolaou *et al.* (2001)
ACA-DC 50109	Stearin	Batch culture, flasks, bioreactor	1.0-6.8	0.26-0.54	Papanikolaou *et al.* (2002)
LGAM S(7)1	Raw glycerol	Batch culture, flasks	0.3-.08	0.05-0.10	Papanikolaou and Aggelis (2002)
		Continuous culture, bioreactor	0.3-3.5	0.08-0.43	
ACA-DC 50109	Stearin	Batch culture, flasks	7.9	0.52	Papanikolaou *et al.* (2007)
		Batch culture, bioreactor	2.5-4.0	0.07-0.16	
ACA-YC 5033	Raw glycerol	Batch culture, flasks	2.0	0.31	André *et al.* (2009)
ACA-DC 50109	Pure glycerol	Repeated batch culture, bioreactor	0.4-1.0	0.12-0.22	Makri *et al.* (2010)
Po1g	Sugarcane bagasse hydrolysate	Batch culture, flasks	6.7	0.59	Tsigie *et al.* (2011)
Po1g	Rice bran hydrolysate	Batch culture, flasks	2.6-5.2	0.30-0.48	Tsigie *et al.* (2012)
MUCL 28849	Glucose, glycerol, volatile fatty acids	Fed-batch culture, bioreactor	12.4	0.40	Fontanille *et al.* (2012)
***Yarrowia lipolytica* ACA-YC 5028**	**OMW-based media**	**Batch culture, flasks**	**1.2**	**0.18**	**Present study**
***Yarrowia lipolytica* ACA-YC W29**			**1.9**	**0.28**	
***Yarrowia lipolytica* ACA-YC 5033**	**OMW-based media**	**Batch culture, flasks**	**0.5-1.4**	**0.10-0.48**	
		Batch culture, flasks pasteurized	**0.9**	**0.26**	
		Batch culture bioreactor	**1.1**	**0.27**	
		Batch culture bioreactor pasteurized	**1.0**	**0.22**	

Concluding, the three strains of the yeast *Y. lipolytica* presented efficient growth when cultivated on glucose-enriched OMWs. In nitrogen-limited fermentations, the presence of OMWs in the medium seemed to favor the accumulation of storage lipids for two of the tested strains. Satisfactory citric acid quantities were produced in nitrogen-limited media while non-negligible biomass production was observed in carbon-limited media. Both nitrogen and carbon-limited fermentations resulted in a remarkable decolorization and a non-negligible reduction of phenolic compounds in the media. Therefore, the tested *Y. lipolytica* strains (and specifically the strains W29 and ACA-YC 5033) can be considered satisfactory candidates for simultaneous OMWs bioremediation and the production of added-value compounds useful for the food industry.

Experiments were conducted in bioreactors in order to assess the biochemical behavior of the microorganism, considering the potential of preserving optimal growth conditions so as to obtain, if possible, better experimental results compared with the shake-flask cultures. Concerning the diversification of the two aforementioned fermentation systems it should be noted that in the present study in aseptic batch bioreactor cultures, biomass production seemed to be insignificantly enhanced (especially by means of X_{max}) compared to the respective shake-flask trials, while *Y. lipolytica* strain ACA-YC 5033 reached its kinetics plateau earlier in shake-flask than in bioreactor cultures. On the other hand, citrid acid production seemed to be slightly decreased in the bioreactor trials, while SCO production presented similar values. A general consideration concerning lipid accumulating microorganisms suggests that optimal conditions for the production of lipids are found in highly aerated and agitated bioreactors (Ratledge and Wynn, 2002). This is obviously in contrast with the results reported in the current thesis. In some agreement with the present report findings, Chatzifragkou *et al.* (2010) studied the ability of *Morierella isabellina* to accumulate lipids when grown on glucose in similar shake-flasks and bioreactor experiments. Bioreactor culture improved slightly the production of total biomass and lipid (L_{max} values 12.7 g L^{-1} in bioreactor cultures against 9.9 g L^{-1} in shake-flasks cultures) whereas as far as glucose assimilation was concerned, not any substantial differences between both culture modes were observed. Comparison between shake-flask and bioreactor experiments for *Thamnidium elegans* growing on glucose revealed that mycelial biomass production in the bioreactor was almost equal with the corresponding shake-flask experiment. However, the duration of the bioprocess was somehow shorter in the bioreactor, while the accumulated lipid in the bioreactor was slightly lower than that produced in the shake-flask trial. Moreover, cultivation in bioreactor was accompanied by a slightly higher uptake rate of glucose compared with the shake-flask experiments (Zikou *et al.*, 2013). Hence, it may be assumed - in agreement with the current study - that both cultivation strategies can be considered satisfactory for investigation and production of SCO.

At the third and fourth part of this study, the ability of *Saccharomyces cerevisiae* strain MAK-1 to grow on and convert glucose-enriched OMWs and blends of molasses and OMWs into biomass and ethanol in aseptic and non-aseptic shake-flask and in aseptic and non-aseptic aerated and non-aerated batch bioreactor cultures under was assessed. The removal of phenolic compounds from the medium and the decolorization of the medium were also tested. Specifically, at trials with OMW-based media, the addition of OMWs to the synthetic medium increased the production of biomass and ethanol compared with the control experiment (no OMWs added), which is an interesting result considering the presence of several inhibitors into the medium. Similar types of observations have been also observed in other types of yeasts (e.g. strains of the non-conventional species *Yarrowia lipolytica*) in which the addition of OMWs to the medium seem to "stimulate" the production of biomass and other metabolites, such as citric acid (Papanikolaou *et al.*, 2008a). Potentially, besides the existence of "inhibiting" compounds (such as phenols) found in the waste, "useful" nutrients (e.g. vitamins, growth-stimulating factors, micro- and macro-elements) may potentially exist, and this could be the reason for the enhancement of biomass and ethanol production in the presence of waste in the medium. Moreover, although low quantities of cellular lipids were produced by *S. cerevisiae* strain MAK-1 throughout aseptic shake-flasks trials, the presence of OMWs in the medium seemed to decrease (by means of monitoring the quantity of lipids produced in dry weight $Y_{L/X}$) lipid biogenesis compared with the control experiment (no OMW added). However, in contrast with this investigation, in the case of *Y. lipolytica* strains (see aforementioned results), when growth was performed in nitrogen-limited media (favoring the accumulation of storage lipids; Fakas *et al.*, 2009), higher quantities of lipids (in terms of principally $Y_{L/X}$) were produced in media enriched with OMWs than in the control experiments. The main cellular fatty acid of *S. cerevisiae* produced during all trials was $^{\Delta 9}$C18:1, but the presence of waste in the growth medium increased the amounts of this FA, while the respective quantities of $^{\Delta 9}$C16:1 and C18:0 drastically decreased, in accordance with results recorded for *Y. lipolytica* in similar trials (Papanikolaou *et al.*, 2008a). The almost complete absence of the fatty acid C18:0 in the cellular lipids of the microorganism in OMW-supplemented media, indicated potential activation of the cellular Δ9 desaturase (catalyzing the reaction C18:0 → $^{\Delta 9}$C18:1) by the presence of microbial inhibitors (e.g. gallic acid, caffeic acid, polyphenols, etc.). This is an interesting result since the addition of "inhibitory" compounds (such as essential oils and cyclopropenic acids) is considered to decrease the activity of cellular desaturases yielding the synthesis of more saturated cellular lipid in these cases in comparison with the control experiments (Moreton and Clode, 1988; Papanikolaou *et al.*, 2008c).

Concerning non-aseptic shake-flask and batch bioreactor fermentations, various amounts of molasses and OMWs were mixed and *Saccharomyces cerevisiae* MAK-1, presented noticeable biomass production (X_{max}=4.6-8.0 g L^{-1}; $Y_{X/TS}$=0.04-0.09 g g^{-1}) in these blends. Non negligible phenolic compounds concentration reduction (up to ~28% *w/w*) and remarkable decolorization (up to 60%) occurred. In non-aseptic shake-flask trials (TS_0~100.0 g L^{-1}), the addition of OMWs into molasses media did not significantly decrease biomass production (higher X_{max}=7.3 g L^{-1}; $Y_{X/TS}$=0.08 g g^{-1}; trial with initial phenolic compounds concentration ~3.94 g L^{-1}), comparing with the control experiment (X_{max}=8.0 g L^{-1}; $Y_{X/TS}$ =0.09 g g^{-1}). The values of total sugars concentration that remained unconsumed (TS_f=5.1-18.3 g L^{-1}) rose proportionally to the addition of OMWs indicating that the presence of the inhibiting compounds of that effluent (e.g. the phenolic compounds) in the molasses negatively affected the metabolism of the strain. As far as non-aseptic batch bioreactor cultures with constant OMWs and increasing addition of molasses were concerned, the addition of molasses into the medium reduced biomass production. *S. cerevisiae* strain MAK-1 did not grow sufficiently at the trial with TS_0~200.0 g L^{-1}, presumably due to increased concentration of melanoidins, mineral salts etc..

Comparing overall, shake-flask with batch bioreactor cultures it could be concluded that in non-aseptic bioreactor fermentations, biomass production was reduced whereas ethanol production was significantly increased. Non-aseptic batch bioreactor conditions could lead to a dramatic reduction in bioprocess cost. *S. cerevisiae* strain MAK-1 tested can be regarded as a satisfactory candidate for simultaneous OMWs bioremediation and the production of added-value metabolites. Moreover, it should be noted that in the present study, no phenolic compounds concentration reduction occurred due to agitation (and aeration) of the culture medium (no presence of *S. cerevisiae* cells), while the color intensity slightly increased (to approximately 5-7%), regardless of the initial concentration of phenolic compounds in the medium, providing evidence that removal of color and phenolic compounds was due to the biological activity of *S. cerevisiae*. It could be supposed, thus, that the phenolic compounds concentration reduction noted in our study could be based on the adsorption of the aforementioned compounds in the yeast cell surface or/and on their partial utilization as carbon source by the microorganism (Chtourou *et al.*, 2004).

As mentioned above, *S. cerevisiae* strain MAK-1 presented notable ethanol production in aseptic and non-aseptic shake-flask and batch bioreactor fermentations, when various amounts of OMWs and glucose or OMWs and molasses were added. Ethanol was produced in significant quantities in the medium despite the fact that growth was performed under aerated conditions (concerning fermentations in shake-flask and in bioreactor under aeration), referring to the Crabtree effect (Ratledge, 1991; Αγγελής, 2007). In the Crabtree (or “glucose”) effect, observed in several

yeast genera (the so-called “Crabtree-positive” yeasts) enzymes involved in the oxidative part of the metabolism (namely the Krebs cycle and the oxidative phosphorylation chain) are subjected to catabolite repression, with the metabolic network being directed towards the synthesis of ethanol via fermentative conversion, despite the significant presence of oxygen in the medium (Ratledge, 1991; Αγγελής, 2007). On the other hand, *S. cerevisiae* cells need oxygen (at least during the first hours after inoculation). Oxygen-dependent reactions led by the yeast cells result in the biosynthesis of sterols, unsaturated fatty acids and phospholipids needed in the formation of cell membranes and thus the continuation of growing (Ratledge, 1991).

Additionally, in the aerated trials rapidly after sugars exhaustion from the medium, ethanol was re-consumed, and new cell material was created. In this so-called “diauxic shift” or “biphasic growth”, the microorganism’s growth is divided into two phases; at the first growth phase assimilation of glucose *via* aerobic fermentation with ethanol and carbon dioxide as the major products occurs (Pyun *et al.*, 1989). When ethanol is available but no sugars are found in the medium and the dissolved oxygen concentration is above the critical level, previously produced ethanol is the only carbon source and serves as a substrate for further yeast growth (Pyun *et al.*, 1989; Zhang *et al.*, 1994). At this point, onset of biosynthesis of the enzymes responsible for the gluconeogenesis occurs, and this takes some time and causes a lag in the yeast growth (Zhang *et al.*, 1994). This phenomenon was observed in the present study as shown in Fig 3.26. (a). According to Piškur *et al.* (2006) the metabolism in Crabtree-positive yeast strains changes after exhaustion of glucose and accumulation of ethanol, with the requirement of certain transcription factors and enzymes. The (ethanol) “make-accumulate-consume” strategy (Fig. 4.2.) is relied on the evolution of *Saccharomyces* against its competitors as ethanol is toxic to most other microbes. It is considered therefore than in a (non-aseptic) sugar-rich environment (as in the current study or in most fermentations performed in the traditional wine-making facilities), *Saccharomyces* kills its competitors by producing ethanol, but can also consume the generated ethanol later. Alcohol dehydrogenase (Adh) catalyzes the acetaldehyde-to-ethanol conversion (during aerobic or anaerobic fermentation) in both directions. Genes *ADH1* (expressed constitutively) and *ADH2* (expressed only when the internal sugar concentration drops) encode cytoplasmic Adh activity.

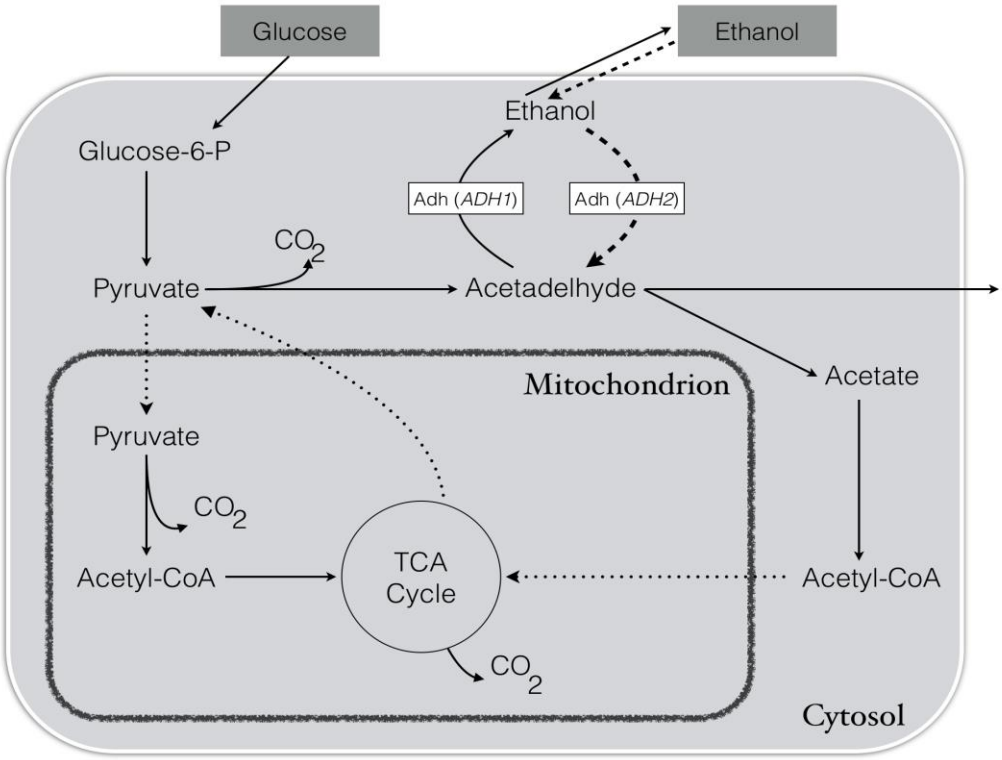


Figure 4. 2. Glucose and ethanol assimilation by *Saccharomyces cerevisiae* under aerobic conditions.

The conversion between acetaldehyde and ethanol is catalyzed by alcohol dehydrogenase (Adh). Gene *ADH1* is expressed constitutively, whereas gene *ADH2* is expressed only when the intra-cellular sugar concentration drops (adapted by Piškur *et al.*, 2006)

S. cerevisiae MAK-1 has been also used previously in simultaneous remediation-detoxification and bioethanol production process; this strain was cultivated on pasteurized grape must in which the fungicide quinoxyfen was added in various concentrations. Significant quantities of biomass were produced (~10.0 g L^{-1}) regardless of the addition of fungicide into the medium. Ethanol was synthesized in very high quantities (~106.0-119.0 g L^{-1}) and the fungicide concentration was reduced to ~79-82% *w/w* (Sarris *et al.*, 2009). Higher initial carbon source concentrations were employed (initial sugars quantities at ~260.0 g L^{-1}) compared with this study. The medium that was used (enriched grape must) was considered as ideal (including all the nutrients needed) for the growth of the microorganism. Thus, it appears that not the high initial total sugars concentration but potentially the high melanoidin (and, in essence, high phenolic compounds) content negatively affected the strain's metabolism in the present study, when growth was done in some of the OMWs/molasses blends (e.g. TS_0~200.0 g L^{-1}; Table 3.17.). Finally, comparing non-aseptic bioreactor with shake-flask fermentations, biomass production was reduced in reactor cultures by means of both X_{max} and $Y_{X/TS}$ values.

OMWs could be proposed either as a diluted and enriched with low-cost glucose or molasses promising substrate ($EtOH_{max}$=52.0 g L^{-1}, $Y_{EtOH/TS}$=0.46 g g^{-1}) or as a promising tap water substitute and substrate ($EtOH_{max}$=52.4 g L^{-1}, $Y_{EtOH/TS}$=0.48 g g^{-1}), for the biotechnological production of ethanol. In various fermentation processes, including production of ethanol, utilization of raw OMWs derived from press extraction systems that contain relatively high initial total sugars concentration (i.e. ~70.0 g L^{-1} or even higher) could be used without supplementary addition of molasses or glucose and thus the cost of the process could be further reduced (Crognale *et al.*, 2006).

As mentioned above, *S. cerevisiae* strain MAK-1 has been used previously in a simultaneous remediation – detoxification and bioethanol production bioprocess [growth on media in which a fungicide was added in varying concentrations (Sarris *et al.*, 2009)]. Ethanol was synthesized in very high quantities (~106.0–119.0 g L^{-1}) and the fungicide concentration was reduced to ~79–82% *w/w* (Sarris *et al.*, 2009). It is evident that ethanol quantities achieved in the above-mentioned study (Sarris *et al.*, 2009) were, in absolute values (g L^{-1}), significantly higher than in the present study, since remarkably higher initial carbon source concentrations were employed (initial sugars quantities at ~260.0 g L^{-1}). Also the medium used in the above-mentioned study (enriched grape must) was considered ideal (including all the nutrients needed) for growth of the microorganism. In any case, the maximum values of conversion yield of ethanol produced per sugar consumed were similar (=0.44 - 0.48 g g^{-1}) to those recorded in the current study. On the other hand, there are only a few reports in the literature suggesting the use of *S. cerevisiae* strains for the treatment of OMWs and the use of this waste fermentation process water directly for the production of ethanol. Bambalov *et al.* (1989) used various *Saccharomyces* strains growing on olive oil extraction effluents containing around 8.0 g L^{-1} of phenolic substances. Three strains did grow only in the 3-fold diluted medium (significantly lower total phenolic compounds compared with the current study) producing ethanol (EtOHmax =10.8-11.7 g L^{-1}; $Y_{EtOH/RS}$=0.38-0.41 g g^{-1}). Zanichelli *et al.* (2007) stated the necessity for removing the phenolic fraction and of the efficient enzymatic pre-treatment of the waste, before inoculating with a *S. cerevisiae* strain. The initial phenolic compounds concentration (after dilution) was 2.1 g L^{-1}, the glucose supplementation of the medium was up to 200 g L^{-1}, the total fermentation time was 65 days and the ethanol production reached 63 – 95 g L^{-1}. Massadeh and Modallal (2008) also proposed the use of OMWs as valuable effluents for the production of ethanol, using *Pleurotus sajor-caju* for the pretreatment of the waste so as to degrade the phenols in the waste. Following the pretreatment, 50% diluted and thermally processed OMW medium was inoculated with a *S. cerevisiae* strain leading to maximum ethanol production of 14.2 g L^{-1} after 48 h of fermentation. Yeasts are the most common microorganisms for the

biotechnological production of ethanol. Among them, numerous *S. cerevisiae* strains have been widely used and reported in literature for the production of high ethanol concentrations when cultivated in various substrates including wastes or low-cost materials like crude molasses (contaminated with fungicides) grape musts, flour hydrolysates, sorghum stalks (Lezinou *et al.*, 1994; Caylak and Vardar-Sukan, 1996; Roukas, 1996; Vallet *et al.*, 1996; Pinal *et al.*, 1997; Ergun and Mutlu, 2000; Navarro *et al.*, 2000; da Cruz *et al.*, 2003; Kiran *et al.*, 2003; Ghasem *et al.*, 2004; Yu and Zhang, 2004; Wang *et al.*, 2007; Sarris *et al.*, 2009). It is of crucial importance to mention that to our knowledge, literature presents no studies at all suggesting the use of mixtures of molasses and OMWs for the production of ethanol through alcoholic fermentation.

On the other hand, molasses (in some cases under completely non-aseptic conditions) have been used for ethanol production. As stated, literature reports the use of various microorganisms grown on molasses for the production of high-added value products such as gluconic acid, citric acid, FOS, pullulan, succinic acid, SCO and erythromycin (Roukas, 1996; El-Enhasy *et al.*, 2008; Liu *et al.*, 2008; Sharma *et al.*, 2008; Chatzifragkou *et al.*, 2010). As far as the production of ethanol is concerned, Baptista *et al.* (2006) have performed immobilization in polyurethane foam cubes in a fluidized-bed fermentor of two *S. cerevisiae* strains for continuous ethanol production using cane black-strap molasses as growth medium ($EtOH_{max}$=40 g L^{-1}; TS=100 g L^{-1}). Kopsahelis *et al.* (2007) performed repeated batch bioreactor trials with *S. cerevisiae* strain AXAZ-1 immobilized in various supports (e.g. delignified brewer's spent grains) with waste molasses utilized as carbon source, and ethanol up to 9.03% *v/v* (yield $Y_{EtOH/TS}$~0.45 g g^{-1}) was reported. Nahvi *et al.* (2002) have used a flocculating *S. cerevisiae* strain for the production of ethanol when growing on beet molasses ($EtOH_{max}$=5.6% *v/v*). Cáceres-Farfán *et al.* (2008) used an inoculum that was composed of the yeasts *Kluyveromyces marxianus* and *S. cerevisiae* grown on henequen (*Agave fourcroydes*) juice and molasses medium resulting in the production of $EtOH_{max}$~5.22% *v/v*. Literature also reports the use of *S. cerevisiae* strains grown on various media for the production of ethanol; ethanol production of around 50 g L^{-1} was achieved during growth of two *S. cerevisiae* strains on blends of molasses with commercial sucrose (Pinal *et al.*, 1997). Caylak and Vardar Sukan (1996) performed bioreactor trials using free or immobilized cells of *S. cerevisiae* strains on sucrose-based media and achieved final ethanol concentrations ranging between 88-110 g L^{-1}. *S. cerevisiae* ATCC 26602 was cultivated with flour hydrolysates being used as the sole carbon and energy source in shake-flask cultures and ~76 g L^{-1} ethanol were produced with ethanol production being optimized through the use of response surface methodology (Wang *et al.*, 2007). Besides *S. cerevisiae* strains, the other potential microbial producer of bioethanol is the bacterial species *Zymomonas mobilis* (Sreekumar and Basappa, 1991; Davis *et al.*, 2006; Lin and Tanaka, 2006; Ruanglek *et al.*, 2006;

Cazetta *et al.*, 2007; Sanchez and Demain, 2008). Sreekumar and Basappa (1991) used *Z. mobilis* strain ZM4 and its mutant *Z. mobilis* strain ZMI_2 cultivated on glucose used as substrate in flask or batch bioreactor experiments and produced 48-78 g L^{-1} of ethanol. The same strain, when grown on a hydrolyzed waste starch stream in repeated batch bioreactor experiments produced 39-54 g L^{-1} of ethanol (Davis *et al.*, 2006), while the respective values reported by Ruanglek *et al.* (2006) (utilization of *Z. mobilis* strain NRRL-B-14023) were 39-54 g L^{-1}. As presented in the previous chapters (see "Introduction") *Z. mobilis* uses the Entner-Doudoroff pathway to break down glucose, resulting in less biomass production, compared with the alcoholic fermentation performed by *S. cerevisiae* that uses the Embden-Meyerhof-Parnas (EMP) pathway (Lin and Tanaka, 2006; Sanchez and Demain, 2008). Biomass production by *Z. mobilis* strains is almost twofold less, but the ethanol yield on sugar assimilated for both microbial sources used is comparable (maximum theoretical ethanol yield $Y_{EtOH/TS}$=0.51 g g^{-1} for both microorganisms used; see a state-of-the art reviews: Sanchez and Demain 2008; Koutinas et al. 2014a). Therefore, although the conversion that is carried out by *Z. mobilis* strains can, in some cases, have as result slightly higher volumetric productivities achieved compared with the yeast fermentation (Lin and Tanaka, 2006), cultures led by *S. cerevisiae* attract interest due to the (higher) concentration of the process by-product (biomass) which can be utilized as animal feed. Moreover, recently the employment of other "non-classical" ethanol producers (e.g. bacteria of the genus *Thermoanaerobacter*) has been successfully performed in several types of substrates (e.g. glucose and/or lignocellulosic sugars) (Brynjarsdottir *et al.*, 2012).

Generally, a viable perspective of industrial-scale bioethanol production (high ethanol quantities, in terms of absolute values in g L^{-1}, conversion yields per sugar consumed in g g^{-1} and volumetric productivities in g L^{-1} h^{-1}) is both the utilization of the appropriate microbial strains and the potential of carrying out the bioprocess under non-aseptic conditions. Significantly lower energy consumption due to the absence of sterilization attributes a notable advantage of this production approach. Roukas (1996) has proposed ethanol production from non-sterilized beet molasses by free and immobilized *S. cerevisiae* cells using fed-batch culture ($EtOH_{max}$=53 g L^{-1}; $Y_{EtOH/TS}$=0.31 g g^{-1}; TS_0=250 g L^{-1}). Kopsahelis *et al.* (2012) have proposed a continuous ethanol production process performed in a multistage fixed bed tower bioreactor, in which trials with immobilized *S. cerevisiae* strain AXAZ-1 were performed with waste molasses (at TS_0=115 g L^{-1}) utilized as substrate. Trials were performed under both aseptic and completely non-aseptic conditions, and in accordance with the current investigation, ethanol production was almost completely unaffected by the non-aseptic conditions employed (ethanol up to 51.4 g L^{-1} with conversion yield $Y_{EtOH/TS}$~0.47 g g^{-1} was reported for the non-sterile trial) (Kopsahelis *et al.*, 2012). Additionally, the mutant strain of *Z.*

mobilis NS-7 has been cultivated on non-sterile glucose-based media. An ethanol concentration of 73 g L^{-1} was achieved with the curves of glucose consumption and ethanol biosynthesis being almost equivalent compared with the sterile media (Tao *et al.*, 2005). Finally Weuster-Botz *et al.* (1993) have developed a continuous fluidized bed reactor operation system for ethanol production by *Z. mobilis* strain ATCC 31821 using hydrolyzed starch without sterilization. The unsterile, 99% hydrolyzed, starch conversion resulted in 50 g L^{-1} ethanol production.

A summary of findings for the conversion of various carbon sources to ethanol in various fermentation configurations, including the current study is given in Table 4.3.. The originality of this part of the present study is based upon the simultaneous presence of molasses and OMWs (not pre-treated) as substrate and the growth of the strain used in completely non-sterile conditions, without contamination problems that could risk decreasing the efficiency of the fermentation. It should be also highlighted that the sole external addition of nutrients was that of yeast extract and $(NH_4)_2SO_4$, while in the current submission, the phenolic content of OMWs was much higher (i.e. c. 10 g L^{-1}) than the typical values found in the literature (i.e. ranging between 2 and 4 g L^{-1} - see: Crognale *et al*. 2006), indicating that OMWs can partly or even completely substitute tap water in the bioethanol fermentation in which molasses are usually used as carbon substrates, without significant negative effect in the performed bioprocess. Lack of salts addition, use of low volumes of water for dilution of the media, no aeration during bioreactor fermentations and no sterilization of the media could lead to tremendous reduction of the bioprocess cost. The tested *S. cerevisiae* strain MAK-1, thus, can be considered satisfactory candidate for bioremediation of molasses and OMWs blends and simultaneous production of added-value metabolites.

Table 4. 3. Strains producing ethanol from various carbon sources and their comparison with the current investigation

	Strain	Carbon Source	Concentration ($g\ L^{-1}$)	EtOH ($g\ L^{-1}$)	Reference
Saccharomyces cerevisiae	27817	Glucose	50.0-200.0	5.1-91.8	Vallet *et al.* (1996)
	L-041	Sucrose	100.0	25.0-50.0	Pinal *et al.* (1997)
	ATCC 24860	Molasses	1.6-50.0	5.0-18.4	Ergun and Multu (2000)
	Bakers' yeast	Sucrose	220.0	96.7	Caylak and Vardar Sukan(1996)
	Bakers' yeast	Sugar	150.0-300.0	53.0	Roukas (1996)
	Fiso	Galactose	20.0-150.0	4.8-40.0	da Cruz *et al.* (2003)
	A3			4.8-36.8	
	L52			2.4-32.0	
	GCB-K5	Sucrose	30.0	27.0	Kiran *et al.* (2003)
	GCA-II			42.0	
	KR_{18}			22.5	
	CMI237	Sugar	160.0	70.0	Navarro *et al.* (2000)
	2.399	Glucose	31.6	13.7	Yu and Zhang (2004)
	24860	Glucose	150.0	48.0	Ghasem *et al.* (2004)
	MAK-1	Grape must	250.0	106.4-119.2	Sarris *et al.* (2009)
	MAK-1	**OMW/glucose**	**115.0**	**52.0**	**Present study**
		OMW/molasses	**135.0**	**52.4**	
Zymomonas mobilis	ZM4 & ZMI_2	Glucose	100.0-200.0	47.6-78.0	Sreekumar and Basappa (1991)
	ATCC 29191	Hydrolyzed starch	120.0 (Glucose)	50.0	Weuster-Botz *et al.* (1993)
	NS-7	Glucose	150.0	73.2	Tao *et al.* (2005)
	ZM4	Hydrolyzed starch stream	80.0-110.0	39.0-54.0	Davis *et al.* (2006)
	NRRL-B-14023	Glucose	100.0	40.0-55.0	Ruanglek *et al.* (2006)
	ATCC 29191	Sucrose	200.0	55.8	Cazetta *et al.* (2007)

Concerning the ability of yeast strains to decrease the phenolic compounds concentration in OMW-based media (blends of OMWs with glucose and blends of OMWs with molasses), confusion exists in the literature indicating that in most cases, phenol removal by yeasts appears to be even a strain-dependent process. Specifically, in the present study, three *Y. lipolytica* strains, capable of producing lipids and citric acid were grown in carbon- and nitrogen-limited, shake-flask, glucose-enriched OMW-based media with various initial phenolic compounds concentration and simultaneous decolorization (up to ~63%) and removal of phenol content (up to ~51% *w/w*) with production of valuable products (X_{max}=12.7 g L^{-1}, citric acid up to 18.1 g L^{-1}) was observed. Moreover, *S. cerevisiae* strain MAK-1 was utilized to simultaneously convert glucose-enriched OMWs into ethanol and yeast biomass (X_{max}=18.9 g L^{-1}; $Y_{X/Glc}$~0.25 g g^{-1}) and perform detoxification (decolorization up to ~63% and removal of phenol content up to ~34% *w/w*) of the employed residue, under aseptic and completely non-aseptic shake-flask and bioreactor experiments. Finally, the same strain grown on molasses and OMWs blends under non-aseptic shake-flask and non-aseptic aerated and non-aerated batch bioreactor cultures, converting them into ethanol and yeast biomass (X_{max}=8.0 g L^{-1}; $Y_{X/Glc}$~0.09 g g^{-1}) as also leading to significant decolorization up to ~60% and moderate removal of phenol content up to ~28% *w/w*.

As mentioned previsously, some confusion exists in the literature concerning the ability of yeasts to perform decolorization and removal of phenolic compounds from media containing OMWs. For instance, *Y. lipolytica* strain ACA-DC 50109, a potential citric acid producer strain (Papanikolaou *et al.*, 2002b; 2008b; Makri *et al.*, 2010) was cultivated on OMW-based media enriched with glucose in which initial phenolics concentration was ~2.0 g L^{-1}, producing ~6.0 g L^{-1} of biomass and reducing the phenolic content to ~15% *w/w* (Papanikolaou *et al.*, 2008a). De Felice *et al.* (1997) and Scioli and Vollaro (1997) cultivated *Y. lipolytica* ATCC 20255 on low phenolic content OMWs (initial phenols at 0.2 g L^{-1}), that had initial concentration of sugars and olive oil of ~21 g L^{-1} and 16 g L^{-1} respectively. Although noticeable biomass (X_{max}=23.0 g L^{-1}) and lipase (700 U L^{-1}) quantities were produced, phenolic content of the treated OMWs was not reduced. Lanciotti *et al.* (2005) used low phenolic content (0.7 g L^{-1}) undiluted OMWs for the growth of many *Y. lipolytica* strains. Some strains lowered the phenolic compounds concentration to ~18% *w/w*, whereas others did not reduce the phenolic content of the waste. Biomass production was negatively affected by the addition of OMWs compared with the control trials. Bambalov *et al.* (1989) cultivated various *Saccharomyces*, *Torulopsis*, *Kloeckera* and *Schizosaccharomyces* strains on olive oil extraction effluents containing ~8.0 g L^{-1} of phenolic substances. None of the strains showed any growth in such high initial phenolic compounds media, whereas five strains did grow in 3-fold diluted medium. Moreover, Ettayebi *et al.* (2003) cultivated *Candida tropicalis* YMEC14 on

OMWs and directed the yeast metabolism towards biodegradation pathways using hexadecane as co-metabolite, resulting in 55% and 69% reduction of polyphenols and monophenols, respectively. *C. cylindracea* NRRL Y-17506 flask-cultured on diluted OMWs enriched with olive oil and salts, presented noticeable biomass and lipase production, whereas simultaneously a phenolic compounds reduction to ~36% *w/w* was observed (D'Annibale *et al.*, 2006). Ben Sassi *et al.* (2008) highlighted the potential of indigenous yeasts in detoxification of OMWs. Many yeast strains, isolated from local olive oil extraction factories, were cultivated on OMW effluents without nutritional supplements. A group of yeast isolates reduced significantly the concentration of total phenols (to ~44%) and COD values (to ~63%) and produced non-negligible biomass quantities (c. 6.0 g L^{-1}). A *Trichosporon cutaneum* strain was capable of completely utilizing phenolic compounds (OMWs ethyl-acetate extracts) when used as the sole carbon and energy source (at phenolic compounds concentration 0.8 - 2.0 g L^{-1}) and removed 85% *w/w* of total phenolic content (initial concentration 6.0 g L^{-1}) (Chtourou *et al.*, 2004). Literature also reports a number of molds (Sayadi and Ellouz, 1992; Fountoulakis *et al.*, 2002; Tsioulpas *et al.*, 2002; Aggelis *et al.*, 2003; Crognale *et al.*, 2006; Aloui *et al.*, 2007; Massadeh and Modallal, 2008; Lakhtar *et al.*, 2010) and bacteria (Piperidou *et al.*, 2000; Lamia and Moktar, 2003; Tziotzios *et al.*, 2007) capable of efficiently reducing phenolic compounds concentration in OMW-based media. Finally, besides the use of microorganisms there are reports in the literature of OMW bioremediation by enzyme treatment (Barakat *et al.*, 2010). A summary of findings for the detoxification of OMW media by various microorganisms, including the current study is given in Table 4.4..

Table 4. 4. Detoxification (decolorization and reduction of phenolic compounds) of OMW-based media and production of compounds (if occurred) by various microorganisms; comparison with the present study.

Microorganism	OMW-media	Detoxification	Products	Reference
Phanerochaete chrysosporium	Diluted OMW (20%)	50% decolorization (6 days)	-	Kissi *et al.* (2001)
Pleurotus ostreatus		50% decolorization (12 days)	-	
Pleurotus spp. strains	Diluted OMW	69-76% phenol removal	Laccase	Tsioulpas *et al.* (2002)
Pleurotus ostreatus strains (LGAM P113 & P115)	Diluted OMW	50-80% phenol removal	Laccase	Aggelis *et al.* (2003)
Panus tigrinus (CBS 577.79)	Diluted OMW glucose enriched	-	Laccase Manganese peroxidase	Fenice *et al.* (2003)
Lentinula edodes strains	Diluted OMW	65% decolorization 75% phenol removal	Laccase Biomass	Lakhtar *et al.* (2010)
Geotrichum candidum	OMW/cheese whey (20:80)	55% decolorization 55% phenol removal	Biomass	Aouidi *et al.* (2004)
Lactobacillus plantarum	Fresh OMW	58% decolorization 46% phenol removal	Lactic acid	Lamia and Morktar (2003)
Lactobacillus paracasei	OMW/cheese whey (10:90)	47% decolorization 23% phenol removal	-	Aouidi *et al.* (2009)
Trichosporon cutaneum	OMW ethyl-acetate extracts	85% (*w/w*) phenol removal	Biomass	Chtourou *et al.* (2004)
Yarrowia lipolytica (ACA-DC 50109)	Diluted OMW glucose enriched	36% decolorization 15% (*w/w*) phenol removal	Biomass Citrate	Papanikolaou *et al.* (2008)
Indigenous yeasts	OMW (no nutritional supplement)	44% (*w/w*) phenol removal	Biomass	Ben Sassi *et al.* (2008)
Yarrowia lipolytica (ATCC 20255)	OMW enriched with olive oil	-	Biomass Lipase	Scioli and Volaro (1997) De Felice *et al.* (1997)

Microorganism	OMW-media	Detoxification	Products	Reference
Candida tropicalis (YMEC 14)	OMW/hexadecane blends	55% monophenols 69% polyphenols removal	-	Ettayebi *et al.* (2003)
Candida cylindracea (NRRL Y-17506)	Diluted OMW enriched with olive oil and salts	36% (*w/w*) phenol removal	Biomass Lipase	D'Annibale *et al.* (2006)
***Yarrowia lipolytica* strains**	**Diluted OMW glucose enriched**	**63% decolorization 51% (*w/w*) phenol removal**	**Biomass Citric acid Microbial oil**	**Present study**
***Saccharomyces cerevisiae* (MAK-1)**	**Diluted OMW glucose enriched**	**63% decolorization 34% (*w/w*) phenol removal**	**Biomass Ethanol**	
	OMW/molasses	**60% decolorization 28% (*w/w*) phenol removal**	**Biomass Ethanol**	

The ability of higher fungi to break down phenolic compounds is based on the secretion of the extra-cellular oxidases (ligninolytic enzymes) laccase, lignin peroxidase and manganese dependent (or independent) peroxidase (Fountoulakis *et al.*, 2002; Aggelis *et al.*, 2003; Crognale *et al.*, 2006; Lakhtar *et al.*, 2010). The secretion of these enzymes is strain-dependent and influenced by various culture conditions (Sayadi and Ellouz, 1992; Aggelis *et al.*, 2002; Tsioulpas *et al.*, 2002; Crognale *et al.*, 2006; Aloui *et al.*, 2007; Massadeh and Modallal, 2008). Non-genetically modified yeast strains (like natural *Y. lipolytica* strains and wild *S. cerevisiae* strains), in general, lack in the mechanisms of producing such types of enzymes (Papanikolaou *et al.*, 2008a), whereas heterologous expression and production of laccases from basidiomycetes in *Y. lipolytica* strains has been achieved (Jolivalt *et al.*, 2005). Thus, the removal of phenol compounds and the decolorization of OMWs that are subjected to fermentation by these yeast species through the use of the above-mentioned enzymes, should be excluded. Rizzo *et al.* (2006) suggested that a potential exclusively physical mechanism involves the establishment of weak and reversible interactions, mainly between anthocyanins and yeast walls by adsorption. It could be supposed, thus, that the phenolic compounds concentration reduction noted in our study could be based on the adsorption of the aforementioned compounds in the yeast cell surface or/and on their partial (potentially very weak) utilization as carbon source by the microorganism (Chtourou *et al.*, 2004).

There are various reports in literature suggesting the use of bacteria (Kumar and Chandra, 2006), yeasts (Tondee *et al.*, 2008a) and fungal strains (Miranda *et al.*, 1996) employed for decolorization of molasses. For instance, the fungi *Cunninghamella echinulata* and *Mortierella isabellina* were cultivated on molasses, and growth was accompanied by non-negligible substrate decolorization, reaching up to ~75% for *C. echinulata* (400 h of culture) and ~20% for *M. isabellina* (200 h after inoculation) (Chatzifragkou *et al.*, 2010). Moreover, Metsoviti *et al.* (2011) used waste molasses (with TS_0 concentrations 20 g L^{-1} and 30 g L^{-1}) as growth medium for *Leuconostoc mesenteroides* so as to produce bacteriocin. Decolorization up to ~27% of this residue was performed. Concerning the OMWs color reduction, Assas *et al.* (2002) cultivated *Geotrichum candidum* on fresh OMWs and achieved decolorization of 75% of this effluent. A *Phanerochaete chrysosporium* strain investigated led in 6 days to more than 50% of color removal when cultivated on OMWs compared to a *Pleurotus ostreatus* strain which reached similar results in the same conditions in 12 days (Kissi *et al.*, 2001). Lamia and Moktar (2003) used *Lactobacillus plantarum* and achieved 58% decolorization of the effluent. Finally, one important point that should be pointed out is that in some cases reported in the international literature, despite significant removal of the phenolic compounds from the growth medium that occurs due to biological activity, it is possible that the remaining phenolic compounds after biotreatment may contain remarkable toxicity (Tsioulpas *et al.*, 20002).

Concluding, the three strains of yeast *Y. lipolytica* used, presented efficient growth when cultivated on glucose-enriched OMWs. In nitrogen-limited fermentations, the presence of OMWs in the medium seemed to favor the accumulation of storage lipids for two of the tested strains. Satisfactory citric acid quantities were produced in nitrogen- limited media while non-negligible biomass production was observed in carbon-limited media. In some trials (using strain W29 and mainly strain ACA-YC 5033), the addition of OMWs in the medium seemed to favor the accumulation of storage lipids suggesting that OMWs seemed to be a “lipogenic” substrate as the presence of OMWs stimulated a reserve lipid accumulation process. Both nitrogen and carbon-limited fermentations resulted in a remarkable decolorization and a non-negligible reduction of phenolic compounds in the media. The tested *Y. lipolytica* strains (and specifically the strains W29 and ACA-YC 5033) can be considered satisfactory candidates for simultaneous OMWs bioremediation and the production of added-value compounds useful for the food industry. *S. cerevisiae* strain MAK-1 presented efficient growth when cultivated on glucose-enriched OMW-based media in aseptic and non-aseptic shake-flask and bioreactor experiments and when cultivated on non-aseptic blends of OMWs and molasses. Satisfactory biomass and ethanol quantities were synthesized. A remarkable decolorization and a non-negligible reduction of phenolic compounds in

the OMW-based media occurred. Non-aseptic batch bioreactor conditions (no contamination problems occurred, that could risk decreasing the efficiency of the fermentation) could lead to a dramatic reduction in bioprocess cost. The sole external addition of nutrients was that of yeast extract and $(NH_4)_2SO_4$ while in the current study, the phenolic content of OMWs was much higher than the typical values found in the literature, indicating that OMWs can partly or even completely substitute tap water in the bioethanol fermentation in which molasses are usually used as carbon substrates, without significant negative effect in the performed bioprocess. *S. cerevisiae* strain MAK-1, can be regarded as a satisfactory candidate for simultaneous OMWs bioremediation and the production of added-value metabolites. The novelty of the present study is based upon the simultaneous bioremediation of OMWs and production of (added-)value products (such as citric acid, single cell oil, ethanol and biomass) in some cases under completely non-aseptic configurations as also in the use of OMWs (not pre-treated) and molasses as blended substrate.

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SHORT BIO

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