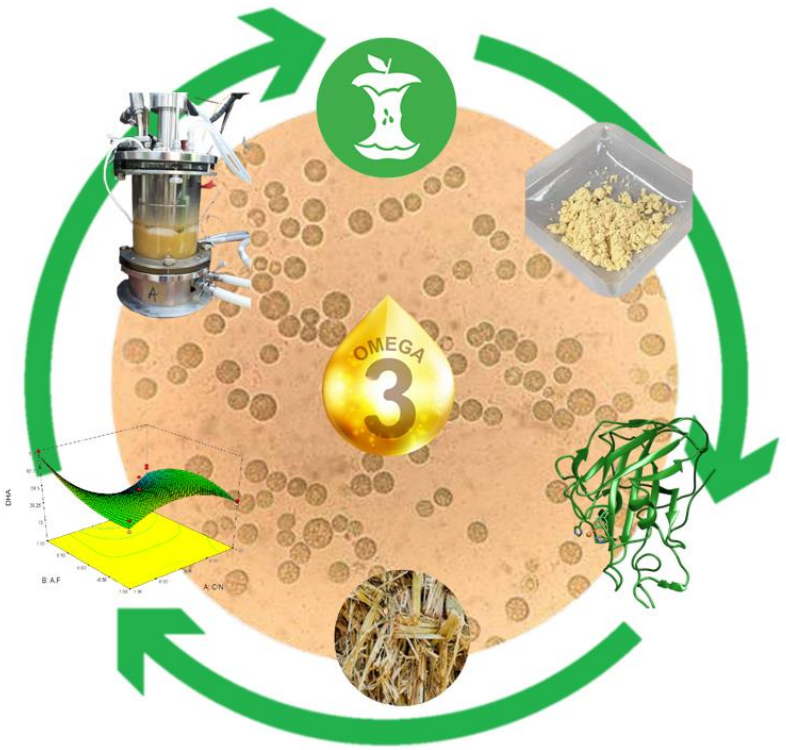
Utilization of biowaste and lignocellulosic biomass for the production of high added-value metabolites from microalgae

Angelina Chalima

Doctoral Dissertation



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Αφιερωμένο στους πιο αγαπημένους και
μακροχρόνιους συνοδοιπόρους μου,
τους γονείς μου Βάσω και Απόστολο
και τον αδερφό μου Θοδωρή

«No thief, however skillful, can rob one of knowledge, and that is why knowledge is the best and safest treasure to acquire.”

L. Frank Baum

«Η έγκριση της διδακτορικής διατριβής από την Ανωτάτη Σχολή Χημικών Μηχανικών του Ε.Μ.Πολυτεχνείου δεν υποδηλώνει αποδοχή των γνωμών του συγγραφέα. (Ν. 5343/1932, Άρθρο 202)».

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Αξιοποίηση λιγνινοκυτταρινούχου βιομάζας και βιοαποβλήτων για την παραγωγή προϊόντων υψηλής προστιθέμενης αξίας με τη χρήση μικροφυκών

Σκοπός της συγκεκριμένης διδακτορικής διατριβής ήταν ο σχεδιασμός και η εγκαθίδρυση βιοδιεργασιών, με προοπτική βιομηχανικής εφαρμογής, που επιτρέπουν την κατανάλωση του οργανικού περιεχομένου ρευμάτων αποβλήτων και την παράλληλη αξιοποίησή του για την παραγωγή μεταβολιτών μικροφυκών, υψηλής διατροφικής αξίας. Τα μικροφύκη αποτελούν μια κατηγορία μικροοργανισμών, ορισμένοι εκ των οποίων είναι γνωστοί και ευρέως χρησιμοποιούμενοι για την ικανότητα συσσώρευσης ωμέγα-3 λιπαρών οξέων. Τα ω-3 λιπαρά οξέα αποτελούν απαραίτητο, λειτουργικό συστατικό της ανθρώπινης διατροφής, ενώ οι πηγές πρόσληψης είναι περιορισμένες. Συνεπώς διακρίνονται από αυξημένη ζήτηση και υψηλό κόστος πώλησης. Η συνδυασμένη μείωση απόβλητων ουσιών, στα πλαίσια της βιοεξυγίανσης, με τη μέγιστη δυνατή παραγωγή ενός προϊόντος προστιθέμενης αξίας αποτέλεσε την κεντρική ιδέα της διατριβής. Για το σκοπό αυτό πραγματοποιήθηκε ενδελεχής βιβλιογραφική ανασκόπηση για την εύρεση και προμήθεια ενός στελέχους μικροφύκους ικανού να αναπτύσσεται σε πληθώρα υποστρωμάτων και να παράγει το ω-3 λιπαρό οξύ, εικοσιδύοεξανοϊκό οξύ, σε μεγάλο ποσοστό του συνολικού λίπους του κυττάρου. Το έλαιο μικροφυκών πλούσιο σε εικοσιδύοεξανοϊκό είναι απαραίτητο συστατικό βρεφικών σκευασμάτων, στα οποία η εναλλακτική χρήση ιχθυελαίου έχει αποδειχτεί βλαβερή. Το μικροφύκος *Crypthecodinium cohnii* ATCC 30772 επιλέχθηκε και αναπτύχθηκε σε διαφορετικά οργανικά οξέα που αποτελούν προϊόν αναερόβιας, σκοτεινής ζύμωσης βιοαποβλήτων, καθώς και σε απλά σάκχαρα που προκύπτουν ως προϊόντα υδρόλυσης γεωργικών υπολειμμάτων. Η επιτυχής ανάπτυξη των κυττάρων, ύστερα από κατανάλωση του συνολικού διαθέσιμου οργανικού φορτίου και εκχύλιση ελαίου, πλούσιου σε ω-3, επιβεβαίωσε την καταλληλότητα του στελέχους. Το εμπόδιο της τοξικότητας των οργανικών οξέων σε υψηλές συγκεντρώσεις παρακάμφθηκε με την εφαρμογή καλλιεργειών ημιδιαλλείποντος έργου σε βιοαντιδραστήρες εργαστηριακής κλίμακας.

Αξιοποίηση λιγνινοκυτταρινούχου βιομάζας και βιοαποβλήτων για την παραγωγή προϊόντων υψηλής προστιθέμενης αξίας με τη χρήση μικροφυκών

Στη συνέχεια, το στέλεχος αναπτύχθηκε επιτυχώς σε διήθημα υγρών κλασμάτων επεξεργασμένου βιοαποβλήτου, προερχόμενα από βέλγικη μονάδα επεξεργασίας λυμάτων, σε καλλιέργεια ημιδιαλλείποντος έργου με συνθήκες σταθερού pH. Ακολούθησαν προσπάθειες βελτιστοποίησης των συνθηκών και της μεθόδου καλλιέργειας με σκοπό την αύξηση της τελικής παραγωγής ωμέγα-3 λιπαρού οξέος. Οι προσπάθειες αυτές βασίστηκαν αφενός στην εξέταση ξεχωριστών παραμέτρων καλλιέργειας και αφετέρου στην μελέτη των συνεπειών της συνδυαστικής μεταβολής ορισμένων εξ αυτών για την εξαγωγή ενός μαθηματικού μοντέλου προσδιορισμού της παραγωγής ω-3, με χρήση της μεθοδολογίας αποκριτικών επιφανειών. Αποδεικνύεται πως ο ρυθμός αερισμού, καθώς και η πηγή και η ποσότητα αζώτου, αποτελούν σημαντικές παραμέτρους για τη συσσώρευση ωμέγα-3 λιπαρών από τα κύτταρα.

Παράλληλα, στα πλαίσια της βιομετατροπής λιγνοκυτταρινούχων αποβλήτων, επιδιώχθηκε η ανάπτυξη του ίδιου στελέχους σε απλά σάκχαρα προερχόμενα από υδρολύματα, προκειμένου να διαπιστωθεί η προοπτική εφαρμογής του *C. cohnii* σε ένα βιοδιυλιστήριο δεύτερης γενιάς. Τα κύτταρα καλλιεργήθηκαν επιτυχώς σε υδρολύματα βιομάζας πεύκου και οξιάς προκατεργασμένων με την τεχνική της υδρολυτικής κλασμάτωσης (organosolv), υπό διαφορετικές συνθήκες, ωστόσο διαπιστώθηκε ευαισθησία στη συσσώρευση παρεμποδιστών. Τα λιγνοκυτταρινούχα υποστρώματα προεπεξεργασμένα με τη χρήση των οργανικών διαλυτών αιθανόλη και τετραϋδροφουράνιο, υπό ήπιες συνθήκες, ευνόησαν την παραγωγή ωμέγα-3 λιπαρών από την κατανάλωση των εν λόγω υδρολυμάτων. Στην περίπτωση όλων των κατηγοριών αποβλήτων που εξετάστηκαν- οργανικών οξέων και υδρολυμάτων-, προέκυψε το συμπέρασμα πως η ενίσχυση του διαθέσιμου οργανικού φορτίου προς κατανάλωση, επιδρά θετικά στην τελική παραγωγή βιομάζας και ωμέγα-3.

Αξιοποίηση λιγνινοκυτταρινούχου βιομάζας και βιοαποβλήτων για την παραγωγή προϊόντων υψηλής προστιθέμενης αξίας με τη χρήση μικροφυκών

Όσον αφορά τα υδρολύματα λιγνοκυτταρινούχων αποβλήτων, η προσπάθεια ενίσχυσης της απόδοσης της υδρόλυσης οδήγησε στην ετερόλογη έκφραση και το χαρακτηρισμό ενός νέου ενζύμου της κατηγορίας των λυτικών πολυσακχαριτικών μονοοξυγενασών από το θερμόφιλο μύκητα *Thermothelomyces thermophila* ATCC 42464. Τα εν λόγω ένζυμα έχουν την ικανότητα να ενισχύουν την ενζυμική υδρόλυση λιγνοκυτταρινούχων υποστρωμάτων με τη δημιουργία οξειδωμένων πολυσακχαριτικών αλυσίδων που διασπώνται στη συνέχεια από γλυκόζυλο-υδρολάσες. Έτσι προκύπτει περίσσεια απλών σακχάρων στο τελικό υγρό, η οποία μπορεί να αξιοποιηθεί για την αυξημένη ανάπτυξη των καλλιεργήσιμων μικροφυκών και την επιπλέον παραγωγή ελαίου. Η πρωτεΐνη επέδειξε την ικανότητα οξείδωσης του β-1,4-γλυκοζιτικού δεσμού πολύ- και ολιγο-σακχαριτών της κυτταρίνης, καθώς και τη δυνατότητα συνεργιστικής δρασης σε συνδυασμό με το ευρέως χρησιμοποιούμενο εμπορικό σκεύασμα Cellic® CTec2. Η ενίσχυση της υδρόλυσης με χρήση νέων ενζύμων αποτελεί κρίσιμο βήμα για την αποδοτικότερη μετατροπή της λιγνοκυτταρίνης σε ω-3 λιπαρά, καθώς αποδείχθηκε πως το εξεταζόμενο μικροφύκος δεν έχει το ίδιο την ικανότητα έκκρισης κυτταρινολυτικών ενζύμων για την κάλυψη των διατροφικών του αναγκών.

Εν κατακλείδι, η διατριβή περιελάμβανε μελέτες βελτιστοποίησης της βιομετατροπής του βλαβερού οργανικού φορτίου διαφορετικών ρευμάτων αποβλήτων σε υψηλής λειτουργικής αξίας, ωμέγα-3 λιπαρά οξέα, βασισμένες, τόσο στην εφαρμογή των κατάλληλων συνθηκών της διεργασίας καλλιέργειας, όσο και στην εξασφάλιση ενός πλούσιου σε θρεπτικά συστατικά ρεύματος τροφοδοσίας, προερχόμενου από επεξεργασία αποβλήτων.

ABSTRACT

The PhD thesis aimed at the development of sustainable biotechnological processes of industrial potential, which will allow the depletion of environmentally polluting organic content of biowaste and lignocellulosic biomass, coupled with the subsequent production of high-added value metabolites from microalgae. Microalgae comprise a vast category of microorganisms, some of which are known for their ability to accumulate omega-3 fatty acids, a necessary component of human diet of limited availability. Therefore, the main idea of the thesis included the optimization of omega-3 production, coupled with the consumption of waste material, through microalgae bioconversion. Initially, the scientific work carried out included the screening of literature for the disclosure of a microalgae species with high potential as means of bioconversion of agricultural and municipal waste to omega-3 fatty acids. The strain chosen, namely *Crypthecodinium cohnii* ATCC 30772, was examined in the laboratory regarding its ability to grow by assimilating different volatile organic acids, present in the liquid fraction of anaerobically pretreated biowaste, or simple sugars released from the enzymatic hydrolysis of lignocellulosic residues. The chosen microorganism responded favorably by assimilating the total available organic content of each waste feed, while the produced microalgal biomass was rich in the omega-3 fatty acid, docosahexaenoic acid, a functional product of high nutritional and economic value. Demand for microalgal oil, rich in docosahexaenoic acid, is especially high in cases of infant formulas production, where the alternative of fish oil derived omega-3 has been deemed harmful and inappropriate. The inhibitory effect of high volatile organic acid concentration inside the cultivation vessel was eliminated by adopting a fed-batch, pH-auxostat cultivation mode in lab-scale bioreactors.

Moreover, the strain was successfully cultivated in fed-batch cultures with a feed of a real biowaste effluent, from an operating Belgian treatment plant. The evaluation of the accumulation of omega-3 fatty acid under different cultivation conditions was carried

out, to enable the researcher to study the responses of the strain to variable cultivation techniques, as well as to different feed compositions. The determination of the optimum fermentation conditions, which allow harvesting of the highest amount of omega-3 fatty acids, was completed both by separate examination of the experimental results of each condition and by preparing a mathematical model to predict the docosahexaenoic acid production under specific fed-batch fermentation conditions, through response surface methodology. The aeration rate and nitrogen source and availability were found to be critical parameters regarding the docosahexaenoic acid accumulation.

At the same time, the assimilation of hydrolysate-derived monosaccharides by the same strain was also evaluated in an attempt to integrate the microalga in a second generation biorefinery concept. The cells were able to grow both on pentoses and hexoses of beechwood and pine hydrolysates, but exhibited a sensitivity to inhibitors released during biomass pretreatment. Organosolv pretreatment under mild conditions with ethanol or tetrahydrofuran was proven more effective in producing a lignocellulosic material with higher potential as substrate for bioconversion. In the case of both waste-derived streams examined- that is the liquid fraction of dark fermentation of biowaste and lignocellulosic hydrolysates- it was concluded, that an enhancement of the carbon content of the feed is favorable for a higher biomass and DHA recovery.

Regarding lignocellulosic biomass as carbon source for the growth of the microalga, attempts to enhance the hydrolysis performance, included also the heterologous production and characterization of a new enzyme from the thermophilic fungus *Thermothelomyces thermophila* ATCC 42464, belonging in the category of lytic polysaccharide monooxygenases. These enzymes are known for assisting the action of glycosyl hydrolases by generating oxidized polysaccharide chains and enhancing the final sugar content of the liquid to be used for bioconversion purposes. The enzyme successfully oxidatively cleaved the β-1,4-bond of poly- and oligo-saccharides of

cellulose and exhibited synergy with the commercial enzyme mixture Cellic® CTec2 in glucose generation from pretreated biomass. Enhancement of the hydrolysis efficiency with newly discovered enzymes is a critical step towards the increased bioconversion of lignocellulose to omega-3 fatty acids, since the examined strain wasn't able to secrete cellulolytic enzymes for itself, to support its nutrition needs by directly assimilating lignocellulosic substrates.

In conclusion, this PhD thesis includes the research conducted in order to optimize the bioconversion of polluting organic content of various waste streams to functional omega-3 fatty acids by microalgae. This was based not only on the application of the best cultivation conditions for each feed, but also on the establishment of waste-derived feeds, rich in nutrients, for microalgal growth.

LIST OF ABBREVIATIONS

β-GL	**β-Glucosidase**
AA	Auxiliary Activity
ACO	Acetone
A.F	Air Flow
AFEX	Ammonia Fiber Explosion
ALA	α-linolenic acid
Am	Ammonium Sulfate
AmNt	Ammonium Nitrate
ANOVA	Analysis Of Variance
ARA	Arachidonic acid
ATP	Adenosine Triphosphate
CBH	Cellobiohydrolase
CBM	Carbohydrate Binding Module
CCD	Central Composite Design
CDH	Cellobiose Dehydrogenase
CE	Circular Economy
CMC	Carboxymethyl-Cellulose
CoA	Coenzyme A
DCW	Dry Cell Weight
DF	Dark Fermentation
DHA	Docosahexaenoic acid
DI	Deionized
DM	Dry Matter
DNS	Dinitrosalicylic acid
DO	Dissolved Oxygen
DoE	Design of Experiments
DPA	Docosapentaenoic acid
DS	Degree of Synergy
EG	Endoglucanase
EPA	Eicosapentaenoic acid
EtOH	Ethanol
FA	Fatty Acids
FAME	Fatty Acid Methyl Esters
FID	Flame Ionization Detector
FPU	Filter Paper Units
GC	Gas Chromatography

HMF	Hydroxy-methyl-furfural
HPAEC	High Performance Anion Exchange Chromatography
HPLC	High Performance Liquid Chromatography
LPMO	Lytic Polysaccharide Monooxygenase
MES	2-*N*-morpholino-ethanesulfonic acid
MS	Mass Spectrometry
Nt	Sodium Nitrate
OVAT	One Variable at a Time
PASC	Phosphoric Acid Swollen Cellulose
PKS	Polyketide Synthase
***p*NP**	*p*-Nitrophenyl-
PUFA/LC-PUFA	Polyunsaturated Fatty Acids/ Long Chain PUFA
RID	Refractive Index Detector
RSM	Response Surface Methodology
SA	Sodium Acetate
SCO	Single Cell Oil
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TAG	Triacylglycerides
TCA	Tricarboxylic Acid Cycle
TFA	Total Fatty Acids
THF	Tetrahydrofuran
TRS	Total Reducing Sugars
Ur	Urea
VFA	Volatile Fatty Acids
VGF	Vegetable Garden Food
YE	Yeast Extract

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Theoretical Background

1. Environmental biotechnology- Basic terms

1.1 Circular economy

The constant depletion of finite earth's resources creates the need for a circular economy (CE), which will ensure the efficient utilization of the waste streams of conventional industrial processes, thus creating "closed" production loops [1]. More specifically, a circular economy is, for many engineers and economists, the solution to the current linear model of industrial production. Although there exist different definitions, the term generally describes an economy model that includes industrial processes exploiting waste streams as feedstock, resulting in an overall minimization of raw-material use. The environmental benefit, therefore, lies not only with the preservation of resources and minimum waste production, but also with avoiding the energy-intensive activities of searching for and harvesting the necessary raw materials [2]. Such "green" processes- that promote environmental stability-should not only accept a feedstock of waste streams, but also ensure the minimum waste production for themselves [3]. Under these circumstances, the bioconversion of waste, utilizing microorganisms, appears to be a very promising solution. Bioprocesses normally result in fewer by-products, thus being easily incorporated in a "green" strategy.

1.2 Bioprocesses

Biotechnology can be broadly defined as the total of the techniques using "living organisms or parts of organisms to make or modify products, improve plants or animals, or develop microorganisms for specific uses" [4]. If we focus on the first part of the definition, a bioprocess can be described as a biotechnological process that utilizes part of or the whole organism for the development of a specific product. Based on this definition it is clear how a bioprocess can be incorporated in a CE plan. Bioprocesses

produce less toxic by-products, if any, and they are even widely used as means of consuming the organic content of a variety of different waste streams, while at the same time producing high added-value metabolites for further exploitation [5–7]. Microbial metabolic activities result in highly specific products and therefore various microorganisms are used for the valorization of raw material to functional commodities. These are called microbial cell factories [8]. Apart from the selection of a specific microbial cell factory, the outcome of a bioprocess greatly depends on the designing of the fermentation mode, as well as on the downstream processing for the efficient harvesting of the final product [8]. The fermentation followed usually for higher product recovery is the fed-batch mode, where a specific feed is provided in a controlled way to maintain cell proliferation. In case of a CE-focused bioprocess a waste-derived feed is desirable.

1.3 Biorefinery

The term of "biorefinery" is strongly correlated with the one of the CE. The approach of utilizing a-so-called-"waste" stream of a process as feedstock for another one reduces the disposal of industrial residues and allows a more efficient utilization of the total amount of the initial raw material. This idea has attracted attention for many years and is summarized in the concept of biorefinery. A biorefinery consists of a number of integrated processes that utilize different fractions of an organic waste, such as biomass, for the production of a series of products related with biofuels, energy, food, chemicals and materials [9]. That way a cascade of processes is formed, which efficiently prevents damage or loss of raw material, by utilizing all its components [10]. Although the idea was initially focused on the production of biofuels-providing therefore a "greener" equivalent of the conventional "refinery"- it was soon clear enough that biofuels cannot yet compete with fossil fuels in terms of production cost. Thus, the only way to make

biofuels competitive was by further valorizing the residues to different commodities [10]. Organic wastes from different sources can be used in processes included in a biorefinery.

2. Organic residues as feedstock

In the case of bioprocesses, where whole microorganisms are used, the waste streams that serve as feedstock must include a substantial organic content. That way they can support the growth of the microorganisms during the fermentation. Normally, the waste to be fed must undergo a special pretreatment, in order for its organic content to become available for assimilation by the cells. Although there are many types of organic-rich waste, two of them are already widely used for valorization purposes; biowaste from municipal solid waste and lignocellulosic biomass. The aforementioned streams include a high content of organic substances, which, under the right management, can be converted to assimilable substrates for microorganism growth. The sum of the processes adopted to render the organic content of biowaste or lignocellulose susceptible to microbial metabolism will be referred to as “treatment” in this thesis, to differentiate from the “pretreatment” of lignocellulosic biomass.

2.1 Biowaste

According to the definition in the European Union’s Waste Framework Directive 2008/98/ EC “biowaste means biodegradable garden and park waste, food and kitchen waste from households, restaurants, caterers and retail premises and comparable waste from food processing plants” [11] .

A characteristic of biowaste is that most of it can be easily collected through source-seperation by the citizens [12]. However, depending on its source, its composition can vary greatly [13].

Composition

Biowaste derived from households, as well as restaurant, supermarket, hotel and bar activity will exhibit different properties with the region or the season. However it always includes a substantial amount of polysaccharides-and specifically starch, cellulose and hemicelluloses, proteins, lipids and sugars [14]. According to analyses of samples from different sources, the carbon content of the total solids of a biowaste stream can vary between 40-51% (w/w), while total nitrogen is around 1-4% (w/w) [15,16]. This characteristically high carbon content makes disposal of biowaste environmentally harmful, while, at the same time, renders it a very good candidate for microbial fermentation.

Treatment for production of fermentable substrates

When a waste stream is to be used for microbial bioconversion, usually, a treatment step is required, in order to render the carbon content of the stream available to the cells. Usual methods for dealing with biowaste in developed countries include composting or anaerobic digestion. In case of residual biowaste, which includes amounts of other garbage, the usual disposal method is landfilling, or incineration for heat [12]. A different treatment, which has gained momentum in recent years, is dark fermentation (DF). DF is an anaerobic treatment that initially follows the same pattern as the classical anaerobic digestion (hydrolysis, acidogenesis, acetogenesis) with the difference that the final step of methanogenesis is inhibited by various methods [17]. As a result, the pattern of the process follows this course; *Hydrolysis*, the first and usually the slowest step of

anaerobic digestion, includes the enzymatic breakdown of long polymeric substances, present in biowaste fraction, to simpler organic monomers such as sugars, amino acids and fatty acids (FA). These monomers are subsequently fermented by acidogenic populations in the *acidogenesis* step, leading to production of volatile fatty acids (VFA), together with an air mixture of hydrogen and carbon dioxide. Lastly, the third step of *acetogenesis* represents the stage where the breakdown of VFA to hydrogen and acetate occurs [18].

According to the above steps, DF leads to hydrogen and VFA accumulation, such as acetic, propionic, butyric, valeric and caproic acid, small production of ethanol and accumulation of lactate [19,20]. Through the conversion of complex organic substances into simple acids, DF succeeds in creating a VFA platform that can supply a microbial culture with readily assimilable carbon. This can be further utilized for the growth of a specific microorganism for industrial applications.

In order for the DF to produce VFA, the consumption of FA by methanogens, which naturally occurs in anaerobic digestion, must be prevented. Therefore, in the above process, the last step of methanogenesis is inhibited, usually either by thermal pretreatment of the waste stream to destroy the methanogenic populations, or by maintaining the pH of the mixture at high values (above 9) that do not allow the growth of the specific bacteria [21,22]. Another solution is the addition of a methanogen inhibitor, such as iodoform [23,24].

Integration in a biorefinery

Apart from serving as a VFA platform, DF is mostly applied as a means of producing biofuels, because the last step of acetogenesis also leads to the emission of hydrogen. In comparison to many other energy-intensive methods, production of biohydrogen from

DF is currently the most sustainable process, since it is more environmental friendly and also has a net energy ratio of 1.9 (>1) [14]. That means that the amount of energy produced- stored in hydrogen- is significantly higher than the one consumed by the DF process. In order to enhance the sustainability and economic viability of the production of biohydrogen, through DF, the utilization of the by-products, namely the VFA, needs to be seriously addressed. So far, attempts of valorization include photofermentation or use of VFA in microbial electrolysis cell for more hydrogen production. However, the first method requires ammonia and oxygen removal from the DF effluent, while the second requires an effective method of suppressing the production of methane during the electrohydrogenesis. Another method proposed for the utilization of VFA by-products is the anaerobic digestion of the effluent for final methane production [25,26]. However methane is a far less effective energy source than hydrogen, with less than half of its efficiency [27]. The production of biobased chemicals, such as biopolymers, has also been suggested [28], resulting in products that necessarily need to have a low price in order to be competitive in the market.

The utilization of the VFA-rich stream as feed for the fermentation of microorganisms of industrial interest is an innovative proposal. Especially cells that accumulate high added-value metabolites justify investing in the expensive process of fermentation and metabolite harvesting, owing to the higher value of the final product [29].

Coupling the production of bio-hydrogen, through DF, with the valorization of the liquid fraction of VFA successfully leads to the establishment of a biorefinery, as defined previously. More specifically, biowaste are effectively used for energy generation, at the same time that the residual organic fraction is converted to functional commodities. The idea is simple, based on already tested methods and allows for the effective utilization of

the total amount of a waste stream, thus reducing raw material needs and promoting a CE.

2.2 Lignocellulosic biomass

Lignocellulosic biomass is the term used to characterize the total of plant residues derived either from agricultural or forest-industrial activity [30]. Lignocellulose is an abundant waste stream and a renewable source of sugars that can be transformed into chemicals for different applications. What is more, as a raw material, it does not include edible compounds that could have been used for human nutrition. For that reason the valorization of biomass residues towards the production of value-added products is attracting increasing interest during the last years [31]. However, until today, it continues to be a rather under-valorized stream, mostly due to the variability and recalcitrance of its composition.

Composition

Although depending highly on the biomass origin, lignocellulose consists always of three main components; cellulose, hemicellulose and lignin. Cellulose is the constituent that exists in higher percentages in plants. It is a linear polymer of D-glucose subunits, linked together by β-1,4-glycosidic bonds. The repeating monomer of cellulose is cellobiose (Fig.1). Cellulose can be hydrolysed to pure, fermentable glucose and therefore it is that component of biomass that can be more readily utilized. Hemicellulose is a heterogeneous polymer of sugars, and more specifically pentoses and hexoses, and sugar acids. Its main building units are xylose, mannose, arabinose, glucose, galactose, glucuronic and galacturonic acid linked together by β-1,4- or β-1,3-glycosidic bonds. Owing to its flexible composition, hemicellulose is more amorphous than cellulose, and thus more easily accessible to hydrolyzing enzymes. However, its

heterogeneity hampers its application in many industrial processes. The third constituent of lignocellulosic biomass is lignin, a complex heteropolymer of phenylpropane units from coumaryl-, sinapyl- and coniferyl-alcohol. These aromatic compounds are linked together by C-C or C-O bonds, forming an amorphous matrix that surrounds hemicellulose and cellulose, offering impermeability and structural support [32,33]. Lignin is the least exploitable component of plant biomass, while its proposed applications are still a controversial subject.

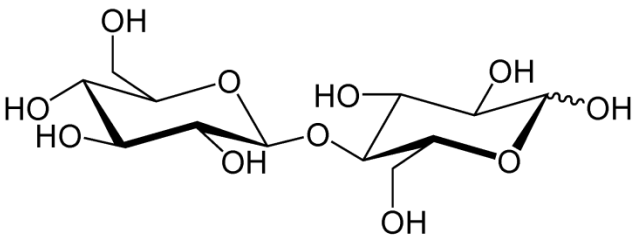


Figure 1. Cellobiose- the repeating unit forming cellulose chains

The three main constituents of vegetal biomass are intertwined together in a complex way that offers the plant stability and resistance against decomposition. It is clear, however, that, in order for the different lignocellulose components to become amenable to industrial utilization for biochemical conversion, their closely associated form must be disrupted [34]. This can be succeeded through various pretreatment techniques, which should not be confused with the broader term “treatment” of a waste stream, as it was previously introduced. More specifically, the necessary “treatment” process to produce fermentable sugars for bioprocesses includes pretreatment of lignocellulose but also another step of saccharification.

Treatment for production of fermentable sugars

a) Pretreatment

Pretreatment of the lignocellulosic waste stream, to render it amenable to bioconversion processes, includes the hydrolysis of cellulose and sometimes hemicellulose to simple sugars. As mentioned above, cellulose is the easiest valorized constituent of plant biomass. Upon the application of efficient fractionation technologies, a cellulose-rich pulp can be produced and serve as feedstock, after enzymatic saccharification, for the production of a wide variety of end-products, through fermentation processes. The release of cellulose from the biomass can be accomplished by physical, physico-chemical, chemical and biological techniques, which are normally described as the biomass pretreatment.

i) Physical methods

Physical methods for cellulose release include techniques such as microwave, ultrasound or mechanical splintering. Their main effect includes the reduction of the particle size of lignocellulose, without exhibiting any efficiency in the removal of hemicellulose or lignin. For that reason they are usually coupled with another chemical or physico-chemical technique, to enhance the cellulose recovery [35].

ii) Physico-chemical methods

Methods that adopt physical processes with addition of substances that aid the alteration of the form of lignocellulosic materials are called physico-chemical. The substance added normally plays the role of the solvent and, therefore, at the end of them, a slurry is collected, where the cellulose-rich solid fraction is separated from the liquid one. The most common physico-chemical process for fractionation is steam explosion. It includes

treatment of the waste stream with high temperatures (160-260^{o}C) and high pressure (20-50 bar). The process duration varies from a few moments until several minutes, before the release of pressure that leads to an explosion and the break-down of the structure of lignocellulose [36]. In most cases, steam explosion is coupled with the addition of a specific agent to increase the efficiency of the fractionation and the degradation of hemicellulose. Depending on the agent, many sub-categories of physico-chemical treatments are applied.

In the case that the only added solvent is water the process is called hydrothermal. It is very successful in the solubilization of hemicelluloses and environmentally friendly, however it requires relatively more time to exhibit substantial yields, in comparison to other solvents. In other cases, the agent can be an inorganic acid, such as suphuric (acid catalyzed steam explosion), or an alkali, such as ammonia (Ammonia Fiber Explosion or AFEX). In the case of AFEX the process requires less harsh conditions of temperature and pressure and therefore is not characterized as steam explosion. Lastly, the process of facilitated explosion can be achieved also with the addition of CO_2, as a "green" solvent, in the form of supercritical fluid. The procedure is called supercritical CO_2 explosion, it is performed in conditions above the critical point of the specific dioxide, and is known to prevent the release of high amounts of inhibitors [35].

iii) Chemical methods

Chemical methods include the treatment of lignocellulose with a chemical substance for the disruption of its structure, the solubilization of lignin or hemicelluloses and the increase of the surface available for hydrolysis. When not combined with steam explosion, simple acid or alkali treatment can be applied. In some cases oxidative methods, such as wet oxidation, in the presence of water, or ozonolysis are adopted.

Oxidative methods, although less examined, exhibit high efficiency in the degradation of lignin and hemicelluloses [30].

A more popular chemical method is called organosolv. It refers to the- normally catalyzed- treatment of lignocellulose with organic solvents, such as ethanol or acetone, under relatively lower temperatures and pressure than steam explosion. Organosolv is extremely effective in separating the lignin fraction, while maintaining almost intact the structure of cellulose in the solid fraction, for further applications. Lastly, other solvents that have been selected for fractionation include the anionic liquids, a technology that is arguably more environmental friendly but still has limited applications due to its high cost [35,37].

iv) Biological methods

A less popular, but also less severe technique is the use of microorganisms that secrete enzymes able to degrade the lignocellulosic structure, such as fungi, bacteria or actinomycetes. In these cases, the selected microbe is cultivated on the lignocellulosic substrate in order to trigger the release of the desired enzymes. The fungal treatment is usually carried out in solid state fermentation where fungi degrade lignocellulose, hydrolyse cellulose to simple sugars and use them as carbon source for growth [35,36]. A basic drawback of this method is the fact that it leads, not only to the degradation of lignocellulose, but also to the enzymatic saccharification and consumption of cellulose, thus making it inappropriate for applications where the further valorization of the isolated cellulose by another microbe is desired.

b) Enzymatic saccharification

The application of a fractionation method allows the isolation of a cellulose-rich solid fraction that may also contain a small amount of hemicellulose or lignin, depending on the selected technique. In order for this cellulose to be fermentable, it must be converted to simple sugars, through hydrolysis. It is true that there exist specific microorganisms capable of directly hydrolyzing this linear polymer to glucose to further support their carbon needs in a single culture [38]. However, enzymatic saccharification is usually adopted as a step of producing a carbon-rich feed, before cultivation. That is normally carried out by enzymatic cocktails consisting of cellulases and sometimes hemicellulases and oxidizing enzymes.

i) Cellulases

Cellulases are a class of enzymes that exhibit high functional diversity. They are mostly hydrolases that can be divided into three basic categories, based on their function; cellobiohydrolases (CBH), endoglucanases (EG) and β-glucosidases or cellobiases (β-GL). CBH are enzymes able to hydrolyse the β-1,4 glycosidic bond of cellulose chains, beginning always from the ends of the chain. They usually exhibit a progressive activity, starting from the end of one chain and moving forward, releasing all the while shorter cellulose chains. That way they can act synergistically with EG. Enzymes categorizes as EG also cleave the β-1,4 bonds, but they act in the interior of cellulosic chains, accumulating shorter poly- or oligo-saccharides, on whose ends CBH can bind. By generating shorter chains, EG greatly enhance the liquefaction of cellulose. The last step of cellulose hydrolysis involves the conversion of its repeating unit, the disaccharide cellobiose, into two glucose molecules. That step is catalyzed by β-GL [39–41].

ii) Lytic Polysaccharide Monooxygenases

Apart from the well-studied hydrolases, another category of oxidoreductases has been discovered to assist cellulose saccharification. Lytic polysaccharide monooxygenases (LPMO) are oxidative, copper-depended enzymes that belong to the category of "Auxiliary Activity" (AA) according to the CAZY database (http://www.cazy.org/AA9.html) and act synergistically with the glycosyl hydrolases mentioned before, for the degradation of polysaccharides such as cellulose. LPMO break the long chain of polysaccharides by catalyzing the oxidation of either the C1 carbon of the one glucose molecule of the β-1,4-bond, or the non-reducing C4 carbon of the other molecule participating in the bond formation. Depending on the carbon atom targeted, the reaction results in the release of a neutral shorter polysaccharide and an oxidized product that is either a lactone, spontaneously hydrolyzed to aldonic acid, or a ketoaldose respectively (Fig.2). Some LPMO can even act on both carbon atoms thus producing a mixture of different oxidized products [33,42].

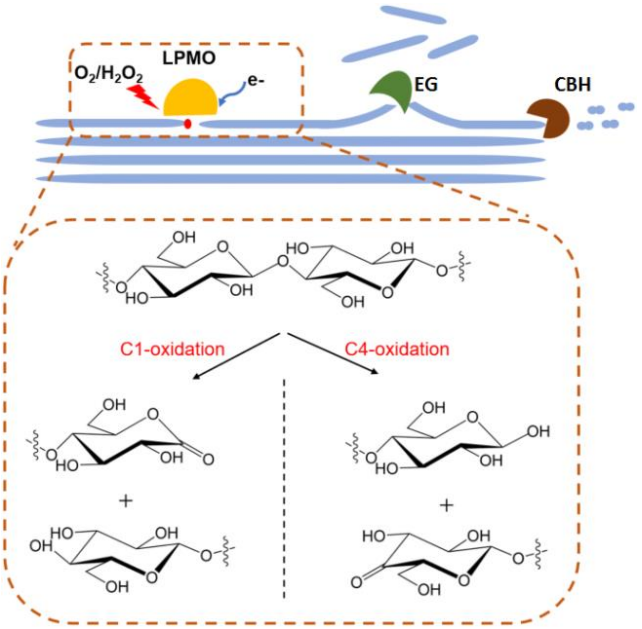


Figure 2. Schematic representation of the LPMO function, adapted from X Zhou et. al. In the upper part the synergistic degradation of polysaccharides by hydrolases and LPMO is depicted. The scheme inside the dotted line exhibits the two possible results of the LPMO catalyzed reaction. [43]

The exact reaction mechanism of LPMO enzymes has not been totally elucidated yet. It is generally accepted that it involves the reduction of the Cu(II) situated in the active center of the enzyme to Cu(I) and the binding of an oxygen atom either from an oxygen, or a superoxide molecule. That activated oxygen species finally hydroxylates the C1 or C4 carbon of the 1,4-glucosidic bond [44]. Although it was a popular belief that this is the mechanism that leads to repeating cycles of oxidation by LPMO, it has been recently suggested that the initial reduction to Cu(I) is just a priming reaction, leading the enzyme to exhibit peroxygenase activity and finally catalyze the oxidation reaction utilizing peroxide as co-substrate [45]. In any case, it is clear that in order for LPMO to perform an oxidative reaction, an electron donor is requested. This donor can be either a constituent of the natural substrate, such as lignin in lignocellulosic biomass, an external

reducing agent that is added, such as gallic or ascorbic acid, or another co-secreted enzyme, such as cellobiose dehydrogenase (CDH). CDH are enzymes that can also catalyze oxidative reaction of cellobiose and some oligosaccharides, but they are specified only on the reductive end, leading to the production of the corresponding lactones. The oxidation results in electron release that can be stored by CDH and further utilized by LPMO [33]. The suitability and effectiveness of an electron donor differs for the different LPMO enzymes and depends, among others, on the potential of the enzyme copper active site [46].

LPMO differ in regard with the polysaccharide substrate that they act on, which can be chitin, cellulose, starch or xylan and derivatives. Depending on their amino acid sequence, they are divided in different sub-categories, or families, in the CAZy database (platform for Carbohydrate-Active enzymes) [43]. There are 5 different LPMO families, namely the AA9-AA11 and AA13-AA15, while recently, another family, AA16, was suggested to exist. The widest family, AA9, includes LPMO active on poly- and oligo-saccharides of β-1,4-linked glucose units, such as cellulose, glucomannan or some types of β-glucan. AA10 LPMO catalyze the oxidation of cellulose or chitin and are isolated from bacteria and some viruses. AA11 and AA13 families consist of LPMO of fungal origin, active on chitin or starch respectively and the recent AA15 includes LPMO of animal or viral origin. Finally AA14 belonging enzymes are active on xylan coating cellulose fibers [44].

The function of cellulases, both glycosyl hydrolases and LPMO, relies highly on their structure. As a general rule, cellulases comprise of a main catalytic domain, while some of them also include a carbohydrate binding module (CBM), connected to the rest of the enzyme. Eukaryotic cellulases are usually glycosylated and therefore contain saccharides connected to N or O atoms of some amino acids [39]. These post-traslational

modifications make heterologous production and characterization of these enzymes harder, since a eukaryotic microorganism is needed to secrete a functional protein. Still in some cases that is not successful. In the methylotrophic yeast *Pichia pastoris*, usually used for heterotrophic expression, a different glycosylation pattern occurs, that may interfere with the functionality of the enzyme. What is more, in the case of fungal LPMO the N-terminal catalytic histidine-a characteristic residue of these enzymes- is methylated, which is not mimicked by the yeast cells [44].

iii) *Hemicellulases*

Finally, taking into consideration that the solid fraction resulting from the treatment of lignocellulose, a material utilized in the specific thesis, may contain a substantial amount of hemicellulose- rich in xylan-, hemicellulases are also employed in specific cases of saccharification. Hemicellulases comprise mostly of xylanases, xylosidases and acetyl-esterases that cleave the acetylated branches from hemicellulosic chains. Of course, taking into consideration the variety of this category, there are many more enzymes that belong to hemicellulases [47]. However, since this category of enzymes is not directly relevant to the present thesis, further analysis of the matter was not deemed necessary.

iv) Reaction conditions

The application of saccharification can include custom made mixtures of the above enzymes, however, the option of a commercial preparation is another popular option. The sugar recovery can vary greatly, depending on the source of lignocellulose, the pretreatment method, the composition of the commercial enzyme mixture applied, as well as the reaction conditions [41]. Usually, enzymatic saccharification is carried out at temperatures between 45-50^{o}C, at a pH around 5, for a duration of 24-72 h, under agitation to allow for sufficient oxygen availability [40]. It is worth mentioning however

that, when using a general purpose enzyme mixture for the hydrolysis of different lignocellulosic materials, it is almost impossible for a hydrolysis reaction of different materials to take place at optimal conditions for all the included biocatalysts [48].

Integration in a biorefinery

Lignocellulosic biomass poses an abundant substantial waste stream from agricultural and industrial processes. The development of fractionation technologies for the collection of its components has long reached a mature state, justifying the effective integration of lignocellulosic waste in a biorefinery. However, in order for such endeavors to be successful, the efficient conversion of lignocellulose to fermentable sugars and the selection of the proper final product are of great importance. Although agricultural waste-derived cellulose can be utilized as fermentation feed for the production of bioethanol, the demand for biofuels cannot be satisfied by the available biomass feedstock [49], let along that they need to compete with the significantly lower cost of fossil fuels. On the contrary, the bioconversion of these components to high added-value commodities is a cost-effective solution that allows for the viable production of specialty goods- required in smaller quantities- totally from renewable sources. Apart from this, the implementation of the proper fractionation method, that allows for the harvesting of intact cellulose, coupled with an efficient saccharification, by an enzyme cocktail, offers the maximum feedstock for the cultivation of the desired microorganism [50].

3. Omega-3

Omega-3 FA are a category of FA that is constantly gaining momentum ever since their benefits for human health became known.

3.1 Chemical composition

The FA belonging in this group may be very heterogenous, still they share a similar chemical characteristic. The term “omega-3” is used to describe all FA that include a double bond between the third and forth carbon atom (ω-3 or n-3 atom) belonging to the FA carbon chain, counting from the methyl end [51]. This definition answers for a very big variety of omega-3 FA that can be monounsaturated or polyunsaturated, conjugated or modified. Still, most of the known and valuable omega-3 FA- also the ones that are going to be mentioned here- are few and characterized by relatively long carbon chains with many unsaturated bonds, thus being part of the group of Long Chain Polyunsaturated Fatty Acids (LC-PUFA). According to nomenclature rules of FA, each acid can be described, not only by its name, but also by a code of the form X:A, where X answers for the number of carbon atoms of the acid, while A is the number of double bonds [52].

3.2 Basic categories

According to EFSA (EFSA-Q-2004-107), there are two main categories of omega-3 FA, which differ in function and requirements; the first refers to α-linolenic acid (18:3, ALA) produced from vegetable oil, while the other includes ω-3 LC-PUFA from marine sources, such as eicosapentaenoic acid (20:5, EPA) and docosahexaenoic acid (22:6, DHA) (Fig. 3). These LC-PUFA have been widely recognized as important bioactive compounds that can be used in the food and nutraceutical industry for the development of functional foods with scientifically sustained claims [53]. What is more, these two acids belong to the essential FA for the human organism, meaning that they are provenly necessary for maintaining the human homeostasis, but cannot be sufficiently synthesized by the organism itself. Therefore they must be uptaken through the nutrition. Another

FA, less known but with equally important function, belonging in this second category, is omega-3 docosapentaenoic acid (22:5, DPA).

Of course ALA is also nutritionally essential and is required for the synthesis of important FA and eicosanoids. However it is more easily acquired through nutrition and therefore there are but few ALA supplements. A small conversion of ALA to EPA and DHA is possible, but depends on several factors, such as the concentration of omega-6 FA and is, in any case, insufficient to cover the daily organism's needs.

Lastly, although not an omega-3, it is worth mentioning one more LC-PUFA. The arachidonic acid (20:4, ARA) is another important FA that belongs to the essential FA for fish and animals, together with EPA and DHA. It is a member of omega-6 FA, exhibiting its first double bond in the ω-6 carbon atom of the acyl chain [51,54].

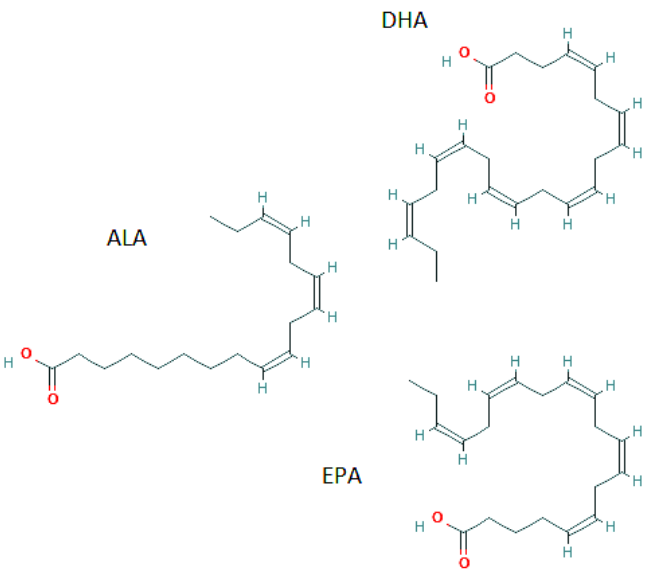


Figure 3. Chemical structure of the 3 main omega-3 PUFA, ALA, EPA and DHA

3.3 Nutritional value

PUFA in general, and also omega-3 FA, present in the human body are components of high functionality and therefore are deemed as ingredients of high nutritional value. Normally they are main ingredients of the phospholipids, situated in the cell and organelle membranes and thus playing a crucial role in the regulation of the membrane fluidity, as well as the modulation of signal transduction between them. Some of them also act as precursors for the synthesis of bioactive molecules, such as prostagladins, eicosanoids, etc.

Owing to their being basic components of human tissues, there have been reported many studies related to health benefits of DHA and EPA. Their vital structural and functional roles in higher organisms are related to prevention of cardiovascular and inflammatory diseases, cancer and diabetes. What is more, they promote the development of ocular function and memory in infants, with DHA specifically enhancing their cognitive function, and are possibly effective against Alzheimer's disease [51,52,54,55] Lately there has even been an association between omega-3 uptake and decrease of the adverse effects of Covid-19 desease in the organism [56].

3.4 Sources

The primary dietary source of EPA and DHA is, even nowadays, fish oil. Nevertheless, the use of fish oil is limited not only due to its fishy, undesirable smell and taste, but also due to its poor oxidative stability and the accumulation of lipid-soluble environmental pollutants, which make difficult the purification of specific FA. Moreover, the massive exploitation of fish leads to serious environmental consequences and depletion of marine stocks. Of course, aquaculture poses a solution to the limited seafood supply, without, however, covering the growing need for omega-3. The reason for this is the fact that fish

lack the enzymes necessary for synthesizing EPA and DHA for themselves and therefore also uptake these ingredients through their diet. Thus, the lipid content of farmed fish reflects that of the food they consume and creates one more need of the industry for omega-3 FA rich feed. On the basis of increasing global fish meal and fish oil costs, it is predicted that dietary fish meal and fish oil inclusion levels within aquaculture feeds will decrease in the long term, resulting in lower levels of useful omega-3 FA in farmed fish [57]. Therefore, new sources and production systems, taking into consideration sustainability and economic feasibility, must be developed for direct human consumption as well as for aquaculture. [58–61].

When considering the above dilemma, it is important to understand that, since fish are incapable of producing them, ω-3 PUFA are accumulated in their tissues through the food chain. Therefore the primary producers of ω-3 must be looked for elsewhere. The truth is that seafood is up taking the necessary lipids from zooplankton consuming ω-3 PUFA-synthesizing microalgae. Microalgae, the almost exclusive primary producers of ω-3, are a heterogeneous group of mostly photosynthetic organisms, the majority of which belong to the oleaginous microorganisms, with the ability to accumulate lipids at a percentage higher than 20% of their dry biomass [62].

The advantages of microalgae oil against fish oil include the synthesis of great amounts of ω-3 PUFA, the absence of cholesterol and contaminants, such as heavy metals, and the satisfactory taste of microalgae [58,60,61]. In addition, microalgae biomass is suitable for extraction and purification of individual PUFA, due to its stable lipid composition. Fish oil always includes a mixture of EPA/DHA, a characteristic that is undesirable for specific applications. In contrast to that, microalgae strains able to produce omega-3, usually accumulate only one of the two essential ω-3 FA. After all, apart from being suitable for more applications, microalgal oil is also suitable for a

broader group of consumers, satisfying the request of vegan or religious groups for animal-free products [55,63]. Lately, the consumers' growing demand for natural bioactive compounds has motivated researchers and industries to intensely investigate microalgae as potential candidates for ω-3 FA production [64,65]

3.5 Applications

Most already established applications of omega-3 FA include their use as food supplements. In such cases, fish oil can be a sufficient option, especially since companies are implementing even stricter controls and protocols, to ensure that they provide a product with a very low cotent of pollutants or heavy metals. Apart from a food supplement or a nutraceutical, a mixture of EPA/DHA with the commercial name Lovaza, produced by GlaxoSmithKline, is now a prescribed treatment for hypertriglyceridemia, categorizing these FA also as medical foods. Medical foods, having the characteristic of being used under the guidance of a physician for the treatment of a specific health condition, belong to a much more expensive category, thus further enhancing the importance and value of omega-3 PUFA [51]. All the above applications are conventionally covered by fish oil products. Nevertheless there exists other applications, where fish oil cannot be used effectively.

DHA is a natural ingredient of human milk and, for the reasons described above, necessary for the early stages of development. Also many researches have highlighted its importance as a component of baby formula. However, when accompanied by EPA, the health benefits of DHA for the infant development disappear. EPA, in contrast to the other ω-3 FA, is a precursor of substances, such as hydroxy PUFA, that are undesired during the early stages of life. For that reason, fish oil, which contains almost always a mixture of these FA, cannot be used for baby formulas supplementation, let alone the

fact that these products derived for infant consumption cannot justify any heavy metal or contaminant presence. To avoid this, more than 70% of the current market of infant foods is supplied by DHA rich-microalgal oil, derived mostly from the microalga *Crypthecodinium cohnii*. What is more, it was proven that DHA in the organism can also be retroconverted to EPA, and therefore, the desirable omega-3 is always accompanied by ARA in the formulas. The presence of ARA fortifies DHA and inhibits its conversion. Two well-known microbial oils used nowadays as formula ingredients is DHASCO (DHA single cell oil) from the heterotrophic microalga *C. cohnii* and ARASCO from the fungus *Mortierella alpina* [51,52,66].

Lately, their contribution to a healthy cardiovascular system is even creating interest into possible applications of omega-3 FA for the fortification of the organism and the prevention of implications provoked by covid-19 disease [55]. Of course such a potential cannot be easily proven, but it might be enough for consumers to lead to an even higher ω-3 demand in the near future.

4. Microalgae

Microalgae comprise a vast category of microorganisms that has attracted industrial attention during the last decades owing to their ability to accumulate valuable bioactive metabolites, coupled with their easy fermentation. They are ubiquitous organisms that are extremely diverse and heterogeneous from evolutionary and ecological point of view, a fact that can be justified by the great range of habitats in which they can be found. Microalgae constitute of prokaryotic cyanobacteria or eukaryotic protists and therefore exhibit variation in their nutritional requirements as well as metabolite production. Apart from cyanobacteria, the biological classification of eukaryotic microalgae is based mostly on the pigments that they produce, resulting in nine

divisions. The main classes of them are Chlorophyceae (green algae), Phaeophyceae (brown algae), Rhodophyceae (red algae), Chrysophyceae (golden-brown algae), Bacillariophyceae (diatoms) and Pyrrophyceae (dinoflagellates) [67].

In terms of molecular biology, they are thought to be microorganisms that have managed to acquire intracellular chloroplasts. In truth, most of the strains are found in saline or freshwater ecosystems and grow autotrophically, absorbing the dissolved carbon dioxide from the water. However, there are still some microalgae that lack the ability of photosynthesis and therefore grow heterotrophically by consuming conventional carbon sources [68,69]. Heterotrophy however doesn't necessarily mean absence of chloroplasts, since specific autotrophic strains will also grow heterotrophically if they are provided a carbon source under dark. Finally a combined fermentation is referred to as mixotrophy.

4.1 Cultivation

Microalgae can be cultivated in various ways. Autotrophic cultivation usually has the lower expenditure demands and can be carried out in simple open ponds that ensure a sufficient light exposure of the cells, by achieving low depth and big surface cultures. It offers the advantage of not requiring arable land and therefore not competing with food crops. Open pond cultivation however can be hampered by changing weather conditions and contamination of the cultures. In such cases, a safer, but more expensive alternative, is photobioreactors. Photobioreactors often consist of a series of transparent tubes that let the light-artificial or not- penetrate into the culture volume and are usually equipped with a CO_2 supplier, which allows air bubbles to move through the tubes. That way, carbon is provided for photosynthesis and simultaneously mass transfer is carried out without the development of shear forces that would damage the cells [70].

Although autotrophic cultivation of microalgae exploits renewable sources, it also exhibits some crucial limitations. The most important one is the low cell growth achieved, due to the inevitable light dependence of the culture. While more cells proliferate in the broth, the light availability in higher depths of the culture decreases. As a result cell multiplication finally ceases. It is characteristic for autotrophic cultivation modes to reach relatively low final cell concentrations (around ca. 0.5-2 g dry cell weight- or DCW- per L), which further hinders harvesting operations [71]. In order to overcome such a problem, heterotrophic cultivation can be adopted, when possible. Heterotrophic microalgal cultures are operated like conventional fermentation bioprocesses for any type of aerobic microbe, in closed bioreactors with the provision of an organic carbon source and appropriate aeration. Stirred fermenters are used to grow microalgal cells on a wide range of substrates such as glucose, acetate, simple sugars or glycerol, but also mixed substrates derived from waste streams [70].

Both autotrophic and heterotrophic fermentation techniques have been developed for microalgae in pilot or industrial units. Although the first category allows for low cost production, being able to utilize resources abundantly available, such as the solar energy and the atmospheric CO_2, heterotrophic fermentation gains momentum, as means of controlling more efficiently the culture conditions and absolving the process from the- otherwise necessary- weather and climate dependence [72]. What is more, this type of fermentation leads to much higher biomass productivities, consequently enabling higher final product yields [73], [74]. In truth, the only drawback of heterotrophic cultures- assuming that proper techniques to avoid any culture contamination are a prerequisite- is the cost of the raw material, that is, the feed of the culture. In order to overcome this constrain, new low-, or even zero-cost, resources are searched.

4.2 Useful metabolites

The reason that microalgae have attracted the interest of the scientific and the industrial community, apart from their easy cultivation, is their unprecedented metabolites that exhibit important biological functions [75]. Nowadays these intriguing microorganisms are cultivated around the world, either for the whole biomass to be used as food, rich in carbohydrates, proteins and lipids, or for extraction of specific metabolites being accumulated in the cells. Depending on species and culture conditions selection, some microalgae strains can produce substantial amounts of short carbon chain lipids (≤18 carbon atoms), carotenoids, vitamins and some LC-PUFA [76–79]. Carotenoids are lipid soluble pigments, with antioxidant properties for the human body, as well as functional role in the membrane fluidity and permeability, when found within the lipid bilayers of the cells [80]. They are an expensive food additive or nutraceutical supplement. Vitamins found in microalgae, although not so well examined yet, belong mostly to the fat-soluble A, D, E and K vitamin [81]. Short chain FA produced by microalgae are not as popular for food applications, since they can be found in many edible components or be *de novo* synthesized by the human body. However they are valuable source of lipid for the production of biodiesel esters [82]. On the contrary, LC-PUFA of microalgal origin, such as omega-3, are of high nutritional value and therefore very expensive commodities. Taking into consideration the nature of the above mentioned substances, it is apparent that the successful production of valuable microalgal metabolites requires a thorough understanding of their lipid metabolism.

4.3 Lipid metabolism

FA can either play a functional role inside the cells, as part of the membrane of the cell or intracellular organelles, or act as storage compounds that provide energy when

needed. Storage lipids are normally produced in the presence of excess carbon source and when cell growth is hampered due to another nutrient limited availability. Under such conditions all eukaryotic cells produce enzymes that convert carbon, received through the food, to energy rich-lipid droplets, usually in the form of neutral, triacylglycerides (TAG) [65].

Basic lipid metabolism in eukaryotic cells

In the case of TAG the accumulation occurs through four main stages. In a first step, a pool of acetyl-coenzyme A (acetyl-CoA) and the molecule NADPH is generated through usual catabolic activities of the cell (Fig.4). These are the main precursors for intracellular FA synthesis, since acetyl-CoA usually acts as initial building block for FA. The elongation of the FA chain by two carbon atoms requires malonyl-coenzyme A (malonyl-CoA), which is formed in the second step by a condensation reaction of acetyl-CoA and a bicarbonate ion. This conversion of acetyl-CoA to malonyl-CoA is catalyzed by acetyl-CoA carboxylase. The newly synthesized malonyl-CoA is connected to the acyl carrier protein, forming malonyl-ACP, which is further converted, by a series of condensation reactions within the FA synthase system, into acyl-ACP. These reactions involve elongation and desaturation of the acyl chain by enzymes, known as elongases and desaturases respectively. Termination of FA elongation is mediated by an acyl-ACP thioesterase, resulting in free FA that are transferred to glycerol-3-phosphate for the formation of TAG, in a third step. The fourth step consists of the formation of intracellular lipid droplets [59,83].

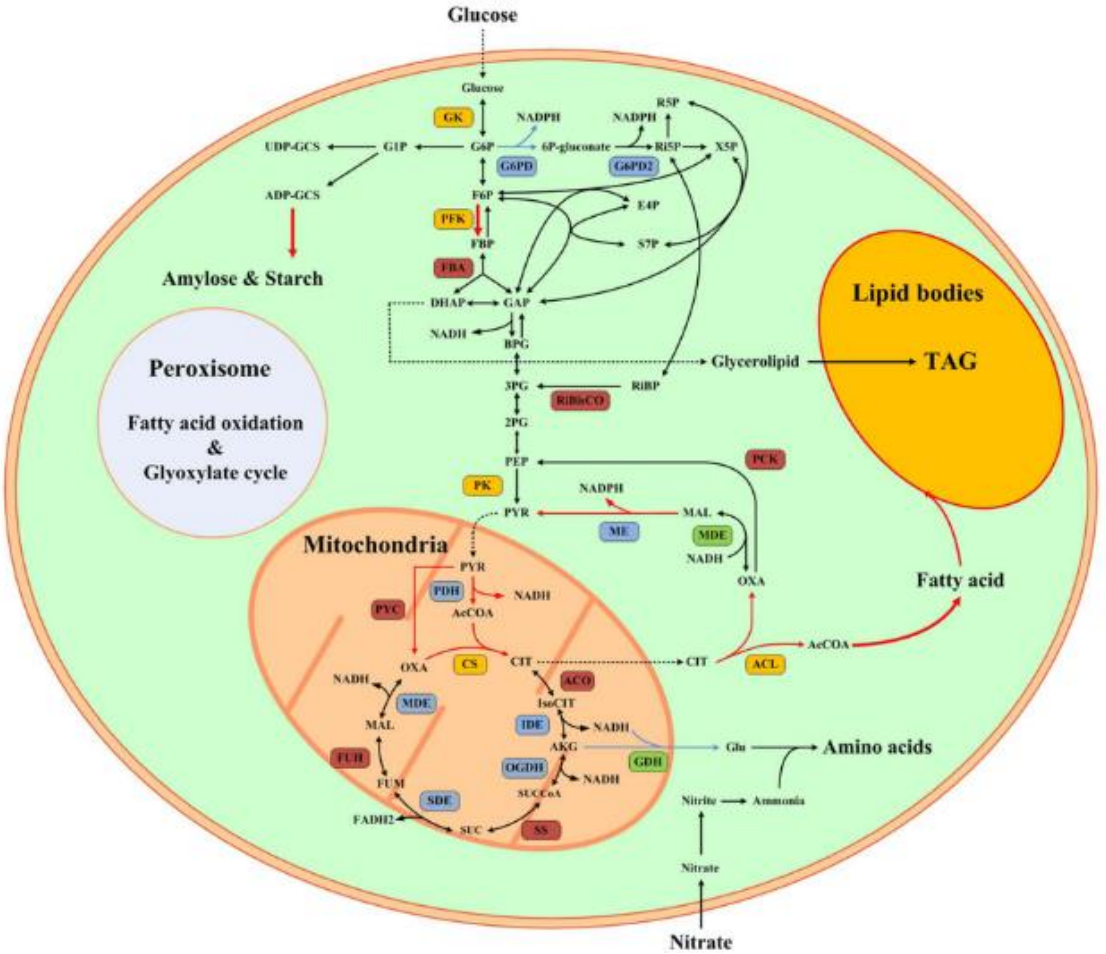


Figure 4. Illustration of the intracellular metabolic activities for lipid biosynthesis [59]

Production of LC-PUFA

The procedure described above leads to aerobic production of main FA, such as C16, C18 and C18:1 in cells. However, the value of certain microalgal strains lies with their ability to accumulate unusual PUFA of high nutritional value and more specifically omega-3 FA, such as EPA and DHA. The conversion of the main lipid precursor, acetyl-CoA, to the desired PUFA has been suggested to follow three different routes,

depending on the lipid-producing microorganism. In animals, it is generally accepted that EPA derives from linoleic acid, through usual elongation and desaturation steps. Afterwards, the all but high conversion of EPA to DHA in the animal tissues follows the Sprecher pathway, where EPA is elongated to 22:5, then to 24:5 and then desaturated to 24:6, before conversion to DHA by β-oxidation (Fig.5).

In contrast to animal metabolism, it is suggested that microalgae normally follow a different route for EPA conversion. This simpler mechanism involves one elongation step to 22:5 and a Δ-4 desaturase activity [67]. Although the second route described has been proved to exist in some microalgal strains, apparently it is not the only possible one for these microorganisms. More specifically, there exist certain strains of the *Schizochitrium* species that have exhibited an alternative anaerobic pathway for DHA production, which involves the activity of a polyketide synthase (PKS). PKS poses a complex enzymatic system that is capable of synthesizing exclusively DHA directly from the acetyl-CoA precursor (Fig. 5) and is present in many bacteria. Transcriptomic research, as well as the lack of intermediate products, leads to the conclusion that specific eukaryotic strains, such as *Schizochitrium* sp., contain this enzymatic system for accumulation of complex LC-PUFA [59,67].

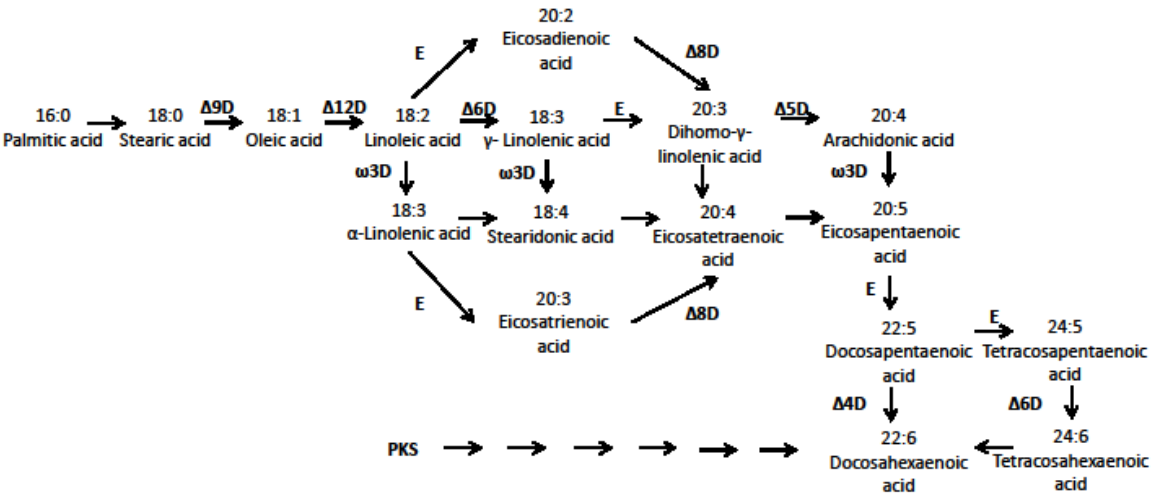


Figure 5. Different metabolic paths for FA synthesis (adapted from Harwood JL. et al) [67]

Lipid metabolism through catabolism of carboxylic acids

As mentioned before, the acetyl-CoA molecule is the main precursor of lipid biosynthesis. However depending on the carbon sources available to the cells, the catabolic activities for acetyl-CoA accumulation differ. Carbon from carbohydrates enhances the acetyl-CoA flux provided to mitochondria and the tricarboxylic acid cycle (TCA) through glycolysis (Fig. 4). This is being converted into citrate in the mitochondria, thereby providing carbon skeletons, energy as ATP, and energy for reduction as NADH.

On the contrary, carbon provided by lipid consumption, broken down to small carboxylic acids, is partly directed to the peroxisome, where the reactions of the glyoxylate cycle occur. The glyoxylate cycle transforms the acetyl-CoA into malate. It is a variant of the Krebs cycle that allows the synthesis of precursor metabolites from two-carbon substrates [84]. The two enzymes specific of the glyoxylate cycle are the

isocitrate lyase and the malate synthetase, which catalyze the formation of four-carbon metabolites from acetyl-CoA. Both enzymes have been repeatedly shown to be induced when cells are transferred to media containing acetate [72].

Heterotrophic growth using carboxylic acids, such as acetic, butyric, propionic, citric, fumaric, lactic, malic and succinic acid, as a substrate, has been demonstrated for microalgae species [85,86]. Acetate and short VFA, as already explained, are a by-product of anaerobic digestion and often accumulate in DF processes. Interestingly, microalgae can easily convert acetate into acetyl-CoA [87]. That is because, eukaryotic microorganisms are able to assimilate acetate via a monocarboxylic / proton transporter protein that aids transport of monocarboxylic molecules across the cell membrane [72]. Once inside, the transferred acetate can acetylate CoA using a single ATP molecule, with acetyl-CoA synthetase catalyzing the reaction [88]. That one-step reaction means that conversion of received carbon to acetyl-CoA is less energy demanding in the case of the carboxylic acids pathway, in contrast to the glycolysis, but on the same time, it lacks the provision of NADH offered by glucose catabolism.

Butyrate is another major by-product of DF, but butyrate assimilation by microalgae has not been studied yet so detailed. Butyrate has relatively higher molecular weight and associated complicity and requires more steps for conversion to acetyl-CoA. Similar to transport of acetate, it can be anticipated that butyrate enters via a monocarboxylic/proton transporter across the membrane. In the glyoxysome, butyrate is converted to acetyl-CoA through β-oxidation [84]. Then the acetyl-CoA is once more partially used for biosynthesis via the glyoxylate cycle, and partially for energy production via the TCA cycle. β-Oxidation is the major source of acetyl-CoA and prerequisite for both processes [89]. Although acetate can be efficiently converted into lipids, butyrate uptake by microalgae is much slower and can reduce the microalgae

growth when both VFA are present. This problem can be solved either by increasing the initial microalgae biomass or by increasing the initial acetate: butyrate ratio [90].

4.4 *Crypthecodinium cohnii*

C. cohnii is a microalga strain of high biotechnological and industrial importance. It has been engaging the interest of researchers since the 1960s and that of the industry since 1990s, but still there are many characteristics of this intriguing microorganism that remain unknown.

Dinoflagellates

C. cohnii is a member of the group of dinoflagellates, eukaryotic unicellular microorganisms of the phylum Dinoflagellata that has rather recently been classified in the kingdom of Alveolates. Dinoflagellates acquire their name from the characteristic pair of flagella they possess. One of the two is folded around the cell, enabling the cell to spin around, while the other extends outward, offering the cell mobility. They are marine protists with impressively long genome, even longer than the human's, which often form endosymbiotic relationships with bacteria. Their DNA is organized in chromosomes, inside a permanent nucleus, however the chromosomal organization lacks the presence of histones and nucleosomes[66,91].

C. cohnii is described by many as a supraspecies, including several biological species of similar morphology. Its cell wall consists of a very thin, cellulosic theca and the cells appear in two different forms; motile "swimming" cells or static cysts. They can reproduce both asexually and sexually- especially in conditions of nutrient deprivation-, but, in order to multiply themselves, motile cells need to shed their flagella and become cysts. The microorganisms are found in many habitats over the globe but they are

mostly described as neritic species that often appear in brackish water where decaying seaweeds thrive [66,91,92].

Nutrition and FA profile

The importance of the specific microalga for research and industrial purposes is derived from its characteristic ability of assimilating various substrates, as well as from its unique FA profile. *C. cohnii* is an obligatory heterotrophic, aerobic organism that can grow under dark, utilizing an organic carbon source for energy and building blocks provision. So far many different carbon sources have been used successfully for the cultivation of the cells, such as glucose, ethanol, dextrose, carob pulp syrup, galactose and acetic acid [93–95]. Equally important for the heterotrophic growth of the alga is the nitrogen source. In the case of *C. cohnii*, cells have grown well with various nitrogen sources such as meat or yeast extract (YE), corn steep liquor, urea or ammonium salts [91].

C. cohnii is classified as an oleaginous microbe, which means that it accumulates lipid at more than 20% of its dry cell weight (DCW), most of which is accumulated as neutral TAG. Apart from FA, *C. cohnii* is known to produce some isoprenoids, like carotene, that are responsible for the yellow to orange color of the cell cultures, as well as secrete extracellular polysaccharides [91]. Still, it is their lipid content that cells are cultivated for.

The microalga accumulates a big reservoir of lipid, around 30-50% of which belongs to the PUFA fraction and more specifically to the omega-3 DHA [96]. *C. cohnii* not only is an excellent producer of DHA, it also produces no other PUFA (to more than 1% DCW), thus simplifying the extraction and distribution of the final lipid product. Most researchers agree that DHA is accumulated in the form of neutral lipids, especially under

nutrient limitation conditions. Still, there have also been examples of DHA being integrated in phospholipids [97]. These differences may be attributed to the different strains, answering for the great variety of microorganisms under the *C. cohnii* name, or the age of the culture when the cells where harvested [91].

Industrial applications

C. cohnii is a microalga long known in the industrial field. Crucial for its use for industrial purposes was the fact that it has no pathogenic effect in human and animals and does not produce any toxins, in contrast to other known dinoflagellates [52]. Strains developed in the laboratory, from a *C. cohnii* strain allocated from UTEX culture collection, are exploited by Martek Biosciences for the production of oil rich in DHA, commercially known as DHASCO (DHA single cell oil). DHASCO received the GRAS certification (Generally Recognized As Safe) in the United States in 2001 [52,66]. Since then it has been widely used, both as an adult supplement and mostly as an additive for infant formulas, for the reasons described before. It can be found either alone or in combination with ARASCO, fungal arachidonic acid produced by the same company. The commercial success of the single cell oils (SCO) was such that Martek Biosciences was bought by DSM (Dutch State Mines) in 2011 [66].

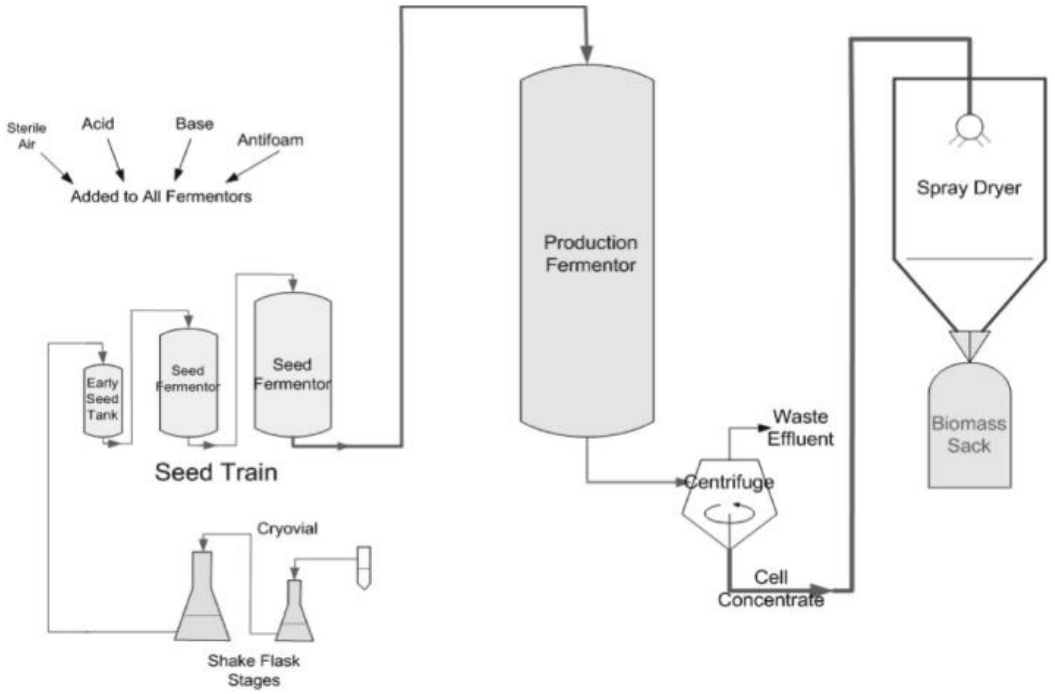


Figure 6. Diagram of *C. cohnii* biomass production and harvesting by Martek Biosciences (until 2010) [66]

According to the company's procedure, *C.cohnii* cells, prepared from a cryovial stock each time (Fig.5), grow according to a two-stage protocol. During the first phase, cells grow normally, reaching a normal lipid content of around 20% DCW, with constant feeding of a carbon source. Afterwards, normally occurring nitrogen depletion leads to a surplus of carbon and accumulation of TAG, rich in DHA. The microbial lipid is extracted and refined similar to commercial plant lipids, before release in the market (Fig.6) [66].

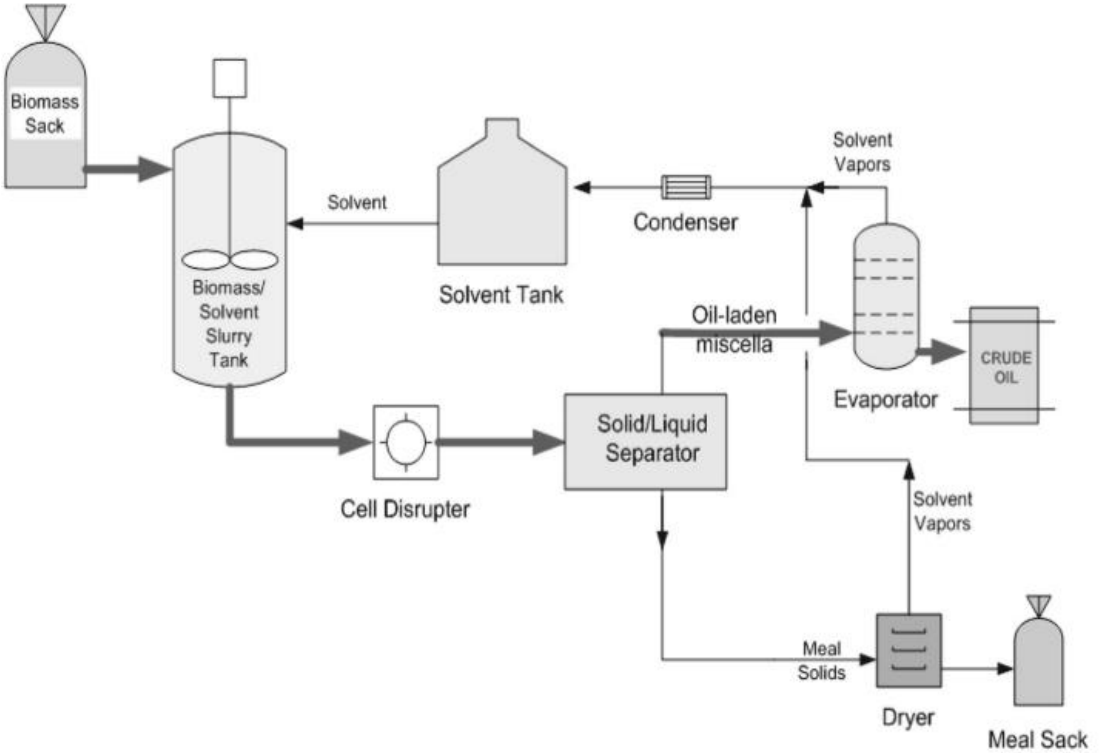


Figure 7. Diagram of lipid extraction from dried *C. cohnii* biomass, according to Martek Biosciences' protocol [66]

5. Fermentation Design

In order to enhance the production of a certain bioprocess product, the selection of the proper conditions and raw material needs to be seriously addressed. In the case of a fermentation process, the most influencing parameters depend on the specific cultivation and they usually belong in the following categories.

5.1 Important parameters

Carbon source

When discussing a heterotrophic cultivation of a microbe, the first think to consider is the carbon source. Carbon sources, apart from offering the most necessary building block for the cell, serve as chemical energy providers. Usually, most fermentation processes are carried out with either a glucose, acetate or glycerol feed [86].

Glucose is the easiest fermentable substrate for the majority of organisms. It provokes high rates of growth and respiration, by offering 2.8 kJ of energy per mol. In general it has a mass conversion ratio to heterotrophic microalgal biomass equal to 40- 64% and a lipid conversion around 19-31% [86]. However, being so easily fixed by the cells, it can lead to catabolite repression. This phenomenon leads to the reduction of the synthesis rate of enzymes, including the ones involved in the utilization of other substrates, thus reducing the uptake of other nutrients [98]. For this reason, glucose is not preferred when consumption of more than one substrate is desired. What is more, glucose may be an inexpensive carbon source, but, due to its variety of applications, it is highly arguable if it should be used for microbial cultivation and not human or animal feeding purposes.

Acetate is an alternative carbon source that leads to the production of lipid precursor acetyl-CoA, without the requirement of excessive energy from ATP. Thus it offers carbon- in the form of malate or citrate from the glyoxylate or TCA cycle accordingly- and secures ATP economy by the cells. Nevertheless, acetate use is limited by the toxic effect that it has on some microorganisms. Contrary to that, glycerol is another popular alternative to glucose, with no usual toxicity [86]. Its use is promoted by the fact that it is a by-product of petrol refining processes and therefore available in large quantities.

Although the aforementioned carbon sources have exhibited good qualities, it must be noted that they are fairly expensive in their pure form. Therefore, their use is limited in the formation of defined media of known composition for laboratory purposes. Large-scale, industrial fermentations are usually carried out with complex, natural media, or waste-derived feeds that sometimes even require a detoxification step prior to utilization [50,98].

Nitrogen source

Nitrogen is the second most important and plentiful nutrient for cell proliferation. It provides building blocks for the formation of amino acids and subsequently proteins. The ability of microorganisms to assimilate various nitrogen sources is strain-dependent and also is determined by the nitrogen form. The reason is that the first, necessary step for nitrogen fixation by the cells is the reduction of N_2 to ammonia. Therefore organic nitrogen in the form of amino acids, or inorganic ammonium, in the readily available reduced form, are usually more easily utilized by the cells [72,98]. Usual nitrogen sources for fermentation purposes are nitrate, ammonium salts, urea, and more complex such as YE or peptone.

Carbon to nitrogen ratio

Apart from the form of it, the nitrogen availability to the microorganisms plays a key role in fermentation processes. Nitrogen, together with carbon provided by the medium, functions as precursor for the synthesis of enzymes that carry out intracellular activities for the maintenance and proliferation of the organism. Subsequently, in case only one of the above is available, cellular functionality is affected. A usual behavior of microbial cells, when carbon is in excess, is the accumulation of storage compounds, such as starch or lipids [99]. For that reason, the adoption of a high C/N ratio, which answers for

a deficiency of nitrogen, in comparison to carbon, is a usual technique for the increase of the lipid content of the cells [100]. In microalgae, a change in the production also of other valuable metabolites has been reported, such as carotenoids and chlorophyll [101,102].

Temperature

Temperature is a parameter to be considered in every fermentation process and is microbe-dependent. Each microorganism has a characteristic temperature range where it can grow better and therefore this ability must be taken into account. Usually temperature is not so crucial in the accumulation of intracellular metabolites but it can have a potent role in the quality of those. In microalgae, for example, temperatures lower than the optimal range for growth result in an increase of the unsaturated lipids accumulated by the cells. This is attributed to the ability of unsaturated FA to enhance the cell's membrane fluidity, which is hampered in a cold environment [103].

Oxygen availability

Availability of oxygen should be seriously addressed, when designing a fermentation process [104]. It can be achieved, either with sufficient stirring, or by securing a high air flow entering the bioreactor. Its importance is based on the necessity of oxygen for the proper function of the enzymes produced by the cell. In the case of *C. cohnii*, previous research has shown a substantial increase in the growth rate, under higher dissolved oxygen (DO) levels. Also DHA percentage was enhanced, according to the researchers [105]. Still the effect of DO in the final DHA percentage is under discussion, owing to the uncertainty about its intracellular production and also to its vulnerability to oxidation. If DHA is accumulated through the normal FAS pathway, which uses elongases and desaturases, its dependence on the oxygen availability is clear. On the

other hand, if an anaerobic, complex PKS enzyme system is responsible for DHA accumulation, then different DO levels are not expected to cause high DHA fluctuations.

Stirring

Stirring is desirable in fermentation processes because it allows the proper circulation of oxygen, the medium nutrients, cells and extracellular metabolites in the total volume of the culture. Thus it ensures the homogeneity of the culture and the availability of nutrients and oxygen. The amount of stirring however should be determined after taking into consideration that excessive mixing may lead to the development of shear forces that can damage the cells. In cases of high-cell density cultures, characterized by high viscosity values, the addition of commercial polysaccharide-hydrolyzing enzymes has been proven to reduce efficiently the viscosity, without the need of developing extremely high stirring velocities [86,106].

pH control

The optimum pH for microbial growth is strain-dependent. Therefore, the control of the pH within the range that is acceptable by the microorganism is a prerequisite for every fermentation. Still, it can sometimes be proven as a useful tool for the feeding of the culture as well. In case of microbial fermentations carried out with an acidic feed- such as acetic acid- the consumption of the acid by the cells leads to pH increase of the medium, which, if not addressed, will eventually inhibit further cell growth. In such occasions, further addition of acid will decrease again the pH and also provide more carbon for the culture. This fermentation technique refers to a fed-batch cultivation mode, where a feed of acid is constantly added inside the bioreactor vessel- referred to as pH-auxostat-, ensuring both the pH control and the carbon availability. The pH-auxostat is repeatedly adopted in *C. cohnii* fermentations with carboxylic acids [85,95].

Cultivation mode

The above parameters can all be categorized as cultivation conditions. The control of these conditions greatly depends on the fermentation method that will be applied in each case. Usually, the submerged fermentation modes adopted in most processes belong to one of the following four categories; batch mode, fed-batch mode, continuous mode or semi-continuous mode.

Batch mode describes a process in a closed vessel where all nutrients and inoculum are situated inside from the beginning of the process, with no streams entering or leaving the vessel during the whole process. On the contrary, fed-batch mode refers to the addition of a stream- usually a feed- during the whole fermentation, but with no removal of material. In continuous mode, an entering and a leaving stream ensure the maintenance of constant conditions inside the fermenter during the whole procedure, while a semi-continuous bioreactor involves the non-continuous, simultaneous addition of fresh medium and removal of a part of the culture liquid at specific times, during the process [86].

Microalgal fermentations are usually carried out in batch or fed-batch modes. In general, a fed-batch mode allows the addition of extra feed in the culture, thus maintaining the proliferation of the cells for a longer period and finally resulting in higher biomass production. Furthermore, it poses a solution in case of substrate inhibition at high concentrations [50].

Apart from opting for a single mode, a combination of methods can also be applied, according to the needs of the bioprocess. A characteristic example is the two-stage fermentation that is adopted when the accumulation of a specific metabolite is desirable. The production of functional metabolites by the cells requires energy and is usually

increased during stress periods, when the biomass growth rate decreases and energy flux is directed to other intracellular functions. For that reason the application of a two-stage cultivation mode effectively allows the accumulation of a higher amount of the final product. During the first stage, the culture conditions favor the biomass proliferation, thus resulting in a high cell concentration inside the fermenter. Afterwards, the conditions change to enhance the accumulation of the favorable metabolite, while the growth is hampered. That way the process results in a culture broth of high biomass concentration, with each cell being rich in the desired substance [86,107–109].

5.2 Scale-up challenge

When designing a bioprocess with industrial potential, the difference between lab-scale and industrial-scale results must be taken into consideration. It is usual that laboratory processes will accomplish a higher product yield than large-scale ones. On one hand, this is the result of a waste of more residual biomass remaining in the bigger industrial equipment, when harvesting is completed. On the other hand, it is a normal course dictated by the different needs of the two systems. For example, a lab fermenter, sterilized in an autoclave, results in higher nutrient concentration, owing to partial evaporation of the liquid. On the contrary, a larger, industrial fermenter, usually sterilized by steam injection, is characterized by a lower concentration of nutrients before inoculation. What is more, nutritional media prepared in the bench are a mixture of pure chemicals of defined composition, while industrial cultivations, due to financial restrictions, include more complex media with less optimized nutrient content [98]. Therefore, the researcher must expect a small difference of the resulting values when scaling up a designed process.

5.3 Model-based bioprocess optimization

As it has already been mentioned, fermentations are widely used in industrial level. However, one basic drawback they have is that they depend on biological systems, which can sometimes be highly unpredictable. In order to standardize as much as possible a fermentation process, it is advisable to try to create a mathematical model describing the outcome of the bioprocess, if specific conditions change. In case the attempt is successful, it is possible also to optimize the process in order to accomplish maximum yields of the desirable products or effect. Optimization of an industrial process begins from the laboratory bench and the last century is mostly aided by focusing on a proper Design of Experiments (DoE) method.

Design of Experiments

DoE is a category of methods that can be adopted for the determination of the experiments that are maximally informative for a chosen mathematical model. Therefore it allows the user to acknowledge and practice the minimum amount of experiments, thus saving time and resources [110]. It is useful when a specific output of a process can be influenced by various parameters, and therefore, both the effect of one parameter and the combined effect of all needs to be estimated. As a technique it represents an improvement of the older “One Variable At a Time” (OVAT) approach that included testing of the effect of one experimental factor at a time, while keeping constrained all the others [111].

DoE is usually applied with the purpose of screening those variables, or factors, whose effect in the desirable outcome is more important, for the optimization of a specific process, or as means of robust design; meaning the reduction of variations in a system, without eliminating the causes [111,112]. In mathematical terms, when an observation *y*

can be related to a number *j* of M different x_j factors by a specific function $y= f(\hat{x})$, where $\hat{x}= (x_1, x_2, \ldots, x_M)$, then DoE determines that design matrix *D*:

$$D = \begin{pmatrix} x_{11} & \cdots & x_{1M} \\ \vdots & \ddots & \vdots \\ x_{1N} & \cdots & x_{NM} \end{pmatrix} \qquad (1)$$

which allows minimization of the *N* number of observations necessary to define the function f. In the matrix, x_{ij} is the j^{th} variable for the i^{th} observation, adapted in order to acquire values between -1 and 1 [110].

DoE methods vary, based on the design purpose, the number of the independent variables and the accuracy that is desired by the model. Full factorial designs take into consideration all different combinations of the different parameters and can therefore be proved infeasible for a high number of factors. In such cases, the adoption of a fractional factorial design, that does not require all the combinations of all the levels of each factor, is preferable [111]. Furthermore, different design methods can also accept a different amount of levels for each variable. Normally, when a model for the optimization of a process is required, through response surface methodology (RSM), the most popular DoE methods are Box-Behnken and Central Composite Design (CCD) [112].

a) Central Composite Design

CCD is a sum of factorial or fractional factorial designs that includes the use of center points, augmented with a group of axial or star points, for the determination of the different values of the independent variables, thus allowing three or five distinct levels for the input variables. Star points are always twice as many as the different factors examined and correspond to the new extreme values that the factors will take. Based on this characteristic, CCD is ideal for curvature estimation and prediction of the responses

at the extremes of the set values. On the contrary, Box-Behnken is suitable when the experimenter is not interested in the behavior of the system in extreme values [112].

Response Surface Methodology

RSM is a collection of statistical methods that combines DoE with Taylor's mathematical theorem for the creation of a model that describes a response of a system when some, important for it, independent variables acquire values within an examined range. Although other orders can also be adopted, RSM is usually using Taylor's 2nd order equation for the description of the mathematical relation characterizing the examined system, the general form of which is:

$$y = \beta_0 + \sum_{j=1}^{M} \beta_j \cdot x_{ij} + \sum_{j=1}^{M-1} \sum_{k=j+1}^{M} \beta_{jk} \cdot x_{ij} \cdot x_{ik} + \sum_{j=1}^{M} \beta_{j+M} \cdot x_{ij}^2 \qquad (2)$$

DoE is the first step of the method and allows the collection of the experimental data. Afterwards, the values collected are fitted in Taylor's equation for the determination of the coefficients β_j. As the name of the methodology itself gives away, the resulting curve from the final equation estimated represents a surface that approximates the response of the system to the different factors [110,113].

RSM is a widely used methodology that has been repeatedly applied for many systems, including microbial bioprocesses [114]. The use of Taylor's second order polynomial allows, not only the determination of the effect of the specific factors, but also the estimation of the quadratic effects and the combined influence of more than one factors. It offers the ability of designing properly or even optimizing a process as long as the following criteria are followed; for application of RSM the critical factors of a process need to be known and acquire values that are continuous. Also there must exist a mathematical function that relates the examined response to the factors, otherwise the

model will have no physical meaning. Lastly, when applying RSM the range, over which the factors are to be tested, must be carefully defined. Too narrow a range may falsely determine a factor as insignificant, because the model won't be able to detect its effect. On the other hand, a very large range can also lead to the same false conclusion, since the equation will not adequately explain its influence. The range evaluated is therefore of high importance and, after all, it also signifies the boundaries within which RSM models can predict a result for the examined system [113].

Optimization, with RSM tools, is carried out by using the Derringer's desirability function [115]. The function combines the influence of different factors in a single equation, based on the idea that the quality of a response, influenced by those factors, is acceptable only if all factors are within some goal values. The evaluation of acceptability, that is the desirability of a response (d), takes values between 0- corresponding to no acceptability- to 1-corresponding to the maximum acceptable option [115]. For k different responses Y_i that need to be optimized, the response of the desirability function is $D=(d(Y_1)d(Y_2)\dots d(Y_k))^{1/k}$, where desirability functions $d(Y_i)$ might refer to minimization, maximization or reaching a specific goal value.

6. Purpose

Current state of the art

Although the use of autotrophic microalgae for wastewater treatment is in general more widespread [116], their use for bioconversion of waste is still on an infantile level. So far, the DHA producing strains *C. cohnii* and *Schizochitrium* sp. have been examined, in lab scale, as means of bioconversion of a few organic-rich waste streams. More specifically, *C. cohnii* has exhibited the ability to assimilate date syrup and carob pulp [117,118], while *S. mangrovei* has been cultivated on food waste hydrolysates [119] and

S. limanicum on sugarcane bagasse and crude glycerol [55,120]. With regard to VFA, Turon et al. were the first that published the idea of DF effluents as carbon substrates to support the growth of heterotrophic microalgae [84]. What is more, they tested the idea with both synthetic and real DF effluent (sterile and non-sterile) for the growth of two *Chlorella* species, *Chlorella sorokiniana* and *Auxenochlorella protothecoides*. The cells grew well, either in axenic cultures or in a bacteria consortium, accumulating FA, which however do not belong to the omega-3 category and can only be exploited for biodiesel production [84,90,121]. To the best of our knowledge, no previous research has been conducted, regarding the heterotrophic growth of microalgae on DF effluents or lignocellulosic biomass hydrolysates for omega-3 FA production.

Purpose of the present work

So far, any valorization of the DF liquid fraction has been limited to "proof of concept" lab experiments, which resulted in the production of short-chain microalga FA, useful only for biodiesel. Furthermore, to the best of our knowledge, the bioconversion of pretreated lignocellulosic waste streams by microalgae has not been examined. With the scope of achieving a more effective utilization of the by-products of existing industrial processes, a bioconversion platform for the valorization of biowaste-derived organic acids or lignocellulosic waste, to high added-value metabolites, is proposed. The conversion of these waste streams to expensive nutritional products, such as omega-3 FA, offers a breakthrough and promising solution for the establishment of a biorefinery that will further support and sustain a CE. What is more, the high cost of the final product can justify the expensive processes of harvesting and lipid extraction from microalgae, that normally pose a disadvantage to other processes such as microalga lipids production for biodiesel [68]. For that reason, this thesis included the examination of the ability of the microalga *C. cohnii*–a known omega-3 producer- to assimilate the

different waste-derived substrates, the determination of the important cultivation conditions and the fermentation mode and finally the endeavor for optimization of the bioprocess for high DHA production. The included attempts for the optimization of the valorization of waste-derived harmful organic content into omega-3 FA were focused, not only on the application of the optimum cultivation conditions, but also on securing a feed, rich in available nutrients and carbon. The purpose was always the establishment of processes with industrial potential, in order also to address the current gap in the literature.

Challenges and difficulties

As in the case of all waste valorization processes, the greatest difficulty of designing a functional fermentation lies with the composition of the waste stream. The pretreated waste may include many different nutrients that vary from batch to batch, as well as other ingredients that can even act as inhibitors to the growth of microbes. Therefore the standardization of such a process, which is to receive a feed of variable composition, is almost impossible. Still such valorization processes are the only solution for the successful implementation of a CE. This thesis provides an elaborated and scientific, step-by-step approach to the design of industrially exploitable fermentation protocols for the different waste streams examined.

Materials and Methods

1. Chemical and biological materials

1.1 Strains

C. cohnii ATCC® 30772™ was obtained from the American Type Culture Collection (ATCC), cultivated and maintained according to the protocols given in the product sheet. Stock cultures of seed cells, with a final volume of 50 mL, were prepared by inoculating fresh, sterilized ATCC 460 medium (A2E6) with 10% (v/v) cells from the previous stock. Also cryopreservation stocks were prepared according to the company's protocol. For the exact recipe of the ATCC 460 medium please refer to Appendix 1.

The methylotrophic yeast *Pichia pastoris* X-33 was used for transformation and expression of the recombinant protein TtLPMO111088 from the genome of the thermophilic filamentous ascomycete fungus *Thermothelomyces thermophila* (or *Sporotrichum thermophile*). For the cloning of the plasmid vector One Shot competent TOP10 *Escherichia coli* cells from ThermoFisher Scientific Inc. (USA) were used.

1.2 Chemicals for media preparation and enzymatic reactions

Chemicals used were purchased either from Sigma-Aldrich-USA (salts, Tris base, inorganic salts, VFA) or Fluka® Analytical-USA (yeast extract, concentrated HCl and H_2SO_4) or Fisher Scientific-USA (glucose anhydrous), while the organic solvents for the experiments were purchased from Fisher Scientific (chloroform, methanol) or Carlo Erba-Spain (n-hexane). Sea salts were of retail commercial origin. Solvents were of analytical grade unless if stated otherwise. Poly- and oligo-saccharides serving as substrates for LPMO reactions were purchased from Megazyme Ltd (Ireland).

1.3 Waste-derived materials used as main carbon feed

The DF liquid fraction was derived from a mixture of Vegetable Garden Food (VGF) waste, provided by Organic Waste Systems NV (OWS, Belgium) and processed by Tecnalia Research & Innovation (Spain). Before delivery, the effluent was ultra-filtrated through a 1200 mm-length and 70 nm-pore size membrane. The different permeate's VFA composition is shown in Table 1.

Lignocellulosic biomass consisted either of a commercially available feedstock of beechwood fibers with a particle size in the range of 150-500 μm (Lignocel® HBS 150-500, JRS GmbH and Co KG, Germany) or of bark-free pine trimmings of *Pinus* sp. as a representative softwood biomass for comparison. Biomass was pretreated by mild-oxidative organosolv pretreatment in the Chemical Process and Energy Resources Institute (CPERI), CERTH. Pretreatment was carried out with a mixture of 50:50 (v/v) of H_2O: organic solvent as a liquid phase at a solid to liquid ratio equal to 1:10. The reactor vessel was pressurized with 100% O_2 and heated up until it reached the desired temperature (175 ^oC). Reaction time varied between 60-120 min, while tetrahydrofuran (THF), acetone and ethanol were used as organic solvents. After cooling down and reactor depressurization, the solid pulp was obtained through vacuum filtration, washed, dried and provided to the Biotechnology Laboratory of the School of Chemical Engineering, NTUA for experiments. The final moisture of the pulps was 5-8 wt. %.

Table 1. Composition of the different DF effluents received

Batch no.	Delivery date	Acetic (g/L)	Butyric (g/L)	Propionic (g/L)	Valeric (g/L)	Caproic (g/L)	Sum of VFA (g/L)	NH_4^+ (mM)
1	4/2018	3.9	2.5	1.8	0.5	1.4	10.1	44
2	9/2018	3.8	1.9	1.7	0.8	0.6	8.8	18
3	2/2019	8.1	3.6	5.7	3.5	1.3	22.2	48
4*	12/2019	51	28.9	29.8	9.8	5.6	125.1	457

*The effluent no. 4 was concentrated by evaporation to achieve high VFA concentration.

1.4 Genes and plasmid vectors

The gene encoding the hypothetical LPMO, situated in the second chromosome of *T. thermophila*, between the nucleotides 1102431 and 1114762 was synthesized and codon optimized for expression in *P. pastoris* by GenScript Biotech Corporation (USA). The plasmid vector pPICZaA of Invitrogen (USA) was used for transportation of the gene and its natural signal sequence in the yeast cells.

1.5 Commercial enzymes

The enzymes or enzyme mixtures used for lignocellulosic hydrolysis and recombinant DNA techniques were of commercial origin. Hydrolysis was performed with Cellic® CTec2 from Novozyme Coorporation (Denmark). All restriction enzymes used, including their reaction buffers, were purchased from TaKaRa Bio, Inc. (Japan).

2. Cell cultivation methods in biowaste derivatives

2.1 Batch cultures

Batch cultures of 50 mL ATCC 460 Medium (A2E6) were prepared in Erlenmeyer flasks as mentioned above in paragraph 1.1 and maintained for 4 days at 23 ^oC without

stirring. These stock cultures were used as inoculum for pre-cultures. Pre-cultures (50mL) contained 9 g/L glucose, 18.7 g/L sea salts and 2 g/L YE, while the volume of the inoculum was 10 % of the final culture volume and the pH of the medium was set at 6.5 prior to autoclaving. Pre-cultures were incubated at 23 ^{o}C, without stirring, under dark for 3 days. They were then used as seed cells for the batch cultures (50 mL), with medium containing 18.7 g/L sea salts, 2.8 g/L YE, 100 mM Tris-HCl buffer and acetate, propionate or butyrate at a concentration of 5, 10, 15, 20 or 30 g/L. A control culture without any acid addition was also examined. Cultures were carried out in duplicates and samples were collected on a daily basis to monitor cell growth. The pH of the medium was set at 6.5, prior to autoclaving. Tris buffer was used in order to control the pH of the culture during incubation time.

In case of optimization experiments, batch cultures with 30 g/L sodium acetate (SA), 18.7 g/L sea salts, 2.8 g/L YE- unless if stated otherwise- and buffered with 100 mM Tris/HCl at pH 6.5 were carried out at an agitation of 160 rpm. Following the approach of changing one parameter at a time, the effect of incubation temperature, nitrogen source, initial C/N ratio and addition of chemical modulators was examined. While keeping the other conditions stable, cultures were carried out at temperatures between 15-30 ^{o}C, different initial C/N ratios between 10-55 (g C/g N), addition of the chemical modulators ethanolamine and salicylic acid and the following nitrogen sources; YE, urea (Ur), $(NH_4)_2SO_4$ (Am), $NaNO_3$ (Nt) or NH_4NO_3 (AmNt) at concentrations that ensured an initial C/N ratio equal to 40 (g C/g N). The different conditions were evaluated by measuring the resulting cell biomass, intracellular total FA (TFA) and DHA produced.

2.2 Fed-batch fermentations (1 L)

Static precultures with a medium of 9 g/L glucose, 25 g/L sea salts and 2 g/L YE were inoculated with 10 % (v/v) of seed cells grown for 4 days in ATCC 460 Medium (A2E6). After incubation for 4 days at 23 ^{o}C, without stirring they were used as inoculum (10 % v/v) for stirred cultures (100 mL final volume) of *C. cohnii* containing a medium of 27 g/L glucose, 25 g/L sea salts and 3.8 g/L YE. The pH was always set at 6.5 prior to autoclaving. Stirred cultures were incubated for 3 days at 27 ^{o}C and an agitation of 160 rpm under dark and used to inoculate 2L pH- auxostats (New Brunswick Scientific-BioFlo 310 Benchtop Fermentor) containing 900 mL of medium with 15 g/L SA, 25 g/L sea salts and 7.5 g/L YE at a pH 6.5. Temperature was set at 27 ^{o}C, unless if stated otherwise, and agitation varied from 300 to 550 rpm in order to maintain the dissolved oxygen at a higher value than 20 % of saturation. Cultivation conditions were monitored and pH was controlled by adding a specific VFA feed for each bioreactor. The feeds examined were the following: 33 % (v/v) acetic acid in autoclaved deionized (DI) water, 25 % (v/v) butyric acid, 25 % (v/v) propionic acid, autoclaved real DF permeate from VGF waste acidified with HCl (Table 1.) and a synthetic medium mimicking the VFA composition of the DF permeate in autoclaved DI water. Also the examination of a two-stage fermentation strategy was realized by alternating between a feed of 33 % (v/v) acetic acid in a solution of Am at a C/N ratio equal to 110 (w/w), for the first 168 h of fermentation, and a feed of solely 33 % (v/v) acetic acid without nitrogen, later. Daily samples of the bioreactors were collected in order to monitor the cell growth. Each fermentation was terminated when the system reached static growth phase.

2.3 Fed-batch fermentations (0.1 L)

Fed-batch cultures in the BioXplorer 100 mini bioreactor system by HEL Ltd. (England) were carried out, for the determination of optimal fed-batch conditions that lead to maximum DHA production. Firstly, fed-batch fermentations with different temperature profiles, or initial C/N ratios were carried out. Initial medium included 25 g/L sea salts, 15 g/L SA and YE or Am according to the desired C/N, while the bioreactors were provided with a feed of 33 % (v/v) of acetic acid. Inoculation was completed similarly as in the 1 L fed-batch cultures. Incubation temperature was either maintained stable at 23 or 27 ^{o}C, or followed a two-stage profile, remaining at 23 ^{o}C for 72 h and later augmented at 27 ^{o}C or reduced to 15 ^{o}C for 96 h more, with the scope of achieving enhanced intracellular lipid accumulation.

The same bioreactor system was applied for a more thorough optimization of DHA production, after DoE, by the determination of a mathematical model using RSM. The independent variables were the initial C/N ratio of the culture medium, as well as the air flow (A.F) provided in the bioreactors, while the final DHA production (mg/L) represented the *y* response measured. The inoculum was prepared as in the case of the 1L fed-batch cultures and used after 3 days of incubation, when the optical density of the stirred culture at 685 nm (OD_{685}) was around 2.3-2.5. Initial medium consisted of 25 g/L sea salts and initial carbon concentration of 7.4 g/L. SA and YE initial concentrations were calculated taking into consideration that carbon content of YE is around 40 % (w/w) and nitrogen content is 11 % (w/w). Fed-batch cultures of a total volume of 100 mL were carried out at a constant temperature of 23 ^{o}C with an unsterilized feed of pure VFA mimicking the composition of the concentrated DF effluent provided by Tecnalia (Table 1). Feeding was continued for 6 days, after which period, the addition of a VFA

feed was stopped and the cultures maintained in batch mode for 24 h more before termination, to ensure the total depletion of the remaining carbon inside the vessels.

2.4 DoE parameters

DoE, RSM and statistical analysis of the resulting model, as well as optimization by the desirability function were carried out by the software of Design Expert Version 7.0.0 with the method of CCD practical, with an *a* value equal to 1.19. For each factorial and axial point, three replicates were carried out, while the center point experiment was repeated five times. The above design leads to the execution of 29 fermentations. The examined range for the independent variables was set between 5 g C/g N and 25 g C/g N for the initial C/N ratio (Factor A) and 50 mL/min to 100 mL/min for the A.F (Factor B) respectively. Scaling of the parameters values between the values of -1 to 1 was carried out, to enable the easier estimation of the effect of each parameter in the response *y*, by the following type:

$$Scaled\ value=(2\cdot Original\ value-Maximum\ value-Minimum\ value)/(Maximum-Minimum) \quad (3)$$

For the estimation of changeable fermentation cost the following values of Table 2 were taken into consideration.

Table 2. Factors influencing changeable fermentation cost based on C/N and A.F

Factor	Value	Source
Purchase cost of YE	5.40 €/kg	Alibaba.com
Purchase cost of SA	0.66 €/kg	Alibaba.com
Efficiency of bubble diffuser	2.0 kg air/ kWh	[122]
Electricity cost	0,21985 €/KWh	Public Enterprise of Electricity Dei S.A

3. Saccharification methods

3.1 Saccharification of pretreated biomass

To achieve efficient saccharification of the cellulose-rich pretreated solid pulps from beechwood or pine, Cellic® CTec2 enzyme cocktail was employed. Protein content of the cocktail was determined by the Bradford assay [123] and found to be 95.6 mg/mL, while the filter paper activity was 102.2 FPU/mL (FPU: Filter Paper Units), according to the protocol from Ghose [124]. Hydrolysis experiments were performed in 100-mL flasks in order to assess different pretreatment conditions and correlate them with the potential to yield sugars that will be subsequently used as a carbon source for microalgae. All reactions took place at 50 ^{o}C under agitation (160 rpm), at an initial dry matter (DM) of 9 (w/v) and an enzyme loading of 9 mg protein/g of biomass, in 80 mM MES (2-*N*-morpholino-ethanesulfonic acid) buffer at pH 5.5. The ratio of total reaction volume to shake flask volume was 1/10. After 72 h of hydrolysis, the supernatant containing the fermentable sugars was collected with vacuum filtration and kept in freezer (-20 ^{o}C) until further use.

3.2 Measurement of diluted sugars

At different time intervals during hydrolysis (8, 24, 48 and 72 h), samples were taken, and enzyme was inactivated after boiling for 5 min. Then, the samples were centrifuged, and the supernatant was filtered from a 0.22 μM pore size membrane and analyzed for the presence of glucose and total reducing sugars. Total reducing sugars (TRS) were determined according to 3,5- dinitrosalicylic acid (DNS) method [125]. Analysis of glucose, xylose and other monosaccharides resulted from hydrolysis was performed in a Shimadzu HPLC (High Performance Liquid Chromatography) system, by isocratic ion-exchange chromatography, using an Aminex HPX-87H column with a micro-guard

column, at 50 ºC (Bio-Rad Laboratories, Hercules, CA, USA). The mobile phase was 3 mM H_2SO_4 at a flow rate of 0.6 mL/min. The total % wt. cellulose conversion into glucose was calculated with the following equation:

$$cellulose\ conversion = \left(\frac{glucose\ \left(\frac{mg}{mL}\right)}{substrate\ dry\ matter\ \left(\frac{mg}{mL}\right)} \cdot \%wt.\ cellulose\ content \cdot 1.11\right) \cdot 100 \quad (4)$$

in which 1.11 represents the conversion factor of cellulose to glucose.

In case of microalgal cultures with hydrolysates, the consumption of glucose by the cells was measured using a rapid colorimetric method. More specifically a commercial glucose oxidase peroxidase solution (GOD-POD, Biosis) was mixed with the supernatant of daily culture samples. After incubation for 15 min at 37 ºC, the absorption of the samples at 510 nm was measured and the glucose concentration estimated.

3.3 Measurement of diluted oxidized and neutral oligosaccharides

The detection of oligosaccharides generated by the LPMO activity was carried out by High Performance Anion Exchange Chromatography (HPAEC). The analysis was conducted wirh an ICS 5000SP system (Dionex, Thermo Fisher Scientific Inc., USA) with a pulsed amperometric detector equipped with a disposable electrochemical gold electrode. The column used was a CarboPac PA1 4 × 250 mm with a CarboPac PA1 4 × 50 mm guard column, at 30 °C. 10 μL of samples were injected and the reaction products were eluted at a flow of 1 mL/min with initial conditions set to 0.1 M NaOH (100 % eluent A). Liquid phase composition changed by a linear gradient toward 10 % eluent B (1 M NaOAc in 0.1 M NaOH) after 10 min and 30 % B after 25 min; a 5 min exponential gradient (Dionex curve 6) to 100 % B followed. Between separate

injections, a 10 min stabilization step with 100 % A was interjected. Integration of chromatograms was performed using Chromeleon 7.0 software.

4. Cell cultivation methods in hydrolysates

4.1 Batch cultures with pure sugars, hydrolysates or lignocellulosic substrates

Cultures with pure sugars (hexoses and pentoses commonly found in lignocellulosic materials) were performed with an initial concentration of 30 g/L sugars, 2 g/L YE and 25 g/L sea salts at a final volume of 50 mL. Also cultures of 15 or 25 g/L of Avicel PH-101 or 20 g/L of carboxymethyl- cellulose (CMC) or corn cob were performed for the examination of the ability of *C. cohnii* to secrete cellulolytic enzymes. Inoculation, in all cases, was performed as described previously for batch cultures in VFA and they were maintained at 27 ^{o}C under 160 rpm agitation. To track the carbon consumption, total reducing sugars concentration was determined with DNS method, while analysis of monosaccharides was performed by HPLC, as described above, or the GOD-POD method in case of solely glucose feed.

Batch cultures on hydrolyzed lignocellulosic biomass were performed in similar conditions as those described above, by utilizing the glucose-rich hydrolysate after enzymatic saccharification as carbon source. After hydrolysis, the liquid fraction was diluted two times, 25 g/L sea salts and 2 g/L YE were added and pH was set to 6.5 prior to autoclaving at 109 ^{o}C, 40 min. The autoclave conditions were those as not to damage the diluted sugars in the hydrolysates. Inoculum posed 10% (v/v) of the final culture volume, as described above. Each experiment was performed in duplicates.

4.2 Fed-batch strategy

Fed-batch examination in hydrolysate cultures was carried out in Erlenmeyer flasks incubated at 27 ºC and 160 rpm agitation. Cultures were inoculated the same way as the batch ones. After 120 h of incubation, when almost total depletion of the main carbon source was detected, a volume of the same hydrolysate (not diluted) was added aseptically, in order for the final sugar concentration of the broth to reach the same value as the initial concentration. After feeding, incubation was continued for 100 h more.

5. Downstream processes

5.1 Microalgae cells harvesting

After termination of the cultivation- either in batch or fed-batch cultures- the culture broth was collected and centrifuged for 10 min at 650 *g*. The supernatant was discarded and the cell pellets were washed with DI water containing an equal concentration of 25 g/L sea salt in order to avoid any membrane destruction due to osmosis phenomena. After another centrifugation, cells were freeze-dried and stored at 4 ºC.

5.2 Cell growth estimation

Daily samples of the cultures were collected and their optical density at 685 nm (OD_{685}) was measured in order to estimate cell concentration. During the pH-auxostat 1L cultures, samples of 10-15 mL in duplicates were centrifuged and washed with salted water as previously described. The cells were freeze-dried and the dry biomass was measured in order to calculate the daily biomass concentration of the cultures. In the case of cultures with lignocellulosic hydrolysates, average doubling times (T_d) between 24-72h were calculated, using the type:

$$T_d = \frac{t_2 - t_1}{(lnOD_{685(2)} - lnOD_{685(1)})/ln2} \quad (5)$$

5.3 Measuring VFA concentration in growth medium

The daily VFA concentration of the culture medium was calculated by HPLC analysis (Shimadzu LC-20AD unit equipped with a SIL-20A autosampler) with an Aminex HPX-87H (300x7.8 mm, particle size 9 μm; Bio-Rad, Hercules, CA, USA) column coupled with a Cation-H Bio-Rad micro-guard column and a refractive index detector (RID). The analysis was conducted at 65 ^{o}C, using 5 mM sulfuric acid as a mobile phase and a constant flow rate of 0.6 mL/min, according to the National Renewable Energy Laboratory (NREL) protocol [126]. The column temperature was maintained constant using a column heater (Merck Millipore, Darmstadt, Germany).

5.4 Lipid extraction and calculation

For the extraction of the lipids from the dried biomass a modified Folch method was developed [127]. A mass of 30-50 mg of freeze-dried cells from each sample were mixed with a chloroform:MeOH 2:1 mixture (analytical grade) and left overnight at room temperature under agitation. For each 25 mg of dried cells, 5 mL of the mixture were added. After the appropriate time had passed and the lipid fraction of the cells was transferred in the organic solvent, DI water was added in the liquid. The volume of the water was equal to 20 % of the volume of the organic mixture. After centrifugation at 650 g for 5 min the upper water phase was discarded and the lower phase was washed twice with 1 mL of a MeOH:H_2O 1:1 mixture. The final extract was collected in pre-weighted glass tubes. After evaporation of the solvent in a vacuum oven (Gallenkump), the total lipids were calculated gravimetrically.

5.5 FA esterification

The dried lipids were diluted in 1 mL chloroform (HPLC grade) and mixed with 2.5 mL of a MeOH:HCl 92:8 (v:v) mixture. Reaction blends were transferred in a waterbath at 60 ^{o}C. After 15 min, the tubes with the FA methyl esters (FAME) were removed from waterbath and 2.5 mL CaCl 5% (w/v) was added. The methyl esters were extracted from the tube mixture with 1 mL n-hexane. The extraction step was repeated 3 times in order to finally collect an extract volume of 3 mL hexane plus 1 mL of the chloroform initially added for FA dilution.

5.6 Lipid profile analysis

FAME identification was carried out using a GC-MS (Gas Chromatography-Mass Spectroscopy) system (Agilent J&W HP-5 Intuvo GC column). Injection temperature was set at 270 ^{o}C, the carrier gas flow was 1 mL/min (helium) and the column temperature rose from 125 ^{o}C to 240 ^{o}C with a rate of 5 ^{o}C/min, where it remained stable for 9 min before the end of the analysis. Samples were injected in the column with a split ratio 50:1. Supelco FAME mixture in dichloromethane (Sigma-Aldrich) was used as standard. FAME analysis and composition estimation was carried out accordingly, using the same analysis protocol in a GC system coupled with a Flame Ionization Detector (FID) with an Equity 5 Capillary GC column. Quantification was carried out by estimating the percentage of each FAME.

5.7 Residual nitrogen estimation for fed-batch fermentations (1 L)

A volume of 5-7 mL of the culture medium selected daily was freeze-dried. The dried samples were weighted and their total nitrogen content was estimated by the kjeldahl

method according to AOAC 984.13. The apparatus used was a Kjeldhal Büchi 321 Distillation unit, Flawwil (Switzerland).

6. LPMO expression and characterization

6.1 Recombinant DNA techniques

The gene of *T. thermophila*, encoding the protein *Tt*LPMO111088, was codon optimized and synthesized in the plasmid vector pPICZaA- containing the AOX1 promoter for methanol-induced expression. DNA was amplified in *E. coli* TOP10 cells exhibiting resistance to zeocin at a concentration of 25 μg/mL. The cloned recombinant plasmid was linearized with the restriction enzyme PmeI and used for the transformation of *P. pastoris* X-33 cells by electroporation, as described in the EasySelect Pichia expression kit instruction manual.

6.2 Protein production and purification

The transformed yeast cells were selected by incubation in YPDS petri dishes with zeocin 100 μg/mL. They were used for the inoculation of 25 mL of BMGY medium in Erlenmeyer flasks. Cultures were incubated at 30 ^{o}C and agitation of 180 rpm for 16-18 hours, to reach an OD_{600} around 2-6. Afterwards, the appropriate volume of the culture was selected and centrifuged for 7 min at 650 *g* for total removal of BMGY medium. Cell pellets were re-suspended in fresh BMMY medium and used to inoculate a culture of 500 mL of the same medium with an initial OD_{600} equal to 1, according to the protocol described by M. Weidner et al. [128]. *P. pastoris* cultures were incubated at 30 ^{o}C and 180 rpm for five days. The induction of the expression of the recombinant protein was realized by daily addition of methanol in a final concentration of 5 mL/L culture.

After the end of cultivation, the medium broth was harvested by centrifugation and filtered through a 0.22 μm pore membrane. The permeate was concentrated ten-fold by ultrafiltration through an Amicon apparatus (exclusion size, 10 kDa; Amicon chamber 8400 with membrane Diaflo PM- 10, Millipore, Billerica, MA). The concentrated crude protein was purified using immobilized metal ion affinity chromatography (IMAC) (Talon, ClonTech) according to the protocol described by Topakas et al. [129]. Tracing of the recombinant protein was carried out by SDS-PAGE electrophoresis, following the steps described in next paragraph.

6.3 Protein characterization

The substrate specificity and also activity of *Tt*LPMO111088 was examined in reactions of 1 mL with the following different polysaccharides at a final concentration of 0.5 % (w/v); wheat arabinoxylan, beechwood xylan, chitin, phosphoric acid swollen cellulose (PASC), avicel cellulose, starch and konjac glucomannan. Ascorbic acid at a final concentration of 2 mM was the electron donor, unless if stated otherwise. The reactions were carried out at 50 ^{o}C under agitation of 900 rpm, overnight, with a buffer of sodium acetate 50 mM at pH 6. Enzyme loading was 12 mg/ g substrate.

In order to examine the activity of the enzyme on soluble oligosaccharides, reactions with 0.5 mg/mL of the following oligo-saccharides were realized; cellohexaose, manno-, xylo-, chito- and cello-pentaose. Enzyme loading and reaction conditions were the same as mentioned above. Reactions were carried out in duplicates and samples were taken at 30, 60 and 180 min.

Temperature and pH optimum were examined in reactions of 0.5 % (w/v) PASC at temperatures between 40-70 ^{o}C and pH between 4-7. For the examination of the effect of different electron donors, the reagents; vanillin, guaiacol, pyrogallol, ferulic, ascorbic,

caffeic, *p*-coumaric and gallic acid were added at a final concentration of 2 mM in 0.5 % (w/v) PASC reactions.

Furthermore, the possibility of synergy of LPMO with other cellulases was tested, and more specifically with Cellic® CTec2 from Novozyme Coorporation (Denmark). The commercial enzyme mixture was added in hydrolyzing blank reactions with a dry matter content of 2.5 % (w/v) of organosolv pretreated beechwood biomass and a protein loading of 6 or 5 mg/g substrate at a final volume of 10 mL MES buffer 80 mM at a pH 5.5. *Tt*LPMO111088 protein was added in the mixture, in order either to substitute part of the protein loading of Cellic (4 mg Cellic-protein:1 mg LPMO per g of substrate) or to complement the Cellic (5 mg Cellic-protein: 1 mg LPMO per g of substrate). In some reactions, also 1 mM ascorbic acid was added as reducing agent for LPMO activation. Hydrolysis was carried out in duplicates for 24 h at 50 ^{o}C with an agitation of 180 rpm. The performance of the enzymes (Cellic and LPMO) was determined by measurement of the TRS with the DNS method, as mentioned previously, as well as, by estimation of the final glucose release by the glucose oxidase/peroxidase (GOD-POD) assay [130].

For the determination of the theoretical characteristics of the molecular weight and the extinction coefficient of the enzyme the ProtParam tool of Expasy was selected. Furthermore, the possible glycosylation sites were discovered utilizing the bioinformatics tools NetNGlyc 1.0 Server and NetOGlyc 4.0 Server [131,132]. The real molecular weight of the produced enzyme was estimated by SDS-PAGE in a Mini-Protean 3 (Biorad) apparatus with a glycine 1 % (w/v) buffer of pH 8.3. Seperation by electrophoresis of the protein samples was carried out by application of current of 35 mA for around 90 min. Afterwards, the gel was extracted and painted with Coomassie Blue for the detection of the protein bands. A standard mixture of known protein

samples (ladder) of Nippon Genetics Europe GmbH (Germany) was used for identification of the molecular weight of unknown bands.

7. Examination of microalgal cellulases production ability

Batch cultures of Avicel PH-101, CMC and corn cob served for the examination of the ability of the microalgal cells to secrete cellulolytic enzymes in the culture broth. Daily samples of the cultures were collected and centrifuged for separation of the supernatant. For all assays, also samples of cell suspensions were examined, to include also the possibility of membrane-bound cellulases. A 5 min-boiled supernatant sample was used for blank reactions. To determine the presence of enzymes the following assays were applied, in duplicates:

7.1 EG assay

Examination of EG activity was realized by reaction of the supernatant with 1 % (w/v) CMC at 50 ^{o}C and 900 rpm for 30 min. The supernatant was 50 % (v/v) of the final reaction volume. Acetate buffer 0.05 M was used at a pH equal to 5. After termination of the reaction, the released reducing sugars were quantified with the DNS method.

7.2 CBH assay

CBH activity was traced by reaction of 20 % (v/v) final reaction volume supernatant with 1 % Avicel PH-101 for 1 h at 50 ^{o}C under 900 rpm agitation. Reaction was buffered with sodium acetate 0.1 M at pH 5.5 and DNS method used for released sugars estimation. Alternatively, 1.7 mM of *p*-nitrophenyl-β-D-cellobioside (*p*NPC) was used as substrate, instead of Avicel, according to the assay of β-GL mentioned below.

7.3 β-GL assay

β-GL assay was carried out with 1.7 mM of *p*-nitrophenyl-D-glucopyranoside (*p*NPG) with sodium acetate buffer 0.1 M pH 5.5. Reaction included supernatant at 6.7 % (v/v) of final volume. After incubation for 20 min at 50 ^{o}C, 1 mL of glycine buffer 0.4 M pH 10.8 was added for 0.7 mL of reaction volume. The amount of released *p*NP was determined by measuring the OD_{430}.

7.4 Xylanase assay

Secretion of xylanases was tested only for corn cob cultures, by addition of 10 % (v/v) of culture supernatant in 0.5 % (w/v) beechwood xylan in citrate buffer 0.05 M pH 5. Reactions were incubated for 1 h at 50 ^{o}C under 900 rpm agitation. Released sugars were quantified with DNS.

Results and Discussion

1. DF effluent as substrate for *C. cohnii* cultivation

1.1 Sole VFA as main carbon source

Batch cultures

The first step towards the utilization of the DF effluent was to ascertain the ability of *C. cohnii* to grow on the main VFA that are present in the waste-derived liquid. The inability of the strain to assimilate certain VFA in the DF effluent would be detrimental, due to a possible growth inhibition through the accumulation in the culture medium. Batch fermentations with acetic, propionic or butyric acid at different initial concentrations not only showed that the strain *C. cohnii* ATCC® 30772™ can utilize the aforementioned carbon sources for its growth, but also indicated the optimum initial concentrations for biomass growth and/or lipid accumulation. The sea salt concentration used was selected for the batch cultures, because it has been proven to be the lowest concentration that didn't influence negatively the growth rate of the cells- at least under glucose feed [133]. A lower salt concentration would inhibit cell growth. On the other hand, a higher than necessary salt concentration is undesirable in processes with industrial potential, due to unfavorable corrosion of the industrial metal equipment. The presence of Tris-HCl buffer is necessary for the pH control, maintaining the growth of the microalga until the complete consumption of the carbon source. When *C. cohnii* assimilates one or more VFA present in the culture broth, the pH of the medium rises as a result of the gradual removal of CH_3COO^- anions and their replacement by OH^- and other anions. This results in the formation of NaOH that is a much stronger base than CH_3COONa, thus increasing the pH [95]. In high pH values, microalga cells tend to aggregate and, consequently, to precipitate.

Optimum initial concentrations for propionate and butyrate -the diluted forms of the corresponding acids used- in terms of biomass growth were 10 g/L and 15 g/L respectively, while the intracellular lipid content that the strain reached appeared higher for both acids at 15 g/L (Fig. 8). The microalga can also grow at higher VFA concentrations, but with a lower growth rate. Both cultures with 20 g/L and 30 g/L of butyrate promoted biomass production, although lower than with 15 g/L. The initial concentration of 30 g/L of propionic acid, however, totally inhibited growth. When acetic acid was used as a carbon source, the optimum initial concentration that favored biomass growth was 30 g/L, while higher lipid content was reached at 15 g/L, as was the case for the other VFA.

To the best of our knowledge, it is the first time that *C. cohnii* is reported to utilize solely propionate or butyrate as carbon source to promote its growth. Experiments with other microalgae so far have showed a relatively slow and selective assimilation of butyric acid. Cho et al. and Liu et al. have cultivated *Chlorella vulgaris* mixotrophically on DF effluents, while Turon et al. have cultivated *Auxenochlorella protothecoides* and *Chlorella sorokiniana* heterotrophically on small concentrations of acetate and/or butyrate, as well as waste effluents [117,134,135]. None of the above microorganisms has been cultivated on propionate or has exhibited any ability to grow heterotrophically on high concentrations of butyrate. *C. sorokiniana*'s growth was inhibited by butyrate concentrations higher than 0.18 g/L, while *A. protothecoides* was demonstrated to grow in a medium that contained 0.46 g/L butyric acid, resulting in almost 0.3 g/L dry cell weight (DCW), but not higher. According to Rumiani et al., butyric and propionic acid were regarded as possible growth inhibitors for *C. cohnii*, even in small quantities, when grown at concentrations of date syrup higher than 17 g/L [117]. However, the acids were found to be efficiently assimilated by the microalga at concentrations as high as 10

g/L for propionic and 15 g/L for butyric acid, without any reduction of the growth rate. Discovering the inhibitory concentration of each acid is of importance for the further implementation of a DF biorefinery. Since all of these acids are being accumulated in the liquid fraction of a DF process in lower concentrations [28], it is suspected that the cultivation of the strain won't be inhibited by the substrates.

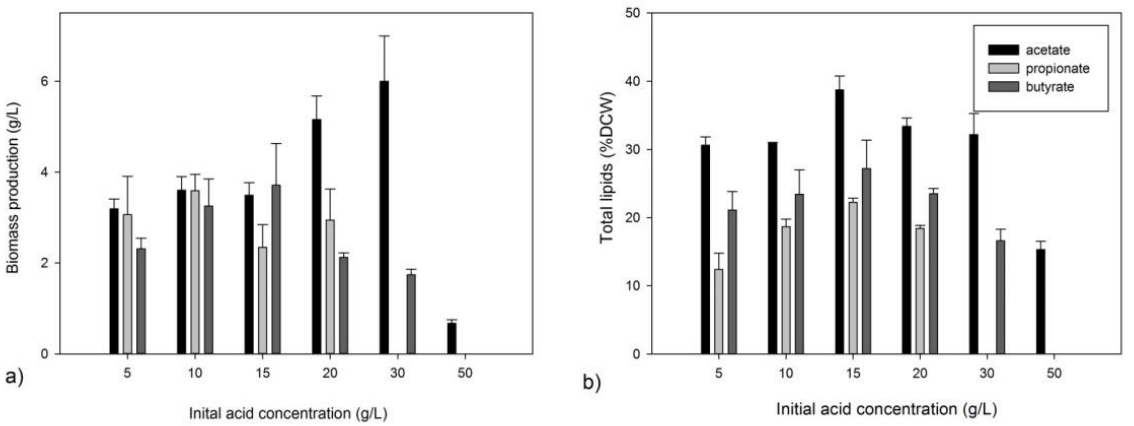


Figure 8. . a) Final *C. cohnii* dried biomass production of the different 50 mL batch cultures containing acetate, propionate or butyrate at specific initial concentration b) Final total lipid content (% dry biomass) of *C. cohnii* cells produced from the batch experiments

Fed-batch cultures

The batch cultures, although able to prove VFA assimilation by *C. cohnii*, could neither afford a high biomass and lipid production, nor enable the consumption of a substantial amount of VFA. The main limitation, apart from the volume of the culture, was the control of the pH of the medium. The buffer could maintain a relatively stable pH for moderate acid concentrations, however after 168 h of cultivation, the pH of the cultures had risen at 7.9-8.9, depending on the assimilation of the initial VFA.

In order to eliminate the feeding limitations posed by pH, the strain was cultivated in fed-batch pH-auxostats with a distinct VFA feed in 1 L final culture volume. Each bioreactor exhibited a different growth pattern. Exponential growth phase for the bioreactor fed with acetic acid was longer in comparison to the propionic and butyric acid and resulted in the higher dry biomass production of 22.1 ± 0.5 g/L. Propionate promoted the production of 14.4 ± 0.3 g/L of DCW and butyrate of 11.1 ± 1.6 g/L, almost 50 % down in comparison to the acetate.

It seems that the degree of assimilation decreases with the addition of carbon atoms in the backbone chain of the substrate. This can be attributed to the higher time and energy needs of the cell in order to break down the acid to its main components and convert it to acetyl-CoA. According to the metabolic regulations of microalgae, although acetate can be converted to acetyl-CoA in a single-step reaction, butyrate's assimilation includes the rate-limiting step of β-oxidation [62]. The same can be assumed for longer-chain VFA such as propionate, valerate etc.

According to Turon et al., the inhibitory effect of butyrate at lower concentrations, may be attributed to the acidification of the cytosol after accumulation of the undissociated form of the acid [84]. However this assumption is contradicted by the transportation mechanism of VFA inside the cells. More specifically, it is expected that the VFA uptake by the cells is based on two mechanisms, namely the diffusion of the acid molecules in their undissosiated form, from the medium towards the cytosol and the active transport of the anionic form through proton transporters, situated on the cell membrane. According to Lacroux et al., when the pH of the medium favors the accumulation of high amounts of the dissociated acid, proton availability for the activation of the second mechanism may be limited. Therefore, at high pH values (>> pKa) the uptake of the acids by the cells is very low, leading to growth inhibition [136].

On the other hand, another theory argues that at very low pH values (<pKa) where the undissosiated form of the acids dominates, such molecules enter the neutral cytosol suddenly, providing high amounts of the anionic form. This process leads to rapid cytosolic acidification, which further affects the normal cellular operations, thus inhibiting cell growth. Therefore, the maintenance of a proper pH appears to be of great importance for the effective microalgae fermentation. Still, in the case of the applied VFA, pKa values (4.76 for acetic acid, 4.82 for butyric and 4.87 for propionic acid) do not differ so much and are substantially lower than the pH of the culture. Therefore an acidification caused by butyric or propionic acid alone is not justified.

Both in fermentations fed with propionic, as well as butyric acid, the initial acetate concentration in the culture medium was totally depleted within almost 80 h from the beginning of the cultivation. Acetate in all cases appeared to be the preferred carbon source and while it was being consumed, the proportion and the relative concentration of the other feeding VFA in the culture medium raised. However, after complete exhaustion of acetate, the microalga began metabolizing the other available organic acid, which caused its concentration in the medium to remain stable or even partially fall (Fig. 9). It is worth mentioning that there is a drawback when the only parameter responsible for controlling the feeding of the bioreactors is the pH. According to Figure 9, none of the fermentors is being operated at the optimum acid concentration, as this has been defined during the batch experiments, but all VFA concentration values are lower. Still, this also implies that none of the VFA concentrations of the medium reaches inhibitory levels during the fermentation. Therefore, it must be concluded that final biomass production from each bioreactor may not represent the highest possible that could be reached if the feeding rate was not pH dependent, regardless of the amount of carbon source utilized. Nevertheless, biomass production by the bioreactor fed with acetic acid

was higher than previous reports. Ratledge et al. have cultivated the same strain of *C. cohnii* ATCC 30772 in a fed-batch pH-auxostat with 50 % (v/v) acetic acid as feed and DO concentration above 30 % of saturation, resulting in a final biomass production of 17 g/L after 140 h, which however was characterized by a very high DHA content of 59 % of TFA [95]. It is possible that a more concentrated feed might lead to higher DHA accumulation, or, most possibly, that a higher DHA content is reached when *C. cohnii* cells are cultivated under higher DO rates. The latter has already been observed for cells grown under glucose feed [105]. However, the purpose of these fermentations was just to establish the ability of *C. cohnii* to grow on different VFA that are present in DF waste permeates and not move on to any process optimization attempt. For that reason, the initial acetate concentration for all experiments carried out in bioreactor was set at 15 g/L of the medium, promoting its quick consumption and allowing the microalga to further utilize the next available substrate.

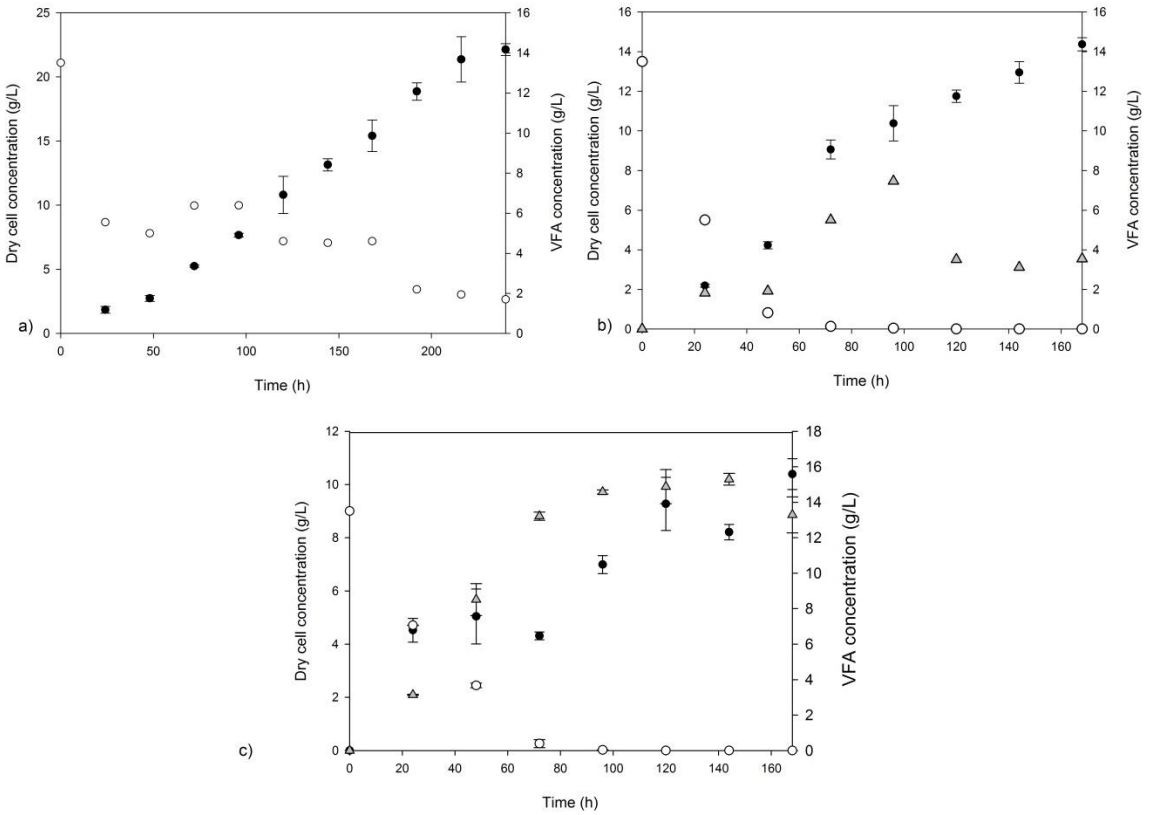


Figure 9. Consumption of (a) acetate, (b) propionate and (c) butyrate by *C. cohnii* under fed-batch fermentation in 1L cultures. The concentration of acetate (white circles), propionate or butyrate (grey triangles) and dry cell production (black circles) are depicted.

More important than the biomass production is the accumulation of lipids and especially DHA. According to Fig. 10 and 11, there is a correlation between total biomass production and final single cell lipid accumulation. This observation appears surprising, since stress conditions that usually favor lipid production result in a decrease in the growth rate of the cells [137]. A plausible explanation is that a higher amount of biomass causes a rapid nitrogen depletion-to support the growing nutritional needs of the cells- which, in turn, triggers the accumulation of lipids [138]. In general, when a nutrient is

limited, such as nitrogen, while there is an excess of carbon in the medium, as in the case of biomass production in fermenters, carbon uptake continues, leading to accumulation of intracellular storage compounds, such as lipids [103].

The biomass production achieved when using the different pure VFA feeds was 42.4 g DCW/L feed for the acetic acid 33 % (v/v). Propionic acid 25 % (v/v) resulted in 40.6 g DCW/L feed, while butyric acid 25 % (v/v) produced only 21.7 g DCW/L feed. The above values were estimated by taking into consideration also the biomass of the samples daily collected. It must be noted here that, although feed of propionic and butyric acid had a lower acid concentration in terms of mass, it does not necessarily mean that the corresponding cultures lack carbon provision. When taking into consideration the molecular weight and the amount of carbon atoms present in each acid, the following carbon composition (% wt.) results for each VFA; 40 % C in acetic acid, 48.5 % in propionic and 54.5 % in butyric acid. The higher percentage of carbon in propionic and butyric acid justifies the lower concentration of these VFA in the feed, in order to obtain comparable results from each fermentation, in terms of carbon availability.

Both batch and fed-batch cultures with pure acid feeds offer the advantage of examining the growth and lipid composition of the cells under each VFA feed. Thus it gives the ability to suggest which DF effluent can be more promising for *C. cohnii* cultivation. Depending on the source of biowaste and the conditions of DF, the composition of VFA produced can vary [28]. By understanding the behavior of the cells under each VFA feeding, the appropriate composition can be designed and achieved by regulating the DF conditions.

TFA composition under different VFA feed

The main FA discovered to be produced by *C. cohnii* included C14, C16, C18:1, C18 and DHA. Although composition altered, depending on the VFA used as feed, each time, there was no change in the type of the FA detected in the extracted lipids of the microalga. These results coincide with previous experiments on *C. cohnii* [105]. DHA was the only PUFA produced by the microalga, thus underlining the commercial value of the lipid extract of the strain [91]. In batch culture experiments, the initial acid concentration that resulted in higher lipid content did not necessarily lead to higher DHA content accumulation; the highest DHA percentage of 28.4 ± 6.2 % TFA was achieved for 30 g/L initial acetate concentration, 38.6 ± 3.1 % for 10 g/L propionate and 21.9 ±5.1 % TFA for 10 g/L butyrate.

Total lipid production and DHA content in fed-batch cultures were daily monitored, in order to determine whether lipid accumulation occurs during a specific growth phase of the culture. FA distribution on 24 h and 48 h is not considered very representative, due to the low lipid and biomass production achieved at this early cultivation stage. The small amounts of biomass harvested for lipid extraction may answer for substantial mistakes during lipid and DHA calculation. After 120 h of the fermentation, FA content and distribution remained relatively stable, and that was observed for all the different VFA feedings. As depicted in Fig. 10, microalga biomass grown on acetate reached a DHA percentage of 33.3 ± 0.2 % (w/w) TFA after 144 h under fed-batch fermentation, which then remained relatively stable with a small decline noticed. Accordingly, higher percentages of DHA for the other fed-batch fermentations were 35.8 ± 0.6 % (w/w) TFA at 96 h for propionate and 31.1 ± 1.0 % (w/w) TFA for butyrate at 72 h. According to de Swaaf et al., butyrate is not used directly by *C. cohnii* as a precursor for DHA biosynthesis. On the contrary, it is first degraded into two units, of two carbon atoms

each, and then integrated in the lipid synthesis [139]. That is a possible reason for the lower DHA accumulation observed under butyric acid feed.

In contrast to butyrate, there are cases where propionate can be directly converted to FA. In the case of acetate, the crucial enzyme for its conversion to acetyl-CoA is acetyl-CoA synthetase (ACS) [140]. ACS activates acetate to acetyl-AMP, which is subsequently converted to acetyl-CoA [141]. In a similar fashion, ACS can also activate propionate to propionyl-CoA. What is more, the presence of a specific propionyl-CoA synthetase has been reported for *Saccharomyces cerevisiae* [142], which also facilitates the activation of propionate to propionyl-CoA [143]. Although its presence in other oleaginous microorganisms has not been verified yet, it may pose an explanation to the increased lipid and DHA production of *C. cohnii* cells under propionic feed.

A small decline of the accumulated DHA was noticed over 100-150 h of fermentation, depending on the feeding VFA, possibly due to the agitation for culture mixing that might cause cell disruption. Allen et al. have found that, when *Coccomyxa subellipsoidea* C-169 strain was cultivated under nitrogen stress, it would accumulate lipid droplets of increasing weight that consisted mostly of triacylglycerols (TAG) against the synthesis of membrane lipids. However, when nitrogen was again supplied in the medium, the droplets inside the cells would shrink rapidly and the cell would produce more membrane lipids [137]. According to Ratledge et al., under high lipid accumulation conditions, DHA is accumulated mainly in the form of TAG [95]. Assuming that the cell disruption causes proteins to dilute into the culture medium, the nitrogen availability rises. This may cause a reduction of TAG and, consequently, of the DHA content, without any reduction of the total lipid content (Fig. 10).

Experimental data from batch and fed-batch cultivations all confirmed that a feed of propionic acid induces a higher DHA accumulation in *C. cohnii*. The use of propionic acid in small concentrations (1 % (v/v)) combined with another main carbon source (acetic acid or glucose) has been verified and patented [36], and it was found to assist a higher final DHA production. Further knowledge of the metabolic paths of the microalga involved in propionate assimilation is needed in order to fully comprehend this result. However, results from the fed-batch cultures, presented in the current study, can provide useful guidelines regarding the effect of effluent composition on biomass and lipid yields of *C. cohnii*. It is safe to assume that a DF effluent with a high acetate content will promote relatively higher biomass growth, while, when coupled with a substantial amount of propionate, it will favor a higher lipid accumulation

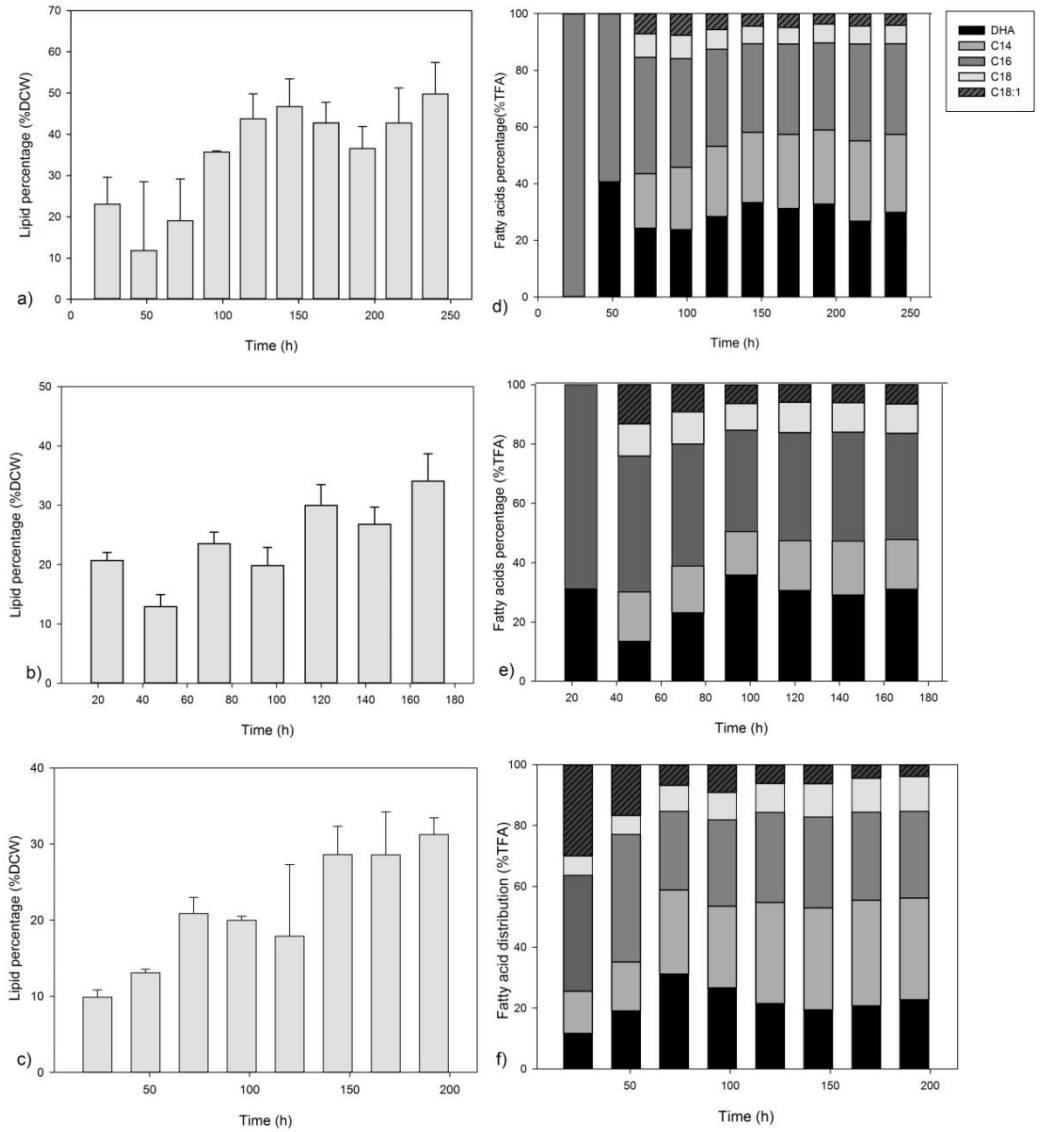


Figure 10. Total lipid content of the microalga cells during cultivation with (a) acetate, (b) propionate or (c) butyrate in a fed-batch bioreactor (1 L working volume). FA distribution in the extracted samples during cultivation with (d) acetate (e) propionate or (f) butyrate.

The role of nitrogen residue

According to Kjeldahl measurements, the initial nitrogen content inside the fed-batch cultures right after the inoculation was determined to be 4.8 g/L. After 24 h of fermentation, the nitrogen concentration in all the bioreactors dropped below 2 g/L, with the exception of the one fed with butyric acid 25 % (v/v), where a value of 2.7 g/L remained. At 48 h of fermentation, nitrogen levels of the cultures were between 1.4- 0.6 g/L, while the bioreactor fed with 33 % (v/v) acetic acid exhibited the lowest nitrogen residue and the highest biomass concentration accordingly (Fig. 3). After another 24 h of fermentation, the nitrogen wasn't traceable with the Kjeldahl method. Therefore, all fermentations that began with an excess of nitrogen, continued under nitrogen limitation conditions for the most part. It can be assumed that the initial high nitrogen content, coupled with a favored carbon source, facilitated a high biomass production at the first part of the fermentation, allowing for a rapid nitrogen consumption and in consequence for a higher lipid accumulation after the first 72 h of cultivation, in accordance to previous reports (Fig. 10) [138]. Thus, our previous hypothesis regarding the connection between high biomass and lipid production is more strongly supported by the Kjeldahl results.

1.2 Waste-derived VFA mixtures as main carbon source

After demonstrating the efficient utilization of the main VFA by *C. cohnii* and developing a specific protocol for fed-batch cultivation, the fermentation with a feed of DF waste effluent was examined. The effluent #1, which was the only available feedstock then, was utilized, in parallel with a synthetic medium mimicking the VFA composition of it. The type of waste from which the effluent was derived, namely VGF, is characterized by a high carbohydrate content, which favors biohydrogen production

and therefore can lead to a coupling of VFA fermentation with the further valorization also of the gas products of DF [28]. It must be mentioned that the DF permeate comprised also of small quantities of iso-butyric and iso-valeric acid (<0.3 g/L each), which were not added in the synthetic medium due to their really low concentration. Although acidified by the addition of the non-fermentable HCl, both feeds had a low VFA concentration, as shown in Table 1. As a result, a large feed volume was necessary to maintain cell proliferation and subsequently the pH of the culture at 6.5, according to the pH-auxostat operation method. After 60 h of fermentation, 500 mL of permeate were consumed and the fermentation process was stopped due to complete depletion of carbon source. Both feeds were found to efficiently support *C. cohnii* growth, with the final dry biomass production for the waste permeate and the synthetic analogue medium to reach 6.5 ± 0.3 g/L and 3.0 ± 0.7 g/L of the culture medium, respectively. In order to determine the ability of the microalga to assimilate the permeate for biomass production, it was calculated that production of 15.0 g DCW/ L feed was achieved for the permeate, while the synthetic medium resulted in less biomass, reaching the value of 10.9 g DCW/ L feed. These values are translated into 1.02 g DCW/g VFA carbon consumed and 0.59 g DCW/ g VFA carbon consumed respectively. The production of biomass by the pH-auxostat fed with the DF permeate is surprisingly high, indicating that each gram of VFA carbon from the permeate gave rise to 1.02 grams of biomass, which is not possible. The results clearly demonstrate that there is a carbon source, additional to the VFA, which is assimilated by the microalga enhancing its growth and promoting the use of DF effluent for *C. cohnii* fermentation. In order to determine this, the organic matter of the ultrafiltrated effluent, excluding VFA, was then estimated by the collaborating company Tecnalia (Spain) and found to be 5.76 g/L. The result verifies the presence of additional carbon sources in the permeate, possibly formic acid, lactic acid, ethanol, or other DF products [144] which however seems to further promote the assimilation by the

microalga without competing or preventing VFA consumption. On the contrary, the additional carbon source promoted biomass growth, thus enhancing the capacity of the culture for bioremediation. The culture depleted the total VFA content of 500 mL of effluent in just 60 h.

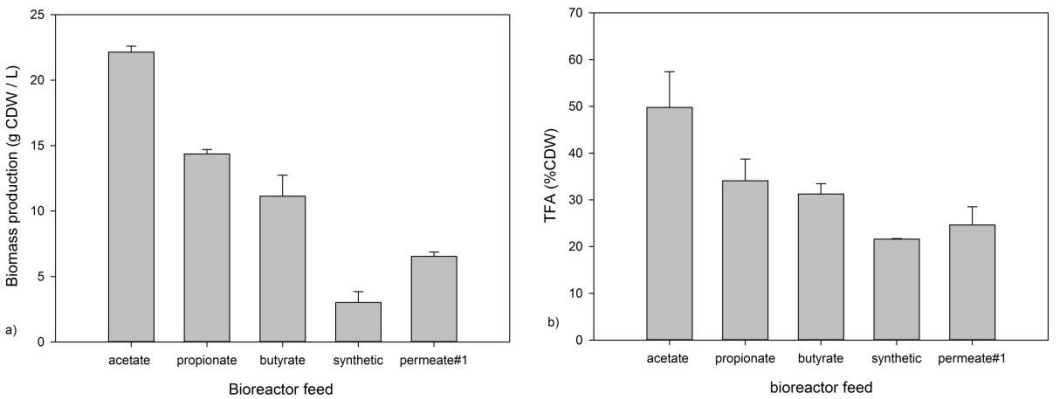


Figure 11. Final (a) dry biomass and (b) total lipid production of the different fed-batch 1L cultures

The relatively low biomass concentration in the bioreactor with the DF permeate feed, in comparison to the pure organic acid feeds (Fig. 11), can be partially attributed to the increased dissolution of the culture medium caused by the high feed volumes needed to maintain the pH of the medium and trigger the cell growth. It must be noted that, despite the similarities of the fermentation process conditions, the volume of the synthetic equivalent consumed by the culture was 330 mL, while in case of the real permeate, 500 mL were required for cell growth maintenance during the 60 h. Therefore, the bioreactor utilizing DF permeate as feeding carbon source demonstrated a higher biomass yield and carbon consumption than the synthetic counterpart. The corresponding total lipid content of the cells reached 21.1 % and 24.6 % of DCW when fed with the synthetic medium and the DF permeate accordingly.

The above results indicate the ability of the microalga strain to grow on a DF effluent and accumulate lipids of great importance as food additives. In order for the above process to be efficiently integrated within the prospect of a biorefinery, it is necessary to utilize, not only the lipid fraction, but the whole biomass, if possible. An interesting study reported in the literature involves the valorization of the microalgae biomass as a substrate for dark fermentation towards hydrogen generation [145]. The latter may offer the possibility to reuse the residual *C. cohnii* biomass, after extraction of omega-3 FA, as a feedstock for DF process (Fig.12).

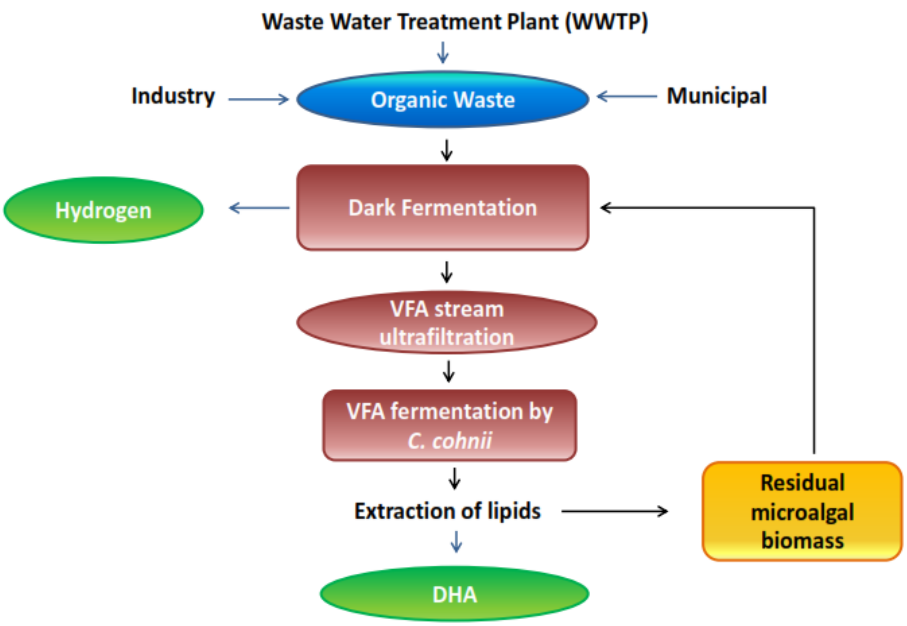


Figure 12. Proposed bioprocess route for *C. cohnii* cultivation and DHA accumulation coupled with biohydrogen production and biomass recycle

After delivery of the other DF effluents, fed-batch cultures were carried out accordingly. In all cases, fermentation was carried out until total depletion of the carbon content of 500 mL of permeate. Effluent No. 4 was used as feed in a fed-batch process of 100 mL

working volume, therefore, less amount of feed was needed. The different process followed was purposeful, due to the limited volume of this material delivered to the laboratory. Final results are depicted in Table 3.

Table 3. Final biomass production and lipid and DHA accumulation in fed-batch cultures (1L) fed with 500 mL acidified DF effluents (or 50 mL in case of #4)

Effluent feed	C/N ratio (g C/g N)*	Final DCW (g/L)	TFA (%DCW)	DHA (%TFA)
#1	8.0	6.5	24.6	29.8
#2	16.8	3.9	14.0	40.8
#3	16.1	4.4	22.7	37.7
#4	9.4	11.7	11.1	28.2

*Calculated based on the carbon provided by the total VFA

In order to properly evaluate the above results, it is crucial to consider the differences of each effluent received and how they are reflected in the characteristics of the final biomass. First of all, according to Table 3, effluents #1-#3 contain a similar final VFA concentration and, therefore, can be easily compared with each other. On the contrary, effluent #4 has been partly evaporated for VFA concentration, before delivery. As a result, it is expected that it will provide a higher amount of carbon and achieve better biomass and lipid yields. Moving on in the performance of the three first effluents as feeds, although fermentation of *C. cohnii* with feed #1 and feed #3 lasted for 60 and 48 h respectively, feed #2 needed 114 h to be consumed by the cells. Still it didn't provide neither the same final biomass production nor lipid accumulation. The above results were confirmed also by other partners of the European Programme Volatile, responsible for cultivating oleaginous yeasts and bacteria on those substrates. It is therefore supposed that effluent #2 included some unknown inhibitory compounds, which affected

negatively the growth rate of the cells. In general, a DF effluent composition depends, not only on the initial fermented substrate and microbial inoculum, but also on operation and environmental conditions [25]. Therefore it is not always easy to predict, or know afterwards, the exact composition of the effluent, unless a thorough analysis is conducted. In the case of feed #2, it is also possible that the low ammonium content (41.0 % and 37.5 % of that of effluent #1 and effluent #3 respectively) leaded to the observed decline of biomass growth. Between feeds #1 and #3, it is clear that #3 promoted more effectively lipid and DHA accumulation. When elaborating on the reason behind this, it is helpful to examine once more the composition of the feeds (Table 1). Apparently feed #3 is richer in propionic acid, which, according to the previous results from pure VFA fermentations, favors lipid and DHA accumulation. More specifically the acetate/propionate ratio of each effluent was the following; 2.17, 2.24, 1.42 and 1.71 for effluents #1, #2, #3, #4 respectively.

Effluent #4 offered a much more concentrated feed, but always at the expense of the total operational costs, since evaporation is an expensive procedure. Owing to its further treatment, the received volume of it was much lower than the previously delivered effluents. Consequently, fermentation of *C. cohnii* with feed #4 was carried out in BioXplorer 100 mini bioreactor system at a total culture volume of 100 mL. According to the analogue of 1 L cultures, 50 mL of #4 were fed in the fermentor. This volume was able to support culture maintenance for 96 h and allow a much higher final volumetric biomass production. It is obvious that, in order for a fermentation with a DF effluent to last longer, a high concentration of VFA is a prerequisite. Also, the avoidance of a neutralization step of the effluent, which is a usual technique that allows its easy transport, should be attempted, as it enables the combination of a carbon source addition

and pH control with a single feed, according to the protocol used in these pH-auxostat cultivations, without the need of any acidification step before feeding.

TFA composition under DF effluent feed

Regarding the DHA content produced from the permeate #1 and the synthetic fed-batch fermentations, this was calculated to be 29.8 ± 1.7% and 6.0 ± 0.2% of TFA. Both biomass growth and DHA content are much higher when cells grow on the DF waste permeate, exhibiting a preference against the synthetic medium. This is a very promising result towards the development of a sustainable industrial process that could utilize DF effluents for omega-3 FA production. Indeed, also DHA accumulation in the case of the other three effluents was similar or even higher than #1 and in any case higher than the one from pure VFA cultures. More specifically, for the effluent batches #2 and #3, intracellular DHA accumulation was higher than the one for #1. A correlation can be detected in Table 3, between higher biomass production and omega-3 storage, but the number of different batches tested is not big enough to securely answer for an existing relationship between the two. Apparently, the complex composition of DF effluents is promoting the intracellular DHA accumulation, but in order to draw a clear conclusion on the matter, a further knowledge of the metabolic system of the strain is needed.

It is worth mentioning that the data collected so far indicates that the accumulation of DHA occurs at the beginning of the static phase (Fig. 10), as it was previously observed [37]. Therefore, it seems possible that the prolongation of both permeate and synthetic waste feeding fermentations for over 60 h, might lead to higher DHA content, since it was not clear whether the exponential growth phase was finished after the 60 h or not. In order to examine that, the obstacle of the low VFA concentration of the feed causing high dissolution of the culture medium must be surpassed. In the case of effluent 4#,

prolongation of the fermentation was achieved, which however wasn't reflected in the final DHA content. Table 3 clearly demonstrates that the extra time may have favored significantly the biomass production, but has resulted in a poor lipid and DHA performance. Also it is observed that a correlation between a high C/N ratio of the feed and a higher intracellular DHA content exists. Still, the different effluents examined do not suffice as a number to suppose this to be a general rule, not mentioning the fact that the estimated C/N ratios take into consideration only the carbon of total VFA of the feed. It has been proven that the effluents contain more unknown, consumable organic matter and therefore, real C/N ratios may be very different. In truth, a more thorough compositional analysis of the liquid fractions is needed to extract solid conclusions.

1.3 Optimization of DHA production under VFA feed in batch mode

Batch optimization of initial conditions

In order to detect the optimum culture conditions for maximum DHA volumetric production, batch cultures with different initial conditions were carried out. Batch culture results cannot provide a final answer regarding the optimization values of a fed-batch procedure, but they might effectively offer a hint for the most important culture parameters and the range of values they should take.

Effect of temperature

The experimental results indicated that the best temperature for biomass growth is found in the range of 20-23 ^{o}C, while DHA accumulation by the cells is maximized at 23 ^{o}C (Fig. 13a). The low temperature of 15 ^{o}C couldn't facilitate cell growth effectively. Previous experiments with another strain, *C. cohnii* ATCC 30555, revealed that the growth of the cells was also inhibited at a very low temperature of 15 ^{o}C [94]. On the

other hand, at this temperature, *C. cohnii* presented the highest total lipid percentage, while in the range of temperatures between 20-30 ºC, 27 ºC was found the second best. Therefore, the strain used in the present study, appears to proliferate at temperatures around 20-23ºC and accumulate lipids at either very low or a slightly higher temperature, as a result of temperature stress. Safdar et al. also examined *C. cohnii* behavior at different incubation temperatures [94]. Their strain was better grown at higher temperatures between 25-30 ºC, but it also accumulated more lipids and DHA at the lower temperature of 20 ºC instead. The induction of the accumulation of lipids at low temperatures is a known technique for oleaginous microorganisms and has been proven effective, not only for *C. cohnii* cells, but also for *Thraustochitrium* strains [146]. The reason for this can be the utilization of lipids by the cell for the increase of the membrane fluidity, which is hampered at low temperatures [147].

Table 4. DHA distribution of the cells grown in batch cultures under different incubation temperatures

Incubation temperature (ºC)	**DHA content (%TFA)**
15	19.3
17	29.7
20	31.0
23	33.9
27	29.3
30	29.4

The results also indicate that the DHA content of the cells doesn't have significant fluctuations with the temperature, although it is obvious that it is partially decreased at very high temperatures (Table 4.). This is in accordance with the general apprehension

that PUFA are favored at relatively lower temperatures [148]. It is possible that the desaturases produced by the microalga cells exhibit a higher activity at low temperatures due to an increased availability of oxygen [149]. By carrying out an analysis of variance (ANOVA) coupled with a Tukey test, it is clear that between the temperature range of 17-23 ^{o}C, the difference of the DHA content (% TFA) of the cells is not significant ($p=0.32$), securing the conclusion that the DHA is indeed favored at lower temperatures. Taking into consideration the final biomass productivity, the highest DHA production, equal to 0.45 g/L was achieved for the incubation temperature of 23 ^{o}C.

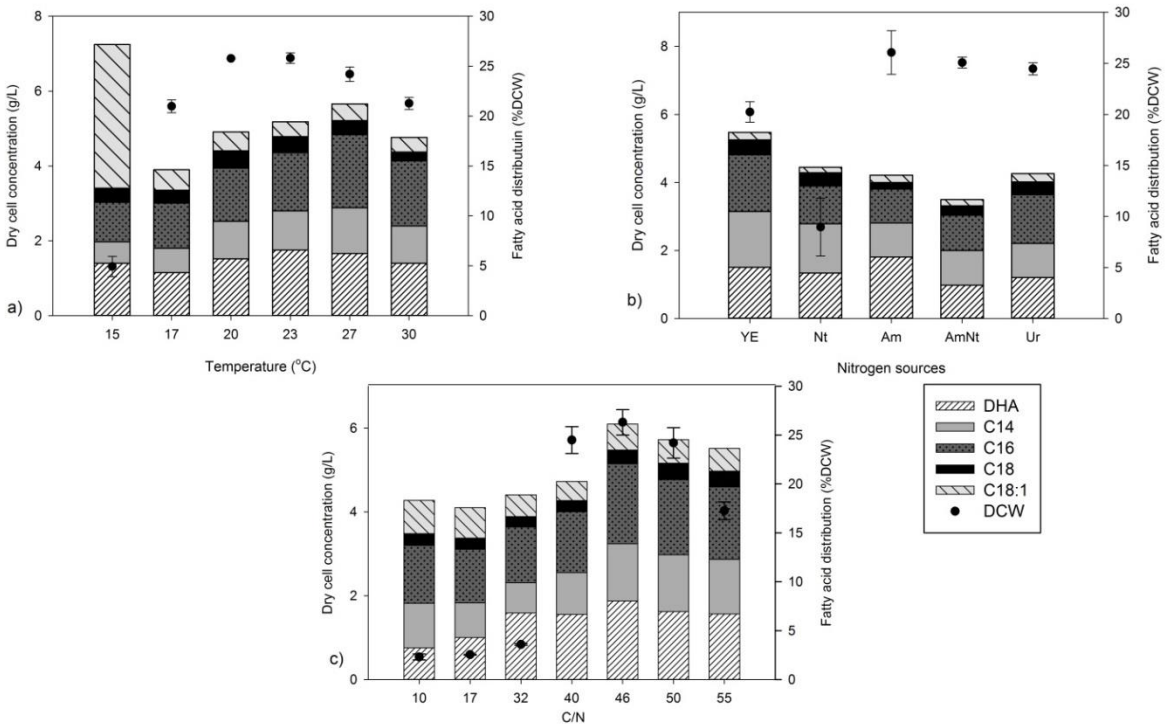


Figure 13. Final dry cell concentration (dots), FA distribution (bars) of the cells grown in batch cultures (a) under different incubation temperatures, (b) different nitrogen sources and (c) different initial C/N ratio

Effect of nitrogen source

Nitrogen is known for playing an important role in lipid production. Apart from its concentration in the medium, the form of nitrogen affects its uptake by the cells, and therefore its availability [72]. For that reason, the examination of the best nitrogen source for DHA accumulation is a necessary step towards the optimization of the process. The batch cultures utilizing Am as nitrogen source demonstrated a relatively higher biomass production, but, most importantly, the highest DHA accumulation by the cells, equal to 43 % of TFA, in comparison to the second-best source of Nt -in terms of PUFA accumulation-, which led to 30 % DHA production (Fig. 13b). In terms of TFA percentage, YE favored the highest total lipid production, which reached 1.12 g/L in batch cultures.

According to typical algal culture techniques, Am is usually the best inorganic nitrogen source, since less energy is needed by the cells for its uptake [72]. That is because the first step of nitrogen fixation is its reduction to NH_4^+. When Am is available, then the reduction step is not required [98]. However, previous research with another *C. cohnii* strain (ATCC 30555), argued that nitrate was the best nitrogen source for DHA and lipid production [138]. The difference may be attributed to the different microalga strain or the use of a different carbon source in the culture medium. Glucose was used as the main carbon source in the research of Safdar et al., instead of acetate in the present study, which is the main component of a DF effluent. Still, a preference for nitrate as nitrogen source is scarcely encountered between aerobic, heterotrophic eukaryotes. So far YE is the favored nitrogen source for most of *C. cohnii* cultivations [150]. In contrast to the current results, another study by A. Mendes et al. has identified YE as a better nitrogen source than the Am when *C. cohnii* was grown on carob syrup. The addition of 5 g/L YE resulted in 19 % higher DHA (% TFA), than the addition of 0.05 g/L ammonium

chloride [118]. However, rather than highlighting the benefit of YE as a nitrogen source, this difference might be the result of the addition of a much higher amount of YE than Am. If we suppose that YE includes 11 % (w/w) nitrogen-according to composition analysis of commercially available YE, then the nitrogen added was more than 10 times higher than in the case of the ammonium. What is more, YE also includes an amount of carbon that is expected to further boost the cell metabolism.

It is interesting to highlight the behavior of the cells when grown at AmNt, which consists of nitrogen in both Am and Nt forms. In this case, the fermentation results in moderate biomass and lipid production (Fig. 13b), situated between the corresponding values of the fermentations consisting only of Am or Nt. When AmNt is dissolved into water, it produces NH_4^+ and NO_3^- ions, whose final concentration in the medium is half of the corresponding concentrations when only Am or Nt is used, provided that the initial total nitrogen content is kept stable. Therefore, it might be hypothesized that nitrogen in the form of NH_4^+ is quickly assimilated by the strain, while NO_3^- is consumed later, resulting in a somewhat lower biomass yield, combining the yields of Am and Nt alone.

Effect of C/N ratio

Lipid production of microalgae is enhanced under certain stress conditions, such as nitrogen deprivation [62]. As a result, the C/N ratio of the culture is one of the most important factors, when lipid accumulation is considered. In the present study, the best initial C/N ratio for DHA production was examined. The initial carbon source concentration was decided to be the same in all batch cultures and equal to 30 g/L, which is the optimal value for biomass production in batch cultures with acetate, according to the previous results [85].

As shown in Fig. 13c, an initial C/N ratio of 46 (g C/g N) favors the highest biomass and also lipid production, which was latter calculated to be 6.1 g/L and 1.6 g/L, respectively, after 168 h of incubation. On the contrary, the DHA content of each cell was found to be optimal at an initial C/N ratio of 40. Nevertheless, due to the higher biomass and lipid content, the final DHA production of the culture was higher at the ratio 46 reaching 0.49 g/L in comparison to 0.38 g/L of the C/N ratio of 40. The preference regarding the C/N ratio seems however to be strain-dependent. Rosa et al. discovered that a ratio of C/N equal to 55 would promote a higher final DHA concentration in a culture of *Aurantiochitrium limacinum* SR21 using glucose as carbon source, when examining different ratios from 10 to100 [108]. This observation, however, can also be attributed to the higher biomass achieved at that ratio. Another group, Ryu et al., proved that *Aurantiochytrium* sp. KRS101 reached the highest DHA content of 34.2 % of TFA at a lower C/N ratio equal to 20, in comparison to higher ratios of 35 or 50 [151].

Among the examined initial C/N ratios, the lipid content is maximized at the high values in the range of 46-55, which qualifies for a low initial nitrogen concentration. This, in general, agrees with the behavior of microalgae under nitrogen limitation. A high C/N ratio coincides with a low nitrogen concentration, which is gradually consumed during the incubation, thus creating nitrogen limitation conditions. The cells respond to that by accumulating lipids. Apart from *C. cohnii*, also other microalgae species have been known to increase their lipid percentage as a result of nitrogen depletion. *Chlorella* sp. accumulated lipids under urea limitation [152], while *Aurantiochytrium* sp. strain T66 increased its lipid content from 13 to 55 % of DCW [153]. The increase of the lipid content under nitrogen starvation has been attributed by some researchers to an accumulation of citrate by the cells when nitrogen is absent from their environment.

Excess citrate is probably acting as a carrier of acetyl units that participate in lipid synthesis [154].

However, it is clear that, when ratios exceed 46 and an even higher nitrogen limitation is accomplished, growth and final lipid production are negatively affected. Cell growth and maintenance require the consumption of nutrients, which should be available above a specific threshold, to allow the culture to survive, let alone produce metabolites. This pattern of *C. cohnii* has also been observed by other researchers. Safdar et al. noticed that, when grown on glucose, *C. cohnii* ATCC 30555 exhibited lower lipid production under extreme nitrogen concentrations [23]. Furthermore, it is clear that extreme nitrogen starvation conditions do not favor DHA production. It has been observed that, although a very low nitrogen concentration is favorable for total lipid accumulation, it usually leads to a higher percentage of saturated lipids [154].

Effect of chemical modulators

It has been observed that specific chemical substances, when added in the culture broth, can act as modulators to induce lipid production in microalgae, as well as specifically in *C. cohnii* cells [32]. Li et al. have concluded that the combined addition of salicylic acid and ethanolamine in cultures of the strain *C. cohnii* ATCC 30556 has led to a 22.5 % increase of the total lipids produced [156]. Salicylic acid acts as signal transducer for cells and has been found to affect lipid metabolism, while ethanolamine belongs to the group of amines, some of which have been successfully used for similar applications [157,158]. In this work, the same quantity of the abovementioned chemical modulators was added in the culture medium. The biomass production between the examined cultures and the control without modulators bear no differences. Therefore, it was concluded that the ethanolamine and salicylic acid added didn’t influence the growth of

the cells. However, no significant difference was also observed in the lipid production, with the ethanolamine and salicylic acid cultures exhibiting only a 2.4 % increase in the final lipid production (p=0.305). More specifically the batch culture with chemical modulators resulted in 1.7 g/L of TFA production in comparison to 1.6 g/L of the control culture. More evident, however, was the effect of the modulators to DHA accumulation, since they resulted in a 29.7 % of DHA in TFA in comparison to 25.8 % of the control culture, resulting in a statistically significant difference between the DHA contents (% TFA) (p=0.031). Still, the differences between the cultures with and without modulators didn't justify their use. They were therefore excluded from further optimization studies.

Examination of experimental optimum initial values

The application of the optimal conditions, found in the above batch cultures, in a fed-batch system, was carried out in a 1L bioreactor fed with permeate #3. According to the results obtained from the series of batch experiments, the "optimum" fermentor included an initial growth medium (ATCC 460) with no glucose added, Am as a nitrogen source at a final C/N ratio equal to 110 that was operated at 23^{o}C. It should be noted that, although the optimum acetate concentration used in batch cultures was 30 g/L, the initial concentration of sodium acetate in the "optimum" bioreactor's growth medium was chosen to be 20 g/L instead. The reason for that was to ensure that the quick addition of effluent feeding, won't result, at any time of the fermentation process, in a final acetate concentration higher than the inhibitory 30 g/L.

The comparative fermentation process employed also a control fermentor which was operated under usual conditions (same as the other 1L fed-batch cultures) at 27 ^{o}C. The optimized strategy compared to standard growing conditions lasted for 68 h. During the first 48 h, 500 mL of the acidified DF effluent was fed in the bioreactors, in order to

maintain a stable pH under pH-auxostat mode. After the end of the feeding step, the fermentations continued for an additional period of 20 h under batch mode, aiming in the complete consumption of VFA present in DF effluent, resulting in an increase of the pH. At the end of the process, oxygen consumption was almost zero, while pH of the culture supernatant had almost reached the value of 9. In previous experiments, HPLC analysis has exhibited the stable consumption of the acetic acid of the effluent, while the other acids were accumulated. A small consumption of propionic acid was also apparent in parallel with the acetic. However, 24 hours after the termination of the feeding, the residues of all the VFA inside the bioreactor were totally consumed by the cells.

It is clear that, again, the fermentation time was shorter in comparison to the fermentation with a feed of acetic acid. Although, *C. cohnii* is capable of depleting the VFA content of the feeding DF effluent, the volume necessary for controlling the pH of the fermentation broth was significant, due to the relatively low concentration of the carbon source presented in the form of the VFA, therefore diluting many critical nutrients. As a result, once more, the necessity of increasing the VFA amount was emphasized [85]. A concentrated DF effluent would increase its capacity to control the pH of the medium and therefore would allow high cell density fermentations that are crucial for the establishment of a process with industrial prospect.

Table 5. Biomass, lipid and DHA recovery from the optimized* and control bioreactor

Bioreactor	**Biomass recovery (g/L)**	**TFA (%DCW)**	**DHA (%TFA)**	**DHA recovery (mg/L)**
Optimized	3.3	9.0	34.2	101.6
Control	4.4	10.4	35.6	162.9

*Based on the batch experiments

In contrast to the results from the batch cultures that indicated the optimal conditions applied in the fermentation, the final biomass recovery from the optimized bioreactor was less than that of the control bioreactor operating under standard conditions (Table 5). This proves the different, constantly changing environment inside a fed-batch bioreactor. The YE as nitrogen source was proven to be more favorable to biomass production than Am, in the case of the fed-batch process. This can be attributed to the fact that the YE is a mixture of various micronutrients, nitrogen and also carbon [34]. More specifically, it is estimated that the carbon content reaches almost 40 % (w/w) of the YE [160]. On the contrary the total nitrogen content of it is almost 11 % (w/w). Therefore, it is clear that the addition of YE cannot count solely as a nitrogen source for the culture. This additional carbon source included in the initial medium of the control fermentor changes the C/N ratio and may answer for the increased microalga growth observed. The DHA content of the cells grown with Am was almost the same as the one of the cells harvested from the control bioreactor, reaching the percentage of 34.2 % of TFA in comparison to 35.6 % of the control. However, due to the lower biomass and total lipid production, the optimized bioreactor resulted in a 38 % lower final DHA production. In order to reach a conclusion about the best conditions for a fed-batch fermentation, more elaborated experiments need to be carried out.

1.4 Fed-batch OVAT approach for DHA production optimization

Considering the lack of success by the utilization of results from batch experiments, the endeavor of optimization was continued in fed-batch fermentations with the BioXplorer 100 mini bioreactor system. Temperature, nitrogen source and C/N ratio were proven, by batch cultures, to play a crucial role in DHA recovery and were the examined parameters. Firstly, the OVAT approach was selected, either to decipher the appropriate

T profile and nitrogen source or to indicate a shorter range for the values of the continuous parameter C/N, on which the DoE should focus.

Effect of temperature profiles

Fed-batch cultures of a final 0.1 L working volume were carried out for 7 days under the following temperature profiles;

- 27const; Temperature was maintained at 27 ^{o}C for the total fermentation duration
- 23to27; Temperature was set at 23 ^{o}C for the first 4320 min before changing in one single step to 27 ^{o}C where it remained stable
- 23const; Temperature was maintained constant at 23 ^{o}C for the total fermentation duration
- 23to15; Temperature was set at 23 ^{o}C for the first 4320 min before changing in one single step to 15 ^{o}C where it remained stable

It must be noted that each fermentation was operated under fed-batch mode for 6 days, while the last day feeding was stopped to enable total consumption of residual carbon inside the vessels. The above profiles were selected to examine also the cell behavior under two-stage fermentation protocols, in which case the initial conditions that favor biomass growth are succeeded by different ones that favor the product accumulation- in the specific case DHA. According to batch experiments, a fermentation temperature of 23 ^{o}C favors cell proliferation, while lower or higher temperatures of 15 ^{o}C or 27 ^{o}C accordingly enhance lipid accumulation. Therefore 27 ^{o}C and 15 ^{o}C were opted as the “second” conditions that would favor lipid production of the already enhanced biomass in a two-stage fermentation.

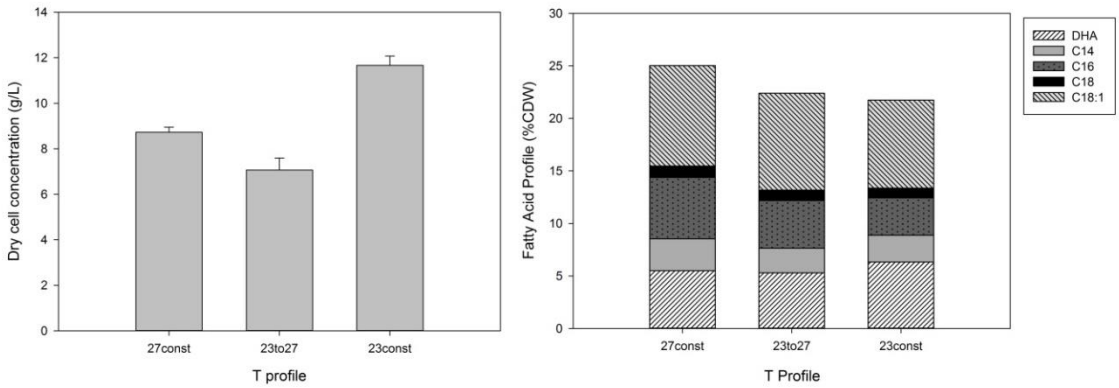


Figure 14. Biomass production and lipid and DHA intracellular accumulation of 0.1 L fed-batch cultures under different T profiles

According to Fig. 14, 23 ºC was again proven to be the best temperature for biomass proliferation for the specific strain. On the contrary 27 ºC possibly induced a small amount of stress in the cells, resulting in lower biomass, but higher lipid content. The behavior of *C. cohnii* under the two-stage fermentations was somewhat surprising. In the case of the 23to15 profile, a very small amount of biomass was generated, not even enough for reliable lipid extraction. Furthermore, the 23to27 profile, although managing to provide a higher cell concentration, didn't give rise to the expectable lipid percentage. Its poor performance, both in terms of biomass and lipid, may be attributed to the necessary adaptation time spent by the cells to recover the temperature change during the cultivation. The higher biomass regained from 23const fermentation resulted in the production of 737 mg DHA/L, in contrast to 479 and 373 mg/L from the 27const and 23to27 fermentations accordingly. Therefore it was concluded that this was the best option both in case of batch and fed-batch cultivation regime.

Effect of nitrogen sources and initial C/N ratio

Nitrogen concentration is constantly changing inside a batch or fed-batch culture. What is more, it has been clear from batch experiments that both the nitrogen source and C/N ratio substantially affect the biomass and lipid production. Therefore these two parameters were further examined in 0.1 L fed-batch cultures with 33 % (v/v) acetic acid feed. The two nitrogen sources examined, selected according to batch results, were YE and Am, while the initial ratio values were 9 –the same as the usual initial medium with SA, YE and sea salts used in fed-batch cultures-or 46 according to previous batch results.

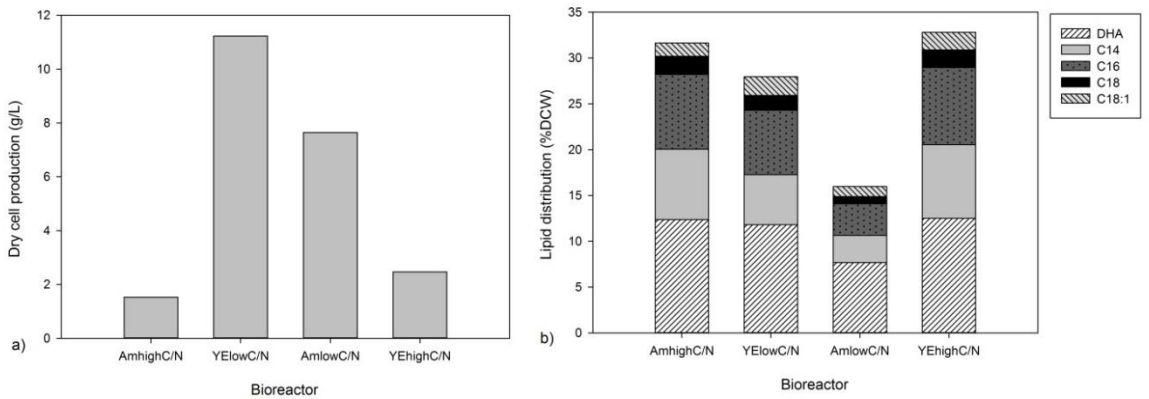


Figure 15. Biomass production and lipid and DHA intracellular accumulation of 0.1 L fed-batch cultures under different nitrogen sources and initial C/N (HighC/N stands for the value of 46, while lowC/N stands for 9)

Experimental results (Fig. 15) clearly depicted that YE is the best option both in terms of biomass and lipid accumulation. This is in accordance with the results from the 1 L fed-batch cultures with feed #3. Similar to the above results, the superiority of YE as a nitrogen source has been highlighted in other researches with *C. cohnii* as well. A.

Mendes et al. have discovered that YE promoted higher specific growth rate of the microalga cells when grown with a feed of diluted carob pulp, in comparison to Am [118]. As already mentioned, YE is a complex nutrient source that also provides the culture with minerals and vitamins, necessary for cell proliferation. In order to recognize why Am had a better performance in batch cultures, more information on the nitrogen assimilation pathways of *C. cohnii* should be acquired. A possible explanation could be the fact that batch cultures for nitrogen source determination were carried out with ATCC 460 medium, enriched with acetate instead of plain glucose, sea salts and the appropriate nitrogen source. This medium already includes the necessary vitamins thiamine and biotin, as well as, a mixture of metal compounds, readily available for the cells. Consequently, batch cultures with Am also included these micronutrients that a complex compound, like YE, can offer. Although YE is a very useful additive for industrial cultivations, owing to its nutritional value, it has the drawback of a much higher cost in comparison to define inorganic nitrogen sources [161].

Regarding the initial C/N ratio, the fed-batch experiments seem to confirm the general behavior of microalga cells under nitrogen limiting conditions. A high C/N ratio, corresponding to nitrogen deprivation, induced more lipid accumulation, while it decreased the total biomass production. DHA intracellular content, in contrast to TFA results, was favored under high nitrogen availability, which is in agreement with the results from batch cultures. Clearly the determination of the C/N ratio that leads to maximum volumetric DHA production needs to establish a combination of a high biomass, intracellular lipid and DHA percentage. The parameter was therefore chosen for further examination.

1.5 Fed-batch DoE approach for DHA production optimization

DoE is an effective approach for the determination of the optimum values of parameters that adopt continuous numerical values, such as C/N. It was opted for the purposes of the current research work to minimize the time and resources necessary for the final optimization of the process, in terms of DHA production. Apart from C/N, the other parameter that was tested was A.F. A.F is critical for oxygen availability, which is known to affect the desaturases activity and consequently the amount of unsaturated TFA. In order to enhance oxygen content inside the culture broth, increase of A.F, or stirring speed is recommended. However, dinoflagellates have been proven to be very sensitive to sheer forces developed under high agitation [161]. Therefore increase of oxygen availability is recommended to be induced with air supply, while A.F inside the vessel is a fermentation parameter that can only be examined under fed-batch mode.

DoE parameters determination

According to previous fed-batch results (Fig. 15) the range opted for C/N optimization was between 5 to 25 g C/g N. When designing a set of experiments for RSM, it is important to choose a range, for the examined factors, that is not too narrow, in order for the effect of the different factor's values to be detectable, but also not too wide, that some effects might go undetected. Following these guidelines, the A.F was chosen to vary between 50 to 100 mL/min for cultures of 100 mL working volume, corresponding therefore to a flow of 0.5 to 1 vvm. Although a flow of 1 vvm is not the highest applied in *C. cohnii* cultures [105], in the specific experiment, higher aeration rates would have been difficultly achieved, owing to the small total volume of the BioXplorer 100 mini bioreactor system used, which would require constant foaming control.

A full factorial CCD was opted for the DoE. The value of a, corresponding to the distance of the axial points from the center point (0,0) was selected equal to 1.19. A value of 1.41 would have ensured that the design was rotatable, however, in that case, the axial point -1.41 for factor A (initial C/N ratio) was translated to a final C/N ratio, lower than that of pure YE. It would therefore require the addition of another nitrogen source and was disgarded as unsuitable. As a result, each factor was examined at five levels in total (-1.19, -1, 0, 1, 1.19). Each combination corresponding to axial or factorial points was carried out in triplicates, while that of the center point was repeated 5 times, resulting in a sum of 29 independent experiments. More repetitions of the center point are useful as measure of the internal error in optimization experiments [114]. The dependent variable *y* correlated to the factors was the final DHA production (mg) from each fermentation.

Model determination and statistical evaluation

ANOVA for the responses of DHA production collected allowed the selection of an aliased cubic model. Unfortunately, a simpler quadratic model with a satisfing fit wasn't obtained. In order to remove the aliased terms A^3 and B^3 from the cubic model, a backward elimination was carried out, with alpha for exit equal to 0.1, resulting in the final, non-aliased model of Table 6. Plotting the response of DHA final production, against factors A and B, resulted in the response surface of Figure 16.

Table 6. Regression coefficients of the final, reduced cubic model exported by application of RSM

Factor (Scaled)	Regression Coefficient
-	+55.23
C/N	-2.10
***A.F**	-25.79
***C/N·A.F**	-12.42
***C/N^2**	+9.55
A.F^2	-3.53
***C/N^2·A.F**	+33.04
***C/N·A.F^2**	-17.81

*Factors that were evaluated as significant after ANOVA ("Prob>F"<0.05)

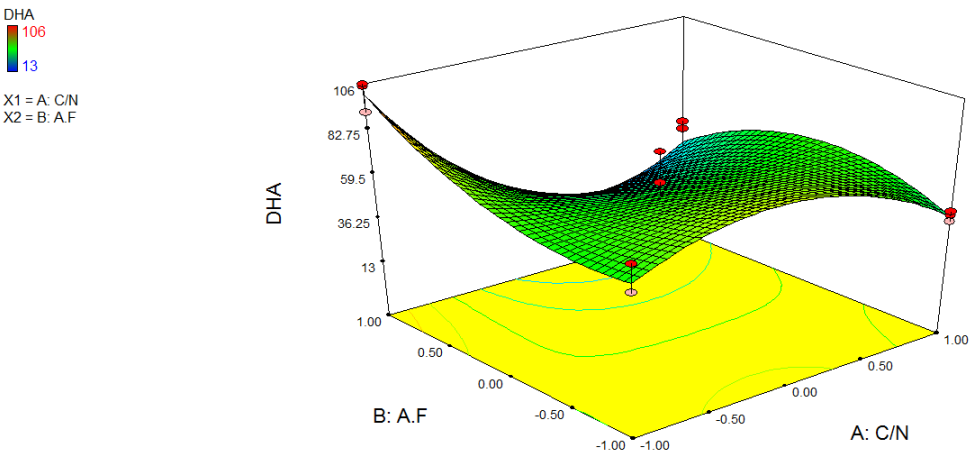


Figure 16. Response Surface of final model for the dependent variable DHA against the variables C/N and A.F

Table 7. ANOVA results for Response Surface Reduced Cubic Model DHA=f(C/N,A.F)

Analysis of variance table [Partial sum of squares - Type III]						
Source	Sum of squares	df	Mean square	F value	p-value Prob > F	Conclusion
Model	14075.56	7	2010.794	19.1967	< 0.0001	Significant
A-C/N	37.5	1	37.5	0.358006	0.5560	Not significant
B-A.F	5642.667	1	5642.667	53.86956	< 0.0001	Significant
AB	1850.083	1	1850.083	17.66242	0.0004	Significant
A^2	810.4462	1	810.4462	7.737189	0.0112	Significant
B^2	110.9442	1	110.9442	1.059165	0.3151	Not significant
A^2B	5425.244	1	5425.244	51.79386	< 0.0001	Significant
AB^2	1577.432	1	1577.432	15.05947	0.0009	Significant
Residual	2199.684	21	104.7469			
Lack of Fit	1.150676	1	1.150676	0.010468	0.9195	Not significant
Pure Error	2198.533	20	109.9267			
Cor Total	16275.24	28				

According to ANOVA results depicted in Table 7, F values highlight the regression model to be significant, with a non-significant lack of fit, thus qualifying for a satisfying model. In order for a factor to be significant it must acquire a *p*-value lower than 0.05, being that the confidence level is 95%. What is more, the factors A and B^2 are characterized as not being significant. Therefore, in case that a simpler model is desired, these two factors can more easily be omitted. The R^2 of the model was calculated equal to 0.8648, indicating that 86.48% of the variability can be described by the model. Similarly, adj. R^2 was 0.8198, providing a measure of the fitness of the final regression model. These values can answer for a satisfying, but not ideally fitted model. It is possible that the nature of the examined system, being an alive, biological entity, might interfere with the better reproducibility and modeling of results.

To further examine the reliability of the model describing DHA production, ensuring its ability to demonstrate the variation of data, different statistical tools need to be utilized. Normal probability indicates a normal distribution of the residuals resulting from comparison of experimental and predicted values. The reduced model exhibits very good fitting of the residuals in the normal % probability plot (Fig. 17a), therefore enhancing the validity of the ANOVA. An undesirable normality of residuals would have resulted in a sigmoidal fitting of the plot points, indicating the necessity of transforming the response of DHA production. The satisfactory normality of residues can also be highlighted by the Box-Cox plot (Fig. 17b) which indicates that no power transformation is necessary for the experimental responses (desirable lamda=1).

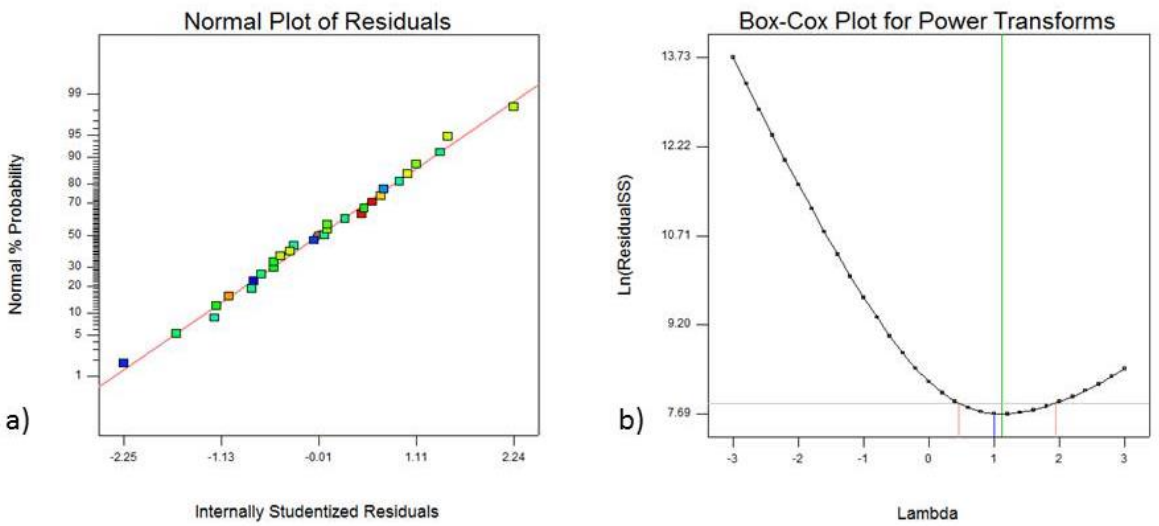


Figure 17. a) Normal Plot of Residuals and b) Box-Cox Plot for the residuals of the final, reduced model

A normal method to ensure that no other variables have influenced the experimental responses, apart from the factors examined, is drawing the residuals versus number of

run plot (Fig. 18b). The plot exhibits a random scatter line, which supports the lack of external influences for the used responses. Furthermore, no abnormal run is identified from the externally studentized residuals (Fig. 18a), since all of them are located inside the range of -3.5·(standard deviation) to +3.5·(standard deviation). Statistical analysis therefore confirms the validity of the model, as well as the suitability of the experimental values. For all the values of the responses, residuals and ANOVA analysis, the reader can refer to Appendix 2 of the thesis.

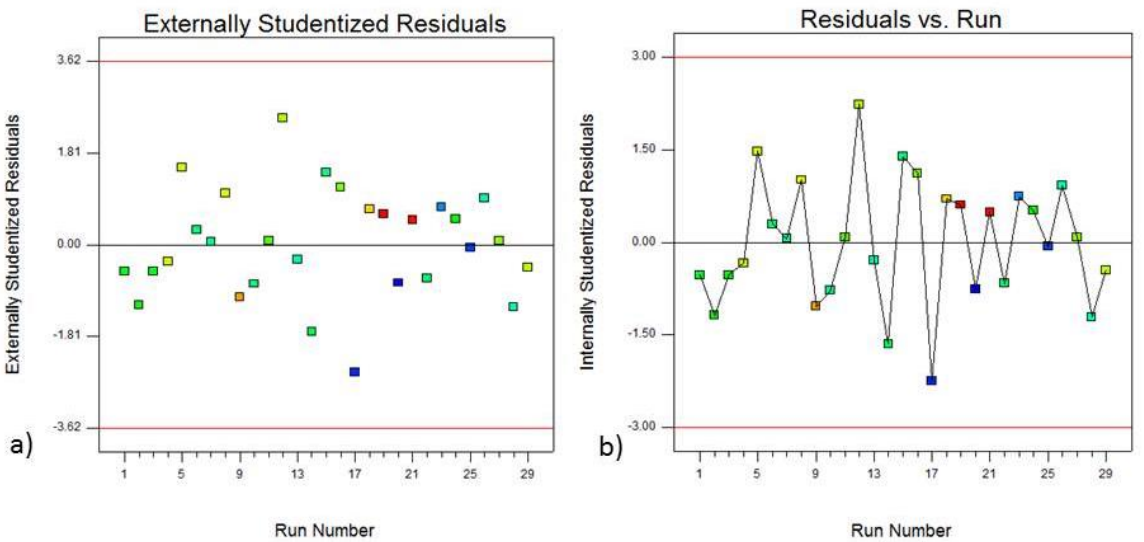


Figure 18. Plot of a) Externally Studentized Residuals and b)Internally Studentized Residuals for each run

Based on the regression coefficients of Table 6, the effect of each factor in the response-that is the DHA production- can be estimated. Apparently A.F has the highest negative influence, while $A/C^2 \cdot A.F$ the highest positive. In case that the two factors of C/N and A.F had not been scaled, the different magnitude of them-5-25 and 50-100 respectively-would have influenced the final regression coefficients and covered the pure effect of

each factor in the response. Between C/N and A.F, A.F seems to affect more prominently, at least directly, the DHA production in comparison to the initial C/N ratio. An explanation for this can be sought in the behavior of microalga biomass and TFA production under different C/N ratios, since many of the previous experiments of this work have shown that, the higher the biomass or lipid accumulation achieved, the higher the final DHA harvest. To unfathom this behavior, RSM was again carried out for the same set of experiments with the responses of volumetric biomass production (g/L) and TFA content (% DCW), since DHA production is affected both by biomass and lipid accumulation. The resulted response surfaces are depicted in Figure 19. In the case of biomass and TFA, the extraction of a specific model and the statistical analysis of its reliability wasn't deemed necessary for the purpose of the thesis. The response surface is used only to help explain the behavior of DHA production under different conditions. By observing Figure 19, a specific trend is visible. In general, conditions that favor biomass augmentation usually inhibit the increase of lipid percentage. For example, a very low initial C/N ratio, translated in a high availability of nitrogen, favors biomass, as depicted in Figure 19a, but results in low lipid content. Furthermore, although the total lipid content appears higher at medium to high values of the two factors, biomass production profile is the exact opposite, exhibiting maximum levels at the limits of the examined range. The combined effect of the two factors in biomass and TFA somewhat smooths the DHA response finally leading to the surface of Figure 16. That is the reason why the favorable effect of a low C/N ratio to biomass production is not reflected in the DHA production.

Another important observation is the effect of A.F in biomass production and lipid content, which was not previously examined in this thesis. Apparently a higher oxygen supply, thanks to aeration, does not necessarily enhance cell growth, as has been

previously suggested for *C. cohnii* cultures [105]. In Figure 19a it is clear that the A.F is not causing any important change in the biomass production, which is rather dependent on the initial C/N ratio. Contrary to that, A.F and oxygen availability is affecting the lipid content of the cells and subsequently the omega-3 accumulation. For example, the preference for a high A.F equal to +1 (scaled value) is clearly depicted in Figure 19b, throughout the range of C/N, but the influence of changing A.F is different for low and high initial C/N ratios. As the oxygen supply is notorious for affecting the activity of various intracellular enzymes, it can be supposed that, under different nitrogen availabilities, *C. cohnii* changes its transcriptomic profile in terms of lipid metabolism. Different enzymes expressed at different C/N conditions, react in other ways to the various oxygen levels. Unfortunately, a more thorough understanding of the metabolism of the cells is needed to explain these results and illustrate their effect in the final DHA production. In every case, it must be noted that, as it happens always with RSM, the model exported has no immediate physical significance. Although it effectively represents the behavior of DHA production within the examined range, it has no use in explaining it.

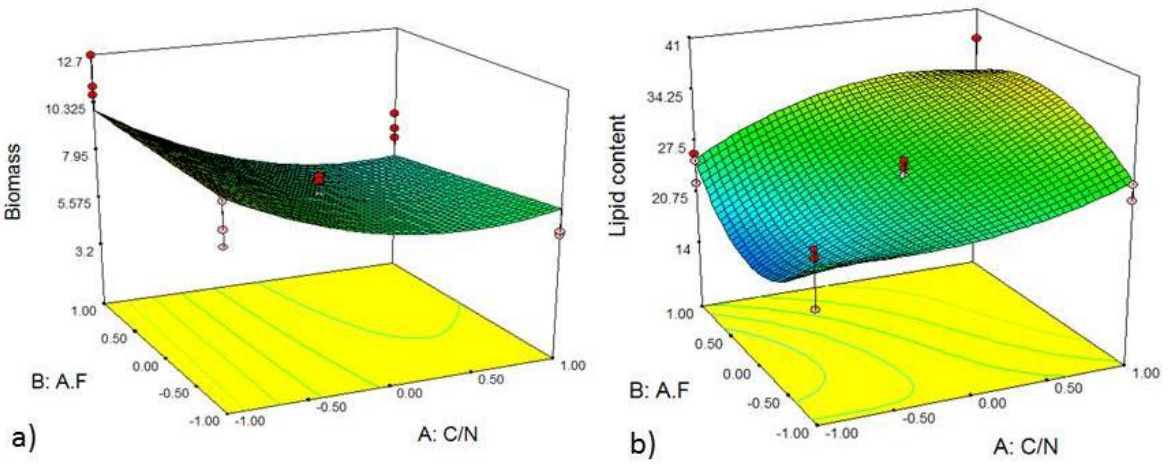


Figure 19. Response surface generated for a) Biomass production (quadratic model) (g/L) and b) TFA content (reduced cubic model) (%DCW) under the influence of initial C/N ratio and A.F. by the set of experiments depicted in Appendix 2, used also for modelling of DHA production with RSM

Changeable fermentation cost estimation

Apart from the final DHA production of a process, which is the main goal of this work, a sustainable process striving for industrial application should also take into consideration the total cost. In case of a fed-batch fermentation, as the one examined, cost is dependent on many parameters, such as cost of raw materials and operational cost of the equipment. For the purposes of the specific research, the cost that is influenced by the different initial C/N ratio and A.F is that of purchasing the necessary amount of YE and SA and the operating cost of the aeration system which was, in this case, a fine bubble diffuser. For each condition-set of C/N and A.F values- experimentally examined, the changeable cost was calculated and the corresponding response of cost was correlated to the factors A and B through RSM, following the same methodology described in the previous paragraph. The determination of a mathematical model that describes the cost

shift doesn't need any statistical method and analysis. Nevertheless the procedure was followed for the drawing of a response surface plot (Fig. 20) that will facilitate the understanding of the cost difference under each condition, as well as, to enable the further attempts of optimization.

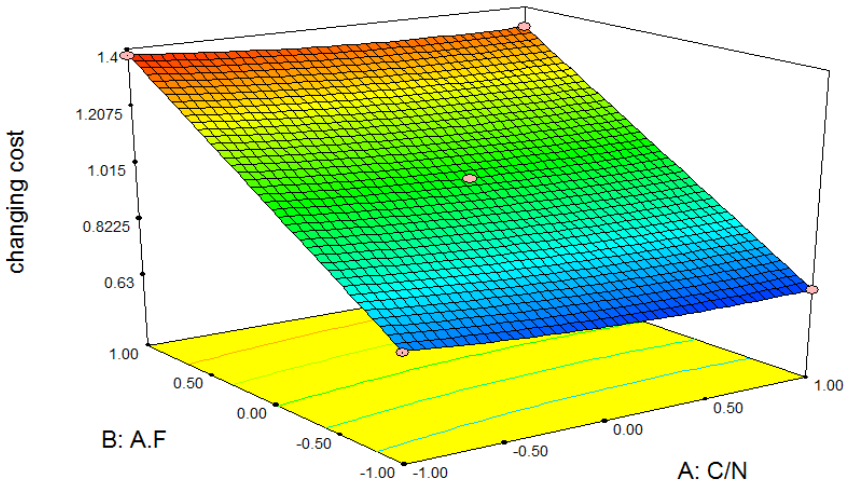


Figure 20. Depiction of the behavior of the changing fermentation cost influenced by C/N and A.F

Apparently, the aeration cost influences more the final fermentation expenses, in comparison to the C/N, owing to the necessary daily energy consumption. In contrast to YE and SA that are added only at the beginning of the fermentation, the air provision inside the bioreactor is a constant, non-stopping, energy-demanding procedure.

Optimization

For the optimization of specific responses, the Derringer's desirability *d* should reach a value as close to 1 as possible, as explained in previous part of the thesis. A suitable model for the maximization of a response should ideally exhibit a maximum inside the ranges of examination. Otherwise optimization is problematic. The reason is that,

although a very good tool, RSM is renowned for its inability to foresee a result outside of the ranges of study. In the case examined, the simultaneous maximization of the DHA production and minimization of cost shift was attempted, using the model for DHA production generated by RSM (Table 6). By examination of the response surface of the model, it is clear that the maximum of the response is not necessarily inside the ranges of the model, thus undermining the successful optimization. This behavior however cannot be necessarily translated to a DoE mistake rather than to a result of the limitations of the fermentation system. According to Figure 16, the response of DHA production tends to increase for high A.F and low C/N, indicating that a proper design should have examined even lower initial C/N ratios and higher A.F values. Nevertheless, the lowest C/N ratio examined-that is the scaled value of -1.19- was in reality equal to the C/N ratio of YE alone- as already mentioned- and was succeeded with the sole addition of YE both as a carbon and nitrogen source. Furthermore, higher aeration rates weren't chosen for the DoE owing to the small total volume of the fermentors which didn't enable a higher toleration for foaming.

The optimization process was therefore continued, despite of the above mentioned system limitations that couldn't be surpassed. Two cases of desirability were examined that included minimization of the changeable cost and also maximization of the DHA production. In the first scenario, and more appropriate one, the values of the factors C/N and A.F were set inside the examined range of scaled variables (-1 to +1). In the second one, the values were allowed to take values outside that range, if necessary, taking into consideration that the ability of the model to estimate the responses is very much hampered. The suggested results of the desirability function are summed up in Table 8.

Table 8. Optimization results for maximum DHA production with minimum changing fermentation cost following the model describing the specific responses exported with RSM

Scenario	C/N*	A.F*	DHA (mg)	Chang. Cost (€/L culture)	Desirability *d*
#1	-0.1	-1.0	78	0.69	0.803
#2	-0.45	-2.13	107	0.34	1

*Scaled values

The above two fermentations were carried out in duplicates, to examine the reliability of the model, while feed was substituted by the real effluent #4. Scenario #1 resulted in the production of 70 mg DHA, while #2 resulted in 79 mg DHA. Apparently the model is mildly optimistic, in contrast to the real results with scenario #1 deviating 10 % from the estimated value and scenario #2 by 26 %. The RSM is notorious for its poor ability to predict the results of variables, depending on factors adopting values out of the examined ranges. Therefore, the inability of the model to successfully predict the behavior of the system at scenario #2 is not surprising. Although a 10 % deviation of the real and predicted value within the ranges of the model is much more satisfying, it also signifies that the model can be further improved. A reason for its inability to make a closer prediction might be the feed of the optimized fermentations. Instead of using an artificial effluent, the real waste-derived liquid fraction #4 was fed in the bioreactors operating under optimized conditions. The more complex composition of the effluent, including also traces of iso-butyric, iso-valeric acid and unknown organic matter, may answer for the 10 % deviation. Since the purpose of the work is organic waste bioconversion and in order to further enhance the reliability of the model, a biowaste-derived feed needs to be used for all the different experiments of DoE. This, of course, requires a much higher volume of effluent, that wasn't available during the present thesis, but it constitutes a future plan of potential.

2. Treated lignocellulosic biomass as substrate for *C. cohnii* cultivation

2.1 Fermentable pure sugars as main carbon source

As a first step of examining the ability of *C. cohnii* to assimilate lignocellulosic sugars- and following the same pattern as the one for DF effluent-, the microalga cells were grown on pure sugars as carbon source at an initial concentration of 30 g/L. The different sugars, namely glucose, xylose, mannose and arabinose, comprise usual monosaccharides, derived from the enzymatic hydrolysis of pretreated lignocellulosic biomass, and were selected for this reason. Galactose, another biomass-derived sugar which has already been reported to support *C. cohnii* growth in the literature, was not examined [133]. As depicted in Fig. 21**,** *C. cohnii*, although able to assimilate partly all the different sugars, prefers glucose as a carbon source over other monosaccharide substrates . After 120 h growth, the glucose batch cultures afforded a production of 9.67 g/L of culture, while xylose, mannose and arabinose supported between 1.40-1.18 g/L. The cells growth on mannose and arabinose seems to be very slow, which is also exhibited in the slow consumption of the carbon source. Average doubling times (T_d) between 24-72h were found to be 8.4 h for glucose, in comparison to the much higher 14.9 h for xylose and 39.4 h for arabinose and mannose respectively.

In contrast to the cell production under different monosaccharide feed, as depicted in Table 9, the differences observed in the % wt. TFA accumulated intracellularly were more limited. Such a behavior can be explained if we consider that all different sugars are expected to be assimilated by the cell in the mitochondria, thus being integrated into the Krebs cycle. Whereas, in the case of a feed of VFA both the glyoxylate and Krebs cycle are set into motion and higher fluctuations in final lipid accumulation are expected

[59]. Still the relative proportion of DHA was much higher in glucose compared to other sugars. In Figure 22, it can be observed that DHA is the major FA present in microalga biomass when glucose is used as substrate; however, this is not the case for the other sugars, where C18:1 is in higher amounts.

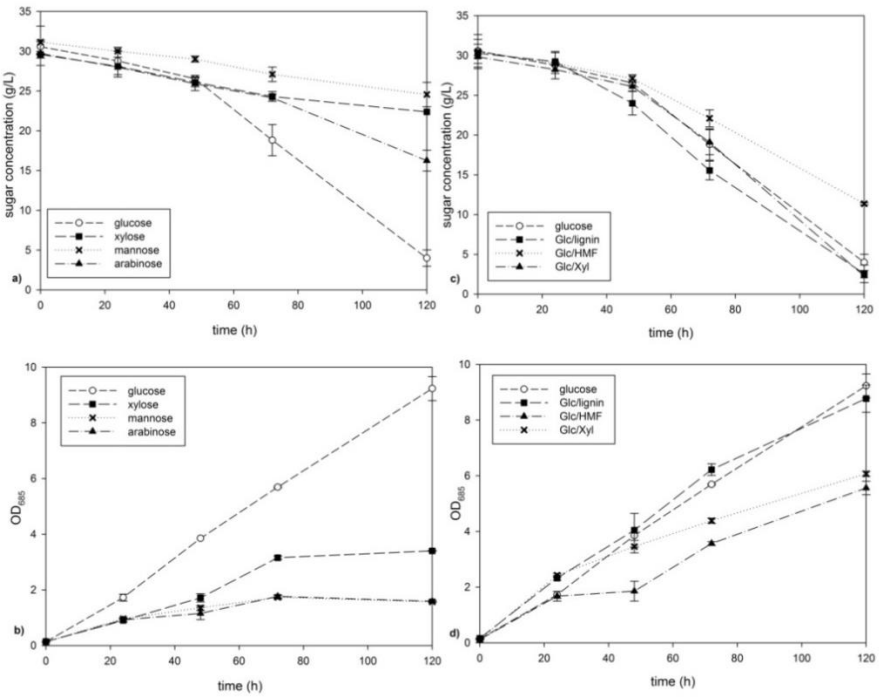


Figure 21. Depiction of the consumption of simple pure sugars with (a) or without (c) addition of chemical inhibitors by *C. cohnii* cells in batch cultures. Monitoring of *C. cohnii* cell growth in batch cultures with simple sugars as main carbon source with (b) or without (d) chemical inhibitors

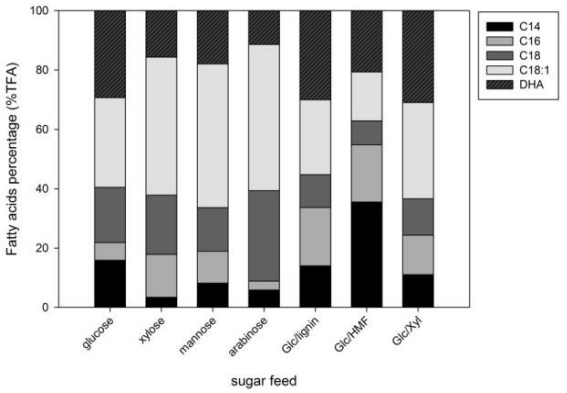


Figure 22. FA profile of *C. cohnii* cells grown with pure sugars under batch mode

Table 9. Biomass, TFA and DHA production by *C. cohnii* cells in batch cultivation in shake flasks, using pure sugars (hexoses, pentoses) as carbon source.

	Biomass g/L	**Biomass (mg/g sugar consumed)**	**TFA (%DCW)**	**TFA (g/L)**	**DHA (%TFA)**	**DHA (g/L)**
glucose	9.67	306.4	43.0	4.2	34.9	1.5
xylose	1.40	312.0	37.3	0.5	5.7	0.1
mannose	1.23	186.8	45.4	0.6	17.9	0.1
arabinose	1.18	125.8	44.3	0.6	11.4	0.1
Glc/lignin	10.86	353.7	47.5	5.2	30.0	1.6
Glc/HMF	7.08	320.2	35.2	2.5	20.6	0.5
Glc/Xyl	4.27	296.5	40.0	1.7	35.0	0.6

The results from pure sugars are encouraging for the application of *C. cohnii* cells as means of lignocellulosic hydrolysates bioconversion. Nevertheless, it must be noted that real hydrolysates include a mixture of monosaccharides, as well as other biomass-derived substances that are known to act as inhibitors to cell growth [162]. To test whether the simultaneous presence of hexose and pentose could have an adverse effect

in the microalga growth and the production of lipids, a mixture of glucose and xylose in a 50:50 (v:v) ratio was also tested as main carbon source. It was immediately observed that the strain exhibited no catabolite repression, as the cells concentration and sugar consumption was equal to the combined effect of these two different sugars (Fig. 21c and d).

Furthermore, the inhibition of the chemical substances lignin and hydroxymethylfurfural (HMF)-present normally in organosolv pretreated agricultural waste- was evaluated in batch cultures with glucose. Acetic acid is another usual inhibitor detected in pretreated lignocellulose. Nevertheless, this acid is a favorite carbon source of the strain, as it was already exhibited, and was not examined as an inhibitor. The presence of lignin didn't result in any adverse effect on the cultivation. On the contrary, lignin addition was proven to affect positively the microalga metabolism for enhanced DHA production. It is speculated that the metabolic boost caused by the presence of lignin could be similar to the one previously proposed for the addition of sesamol in *C. cohnii* cultures, owing to its antioxidant properties and ability to scavenge intracellular reactive oxygen species, thus protecting the microalga cells [163]. In contrast to lignin, the presence of HMF seems to inhibit both cell growth and lipid accumulation, as well as strongly influence the FA profile, as seen in Figure 22. A high accumulation of C14 over other FA is observed, suggesting that HMF interferes with the lipogenic mechanism of the microalgal cells. The inhibitory effect of HMF is based on its reduction to 5-hydroxymethyl furfuryl alcohol, the presence of which causes a series of further adverse effects in the cell physiology and metabolism [162]. It is not the first time that a growth inhibitor is known to have an effect also in the FA profile of the cells. M. de Swaaf et al. have exhibited that, when grown with norflurazon as an inhibitor, the fungus *Mortierella alpine* produced a higher amount of C16:0 and C18:1 lipids, while the accumulation of the polyunsaturated arachidonic acid was hampered [139].

According to the same group, which has conducted ^{13}C-labelling experiments of *C. cohnii*, the FA synthesis in the microalga cells follows three different metabolic paths The first one, responsible for saturated acids biosynthesis, involves cytosolic reactions, the second one answers for the desaturation of saturated FA to monounsaturated and the third one is solely for the production of DHA. This is in accordance with the general idea of a PKS system in *C. cohnii* cells, responsible solely for DHA synthesis (Fig. 4). Since the percentage of C18:1 increase under a feed of other sugars, apart from glucose, it is possible that these substrates have an inhibitory effect on the enzyme complex responsible for de-novo DHA synthesis. Therefore the available carbon and energy flux are directed towards the sum of elongase- and desaturase-reactions leading to C18:1 formation.

2.2 Lignocellulosic hydrolysates as main carbon source

Batch trials

The successful utilization of pure monosaccharides paved the way towards the examination of microalga cultures with hydrolysates from organosolv pretreated biomasses. Commercially available beechwood sawdust with a particle size of 150–500 μm (Lignocel® HBS 150–500, JRS GmbH and Co KG, Germany) and bark-free pine trimmings of Pinus sp.-for comparison, as representative of softwood- were used. Biomasses were treated with aqueous solutions of the different organic solvents ethanol (EtOH), acetone (ACO) and THF, at different operation conditions (type of organic solvent and reaction time) under an overpressure of O_2. According to the results depicted in Table 10, pretreatment at 120 min was more efficient compared to 60 min regarding the fractionation process, for all three different solvents used. What is more, most of the pulps obtained after pretreatment had a lignin content of < 10 wt%, which greatly

enhances their further enzymatic saccharification and sugar release in comparison to the untreated material. Lignin can affect enzymatic hydrolysis in mainly two ways; namely by causing restriction of the cellulose accessibility to enzymes and non-productive binding of the enzymes to aromatic substances [164]. Oxygen facilitates lignin depolymerization through the breakage of ether linkages and alter the lignin structural properties [165], while the presence of organic solvent enables the dissolution and removal of lignin fragments in the liquid fraction. The use of organic solvents also affects the cellulose surface and causes cellulose swelling [166], thus rendering the substrate more amenable to enzymatic hydrolysis. Further examination of the effect of pretreatment conditions to the final pulp composition wasn't deemed relevant to this thesis purpose.

Table 10. Compositional analysis of the biomass pulps used for enzymatic saccharification and harvest of simple sugars for *C. cohnii* growth

No.	Pretreatment conditions	Wt.% cellulose	Wt.% hemicellulose	Wt.% lignin
1	H_2O/THF (50/50%), O_2 12 bar, 175ºC, 120min	80.1	11.4	5.8
2	H_2O/ACO (50/50%), O_2 16 bar, 175ºC, 120min	80.3	12.3	1.6
3	H_2O/ACO (50/50%), O_2 12 bar, 175ºC, 60min	73.8	16.3	3.9
4	H_2O/EtOH (50/50%), O_2 12 bar, 175ºC, 60min	71.6	18.0	6.4
5	H_2O/THF (50/50%), O_2 12 bar, 175ºC, 60min	73.5	14.6	7.9
6	H_2O/THF (50/50%), O_2 16 bar, 175ºC, 120min	64.2	19.9	10.7
7	H_2O/EtOH (50/50%), O_2 16 bar, 175ºC, 60min	77.1	15.7	2.7
8*	H_2O/EtOH (50/50%), O_2 16 bar, 175ºC, 60min	77.6	13.2	7.2
9	Untreated beechwood	40.1	19.1	23.6
10	Microcrystalline cellulose (Avicel)	95.5	2.5	0.8

**No. 8 refers to pine biomass, while No.1-6 to pretreated beechwood*

The above mentioned pretreated pulps were treated with the enzymatic mix CellicCTec 2 for the release of simple sugars. It must be noted that the available pretreated beechwood biomasses covered a broader spectrum of organosolv conditions than the ones appearing in Table 10. However, only the beechwood samples which exhibited the

higher % cellulose conversion (>80%) (Table 11) were used for the cultivation of *C. cohnii* and are therefore included in the present thesis.

Enzymatic digestibility of run No.2 was significantly high, resulting in 88.9 g glucose per 100 g of biomass. In case of materials pretreated with EtOH and ACO, a pressure increase from 12 to 16 bar resulted in higher delignification rates, higher % cellulose recovery in the pulp, but did not have a positive effect on enzymatic hydrolysis (Table 11). On the contrary, harsher pretreatment conditions, such as higher pressure or longer treatment, led to lower conversion of cellulose. This observation can be attributed to the increased release of inhibitors by severe treatment of the lignocellulose, which in turn do not allow the hydrolytic enzymes to act so efficiently [164]. The results from pine biomass (run No.8) underline the effectiveness of the acid-free mild oxidative organosolv fractionation in softwood biomass, which is considered to be more difficult to process compared to hardwood; pretreatment resulted in a highly-delignified solid fraction (7.2 wt% lignin) with 77.6 wt% cellulose which exhibited a high cellulose conversion rate of 70.5% after enzymatic hydrolysis (Table 11).

Table 11. Saccharification yields of pretreated beechwood and pine lignocellulosic pulps after enzymatic hydrolysis. Untreated beechwood biomass and purchased Avicel PH-101 were also used as controls

Run No.	glucose (g/L)	% celullose conversion	glucose (% wt. of substrate)	TRS (g/L)	TRS (% wt. of substrate)
1	77.9 ± 0.9	97.3 ± 1.1	86.5 ± 1.0	88.2 ± 2.5	98.0 ± 3.1
2	80.0 ± 0.7	99.7 ± 1.1	88.9 ± 1.0	89.1 ± 0.9	99.0 ± 1.1
3	76.2 ± 2.0	100 ± 2.6	84.7 ± 2.2	88.9 ± 2.1	98.8 ± 2.3
4	72.6 ± 1.5	100 ± 2.1	80.7 ± 1.8	85.1 ± 1.8	94.4 ± 2.1
5	67.3 ± 1.1	91.6 ± 1.5	74.8 ± 1.3	77.2 ± 0.3	85.8 ± 0.3
6	54.8 ± 3.5	85.4 ± 4.8	60.9 ± 3.9	72.3 ± 1.9	80.4 ± 2.1
7	65.1 ± 2.0	86.5 ± 3.4	72.4 ± 3.1	83.3 ± 1.7	92.5 ± 2.2
8	54.7 ± 0.3	70.5 ± 0.3	60.8 ± 0.3	66.8 ± 1.0	74.2 ± 1.1
9	10.4 ± 1.2	26.4 ± 3.1	11.6 ± 1.5	12.5 ± 0.9	13.9 ± 1.1
10	59.3 ± 2.5	62.1 ± 3.0	65.9 ± 3.3	60.1 ± 0.4	66.8 ± 1.2

Produced sugars were used as carbon source for *C. cohnii* growth in batch cultures. For the medium preparation, the hydrolysate was diluted 2-times, previously to other nutrients addition and autoclaving, in order for the initial sugar content to be no higher than 36 g/L. That way the inhibitory effect of high glucose concentration on microalgae growth is alleviated. The hydrolysates were therefore evaluated regarding their ability to support cells growth per volume of sugar solution produced, since not all cultures had an initial sugar concentration. All beechwood pulps could efficiently support the growth of microalgae and the synthesis of FA (Fig. 23), while growth was higher for pulps pretreated at 60 min, compared to those pretreated at 120 min, following the same trend as enzymatic hydrolysis. Especially materials No.1 and No. 2 that even provided hydrolysates of high glucose concentration weren't very effective as carbon source. It is a fact that at high temperatures, such as 175^{o}C, hemicellulose depolymerization and

dehydration can partly occur, thus leading to the formation of furfural and HMF [167]. At longer treatment periods these inhibitors might accumulate in the material. This suggestion can be further supported by the low final amount of hemicellulose detected in those specific biomasses (Table 10). A high concentration of HMF, however, was proven to be harmful for *C. cohnii* cells and might answer for the low performance of the materials of longer pretreatment.

When EtOH was used as an organic solvent, the beechwood hydrolysates resulted in higher microalgae growth, accompanied by higher wt. % TFA accumulation and higher relative intracellular amount of DHA, both after 60 and 120 min of hydrolysis, compared to ACO and THF. What is more, their initial TRS concentration was not the highest between the hydrolysate, so as to answer for this development. It is a fact that *C. cohnii* has been repeatedly proven to assimilate EtOH for carbon provision [93]. Still it is almost impossible to expect that any EtOH from the pretreatment will have remained after the enzymatic saccharification. It may be that an EtOH pretreatment secures a better quality of lignocellulose, whose hydrolysate performs better as carbon source, lacking toxic compounds. In terms of sugar assimilation for biomass production, beechwood materials treated with THF and EtOH promoted the microalga biomass production better that other hydrolysates. The very good performance of the untreated beechwood biomass is rather surprising and may be an experimental error due to the very low available amount of sugars and microalga biomass. Another explanation is the high amount of lignin present in the untreated material. It has already been proven that lignin, instead of inhibiting, is aiding both biomass and lipid accumulation (Table 9). After all, according to the values depicted in Table 12, the hydrolysates that resulted in higher microalga biomass per sugar consumed were those that included a relatively high percentage of lignin after the organosolv pretreatment. This observation validates the beneficial effect of lignin in the strain’s growth. Further research to understand the

mechanism of this behavior is needed. *C. cohnii* could also utilize pine hydrolysate as carbon source, however achieving lower microalgae biomass yields of 5.5 g/L, 38.7 wt. % TFA accumulation and 24.8 wt. % DHA. HMF inhibition might be able to justify the low preference of the cells for pine hydrolysates. Under treatment with THF, pine was proven to release higher amounts of HMF, in comparison to other biomasses, in previous research [168]. Although pretreated with EtOH, in this work, presence of HMF in pine hydrolysates is again very possible.

It is interesting that, when cultivated under pure sugar substrates, *C. cohnii* accumulated equal, or a bit higher, amount of DHA in the presence of both glucose and xylose, and occasionally lignin. Accordingly, it was expected that a biomass hydrolysate that consists of all three, triggers a higher DHA content than the Avicel hydrolysate, which consists solely of glucose. However, that was not the case, since the utilization of Avicel resulted in the highest DHA content of 45.7 wt. % (Table 12). It is possible that the presence of inhibitors in the hydrolysates from the pretreated lignocellulosic biomass might decrease DHA production conquering the beneficial effect of lignin and hemicellulose presence. *C. cohnii* has been reported to grow and accumulate DHA utilizing other cheap, renewable sources, such as carob pulp syrup in a 1:3.5 (v/v) dilution (corresponding to 26.3 g glucose/L), achieving a DHA yield of 45.4 % [118] which is close to the yield obtained with Avicel hydrolysate in this study (45.7 %). Considering that the microalga biomass left after extraction could be utilized for aquaculture, poultry and animal feed, as summarized in the study by Mendes et al. (2009), the overall process of hydrolysates utilization is sustainable and economically viable with low carbon source costs. Moreover, it is exhibited that *C. cohnii* can grow on pentoses, although slower, which is a great advantage for the production of DHA from biomass compared to other DHA producing organisms, such as thraustochytrid marine protist *Aurantiochytrium* sp. that cannot utilize xylose [169].

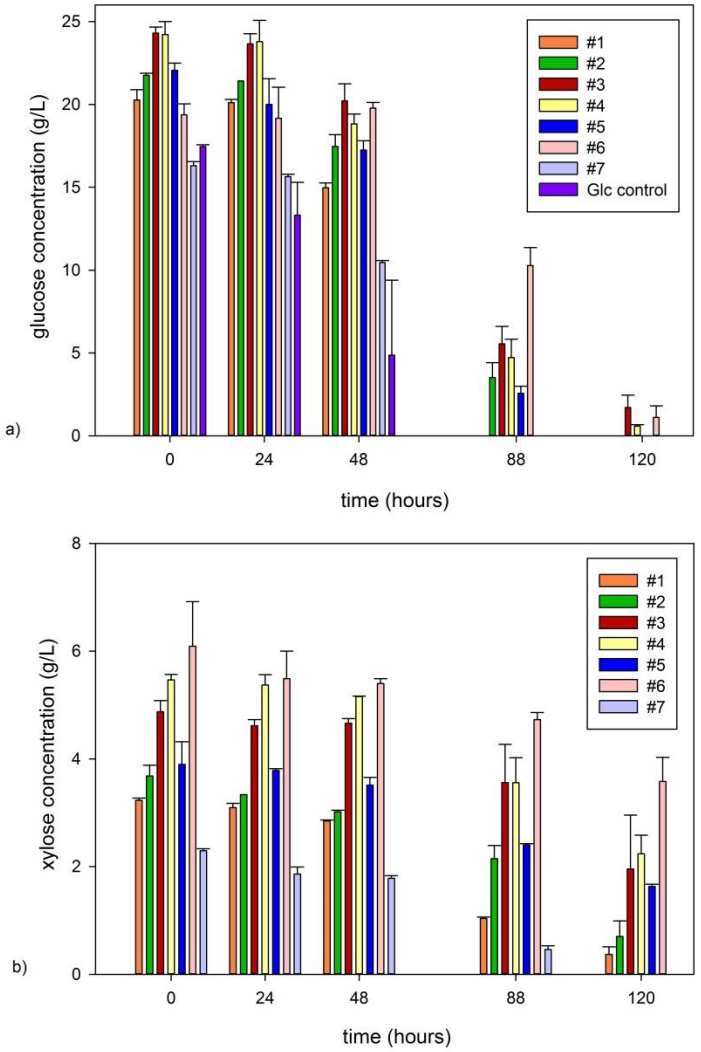


Figure 23. Beechwood hydrolysate-derived a) glucose and b) xylose consumption in batch cultures of *C. cohnii*

Table 12. Biomass, TFA and DHA production by *C. cohnii* cells in batch cultivation in shake flasks, using hydrolysate-derived sugars as carbon source.

No.	Biomass g/L	biomass (mg DCW/g sugar consumed)	TFA (% DCW)	TFA (g/L)	DHA (% TFA)	DHA (g/L)
1	7.39	320	44.2	3.3	27.2	0.9
2	7.71	312	38.2	3.0	22.0	0.7
3	7.76	304	33.5	2.6	29.0	0.8
4	8.72	325	39.1	3.4	28.2	1.0
5	7.98	328	35.7	2.9	29.5	0.8
6	7.90	373	54.3	4.3	29.4	1.3
7	8.61	345	48.0	4.1	29.5	1.2
8	5.51	294	38.7	2.1	24.8	0.5
9	0.99	391	36.0	0.4	14.7	0.1
10	5.57	355	23.3	1.3	45.7	0.6

Trials with fed-batch strategy of hydrolysates

Although a feed of hydrolysate is characterized by a low raw materials cost and allows lipid and omega-3 accumulation, its benefit under a batch mode is limited. Lignocellulose-derived monosaccharides are quickly consumed and consequently the growth ceases. Still, taking into consideration the similar final DHA production values (g/L) achieved in Table 12 and those obtained with a synthetic DF effluent in the 100 mL fed-batch cultures, it seems that hydrolysates have high potential as bioconversion substrate. In order to increase the accumulation of lipids by providing more carbon and applying nitrogen limitation culture conditions, a fed-batch strategy was employed using the run No. 7 (H_2O/EtOH (50/50%), O_2 16 bar, 175^oC, 60min) and No. 6 (H_2O/THF

(50/50%), O_2 16 bar, 175^{o}C, 120min) hydrolysates as carbon source. After all, these two were able to support the highest biomass production per sugar consumption and volumetric DHA production. Owing to a lack of automatization in the feeding technique, the addition of hydrolysate was carried out manually. During cultivation the lignocellulose-derived sugars were almost totally depleted after the first 120 h of cultivation, when more hydrolysate was added, to achieve a sugar concentration equal to the beginning of the culture (t =0 h). That resulted also in a dilution of the cells in the culture, as it is depicted in Figure 24, while after the addition of sugar feeding, growth and carbon utilization was slower for the following 120 h, resulting in a slower growth translated into lower doubling time. There are two possible explanations for this; either nitrogen limitation after 120 h resulted in reduction of cell growth or the accumulation of degradation products originating from the pretreatment process caused inhibition of cell growth. The lack of a nutrient source has been reported before as a growth limiting factor of *C. cohnii* grown on glucose or acetic acid under fed-batch high-cell density fermentation [106]. In that case the exponential growth of cells was later substituted by a linear growth, a fact that was attributed to the consumption of another necessary nutrient, since carbon was always provided in the cultures. Fed-batch cultivation led to an increase of the final biomass concentration obtained, reaching the value of 10.28 g DCW/L for the material #6 and 12.71 g DCW/L for #7, but also to a reduction in wt. % TFA. It is important that the % DHA content was increased, from 29.5 % to 38.7 % for Run #7 and from 29.4 % to 43.5 % for Run #6. The decrease of wt. % TFA can be an indication that the slower growth observed is a result of the accumulation of inhibitors, rather than a nitrogen limitation. In case of nitrogen deprivation the lipid content would have probably risen. Having that in mind, it must be mentioned that, although final biomass production was higher, the total productivity of the cultures wasn't much altered under fed-batch mode. More specifically, for sample No. 7 productivity had risen

only by 3% changing from 1.79 mg/h for batch to 1.85 mg/h for fed-batch mode. In the case of sample No. 6 the productivity even decreased a bit from 1.65 mg/h to 1.50 mg/h. This is expected after observing the decreased growth rate of the cells after the feeding and clarifies that higher final cell concentration is only a result of the prolongation of the cultivation time and not of the establishment of more favorable, to the cells, culture conditions. This leads to the conclusion that a better productivity might be more easily succeeded through a higher sugar concentration in the initial hydrolysate, which will enable the addition of lower feeding volume without a substantial amount of inhibitors.

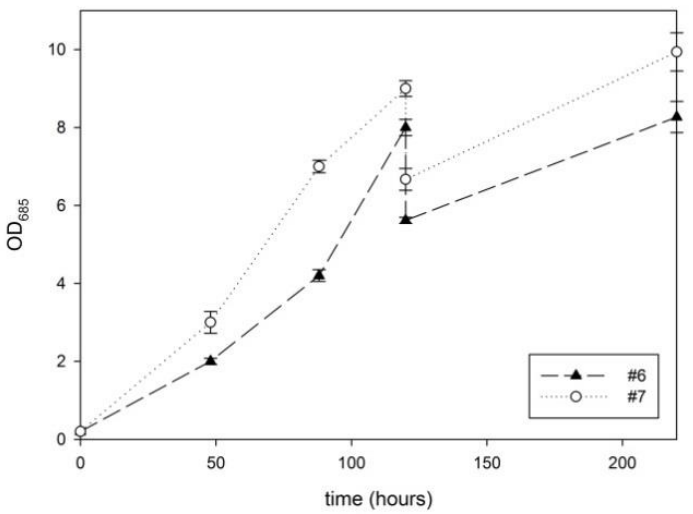


Figure 24. Study of *C. cohnii* cell growth under fed-batch cultivation with a feed of hydrolysates #6 or #7

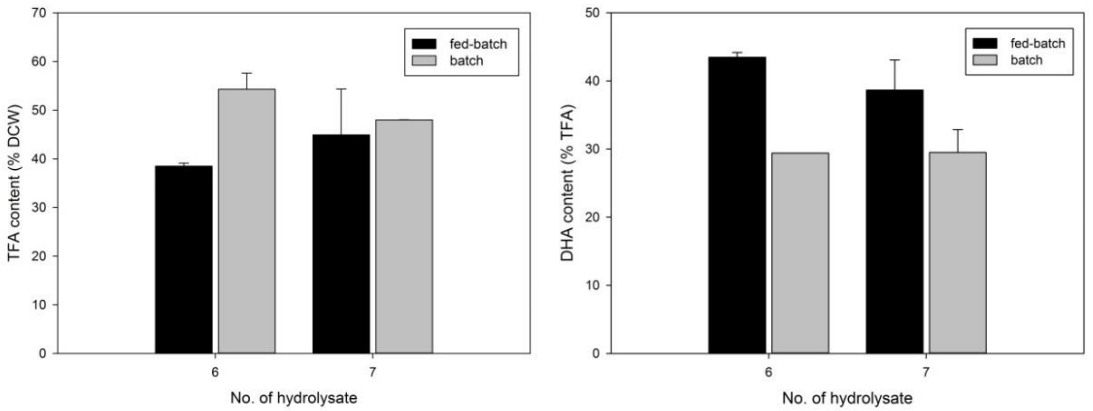


Figure 25. Differences in TFA and DHA accumulation of *C. cohnii* cells cultivated under batch and fed-batch mode with a feed of hydrolysates #6 and #7

2.3 Secretion of cellulolytic enzymes from *C. cohnii* cells

Apart from the cultivation of *C. cohnii* in sugars derived from hydrolysis of lignocellulosic biomass, the ability of the cells to grow directly on lignocellulosic substrates was examined. This ability requires that the cells can produce and secrete enzymes that hydrolyze the biomass for themselves. In an attempt to trigger, if possible, the secretion of hydrolyzing enzymes by *C. cohnii*, the cells were cultivated in cellulose-rich substrates, and more specifically microcrystalline cellulose (Avicel PH-101), corn cob and carboxy-methyl-cellulose (CMC). The culture supernatants were harvested and assayed for the appearance of cellulases.

Assay results revealed that *C. cohnii* wasn't able to produce free or membrane-bound cellulases with the potential of further industrial exploitation. More specifically, no CBH or β-glucosidase activity was detected in the supernatant with or without cells. EG activity was present in traces and only for the first 48 h of cultivation, when cells were cultivated with CMC or corn cob as main carbon source (Fig. 26).

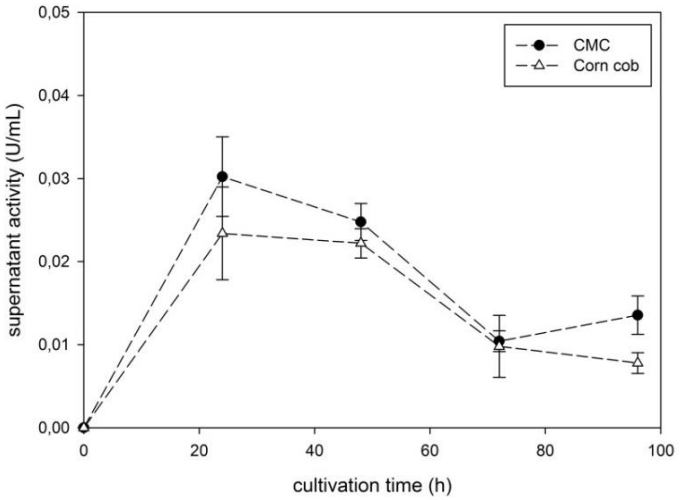


Figure 26. EG activity of *C. cohnii* culture supernatant, during cultivation with different main carbon sources (1 Unit refers to the amount of enzyme needed to release 1 μmol/mL reducing sugars as glucose equivalents with DNS method)

Apparently, the EG activity detected in the culture broth is too low to excite any hopes for further industrial exploitation. Nevertheless, it can be linked to the cell cycle of the microalga. Dinoflagellate cells are known of being surrounded by a complex theca, composted either of glucans, or cellulose [170]. More specifically *C. cohnii* has a delicate, thin cellulosic theca, present both in motile and non-motile cells [91]. In order for the cells to grow and divide, this cellular wall needs to be decomposed and composed many times, as in the case of plants. Therefore, it is suspected that such microalgae species are capable of producing cellulases, and more specifically EG, for the decomposition of the old thecas. In previous research, Kwok et al. have found that a *C. cohnii* strain could exhibit EG activity during specific periods of the cell's life cycle, when the development of new cell wall occurs [171]. According to the researchers, the activity was due to a wall-bound cellulolytic enzyme. In contrast to that, in the present work, no difference of enzymatic activity was observed in the case of the broth with or

without the cells, signifying that the EG enzymes were secreted in the supernatant instead.

The present results, coupled with the above information, indicate that the EG secreted by the cells is not associated with the cellulosic substrate on which *C. cohnii* was cultivated. It is rather concluded that the strain is not able to assimilate the substrates. More evidence to that is the negligible cell productivity, as well as the limited EG activity during the first 48 h. The culture media, although providing carbon mainly in the form of a non-assimilable polysaccharide, it also contains small amounts of YE, a nutrient rich in both carbon and nitrogen. It can, therefore, be assumed that the small growth, linked to the appearance of EG activity, during the first cultivation hours, is solely due to the YE consumption. Further growth would have been accompanied with constant high EG activity for thecas decomposition and would have been possible only if CMC or corn cob could have been assimilated by the strain.

2.4 Boosting the enzymatic hydrolysis of lignocellulosic biomass

Heterologous production of LPMO in P. pastoris from Thermothelomyces thermophila

Apparently, a higher hydrolysis yield is favorable for the further valorization of the released sugars, according to the above results. In order to enhance the hydrolysis of lignocellulosic biomass the recombinant *Tt*LPMO111088 was produced, characterized and examined for its potential to act synergistically with other cellulases for the more effective release of sugars from pretreated agricultural waste.

*Tt*LPMO111088 (NCBI ref. seq. XP_003661661.1) is a member of the AA9 family and was chosen because it exhibited the highest sequence homology with the AA9 LPMOs *Nc*LPMO9C from *Neurospora crassa* and *Pa*LPMO9H from *Podospora anserina* (67%

identity to the first and 78% identity to the second). Both of the above characterized enzymes exhibit a C4 oxidizing activity- with *Pa*LPMO9H exhibiting also C1 activity- in a varied spectrum of substrates [172,173]. What is more, the selected enzyme seems to carry also a carbohydrate binding module (CBM), which is speculated to enhance the protein's binding ability to different substrates, and therefore its activity [174]. The possible *N*- and *O*-glycosylation sites of the enzyme were determined using the NetNGlyc 1.0 Server and NetOGlyc 4.0 Server accordingly. Although no *N*-glycosylation sites were found, 4 possible *O*-sites, at amino acids situated at places 234, 244, 246 and 248, were defined.

The heterologous production of *Tt*LPMO111088 in 1 L culture of *P. pastoris* yielded a final production of 14 mL of 1.0 mg/mL purified protein, under methanol induction. The gene inserted in the yeast cells was codon optimized for *P. pastoris* (the reader is directed to Appendix 3 for the sequence), while expression was carried out using the native signal peptide, instead of the α-factor of pPICZaA vector. The reason for that was to ensure that the native N-terminal sequence of the enzyme will remain unaffected during heterologous expression. It is a fact that, in LPMO amino acid chain, the N-terminus, includes a histidine (His) residue, that participates in the coordination of the copper atom in the active center of the protein [172]. Analysis of the enzyme solution received after Talon chromatography with an SDS-PAGE resulted in Figure 27:

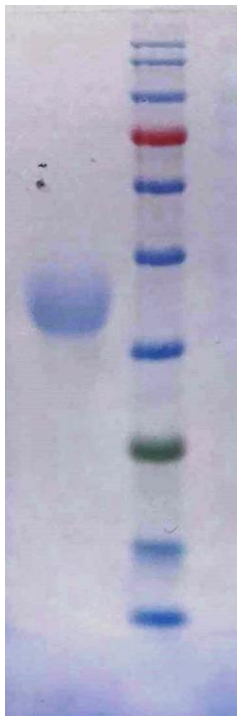
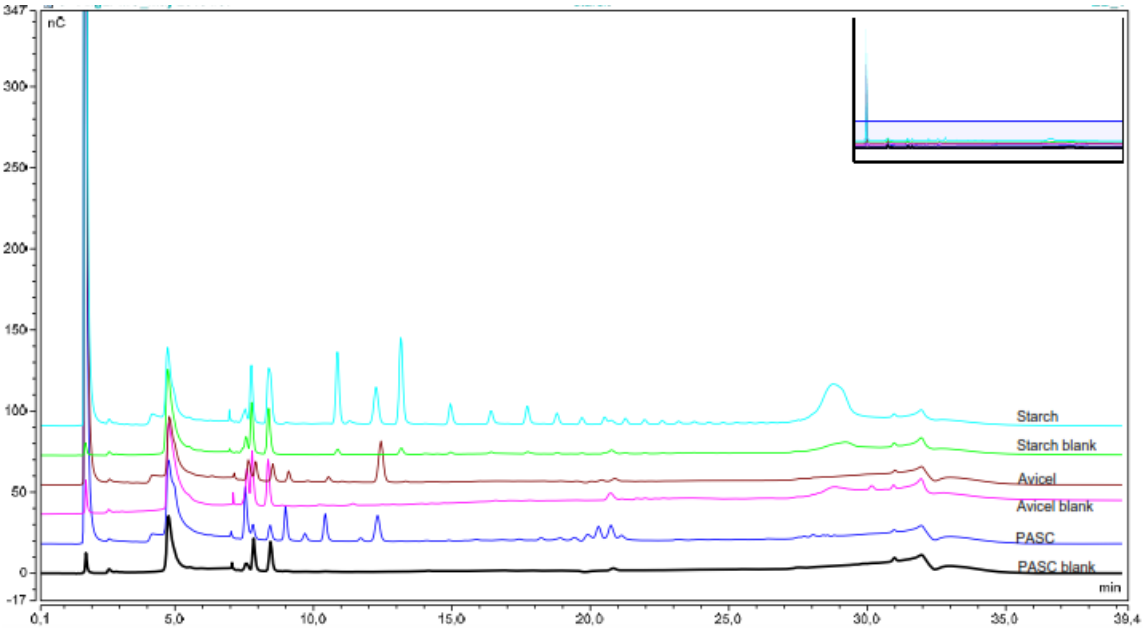


Figure 27. SDS-PAGE of the purified *Tt*LPMO111008. On the left the purified protein is situated between the bands of 48 and 35 kDa of the standard mixture that is visible on the right

The theoretical molecular weight of 32384 Da is lower than the one observed after the SDS-PAGE. Apparently, the enzyme is heavily glycosylated during post-translational modifications of the yeast. The *O*-glycosylations couldn't be hydrolysed by enzymatic treatment of the protein, neither with Endo-H, nor with O-glycosidase (New England BioLabs Inc.). This was also the case in a previous work of producing 4 *Neurospora crassa* LPMOs. R. Kittl et al. observed, after isolating the proteins, that 3 of the 4 enzymes carried more than a few *O*-glycosylations, which the authors didn't successfully hydrolyze with α-mannosidase, resulting in an SDS-PAGE with diffused bands of a substantially larger size than the theoretical one [172].

Substrate specificity and regioselectivity

The polysaccharides PASC, Avicel PH-101, xyloglucan, a-chitin, starch, xylan, konjac glucomannan and arabinoxylan, together with xylo-, chito-, manno- and cello-oligosaccharides were examined as possible substrates for the new enzyme and their reaction products were analysed with HPAEC (Fig.28).


nC
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300
250
200
150
100
50
0
-17
Starch
Starch blank
Avicel
Avicel blank
PASC
PASC blank
min
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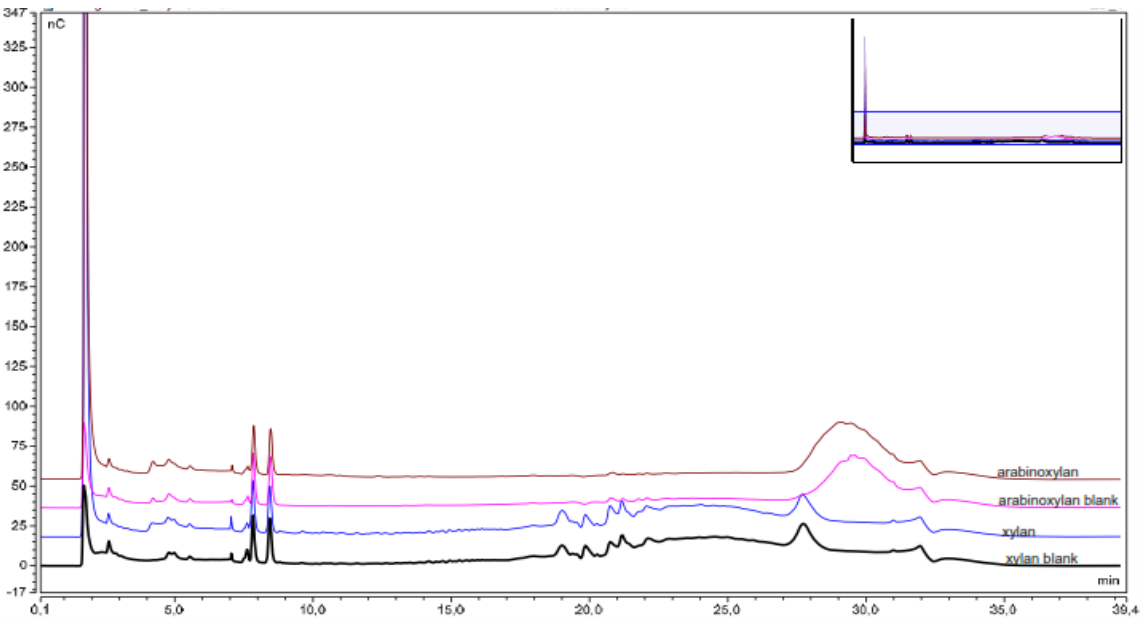
15,0
20,0
25,0
30,0
35,0
39,4


nC
347
325
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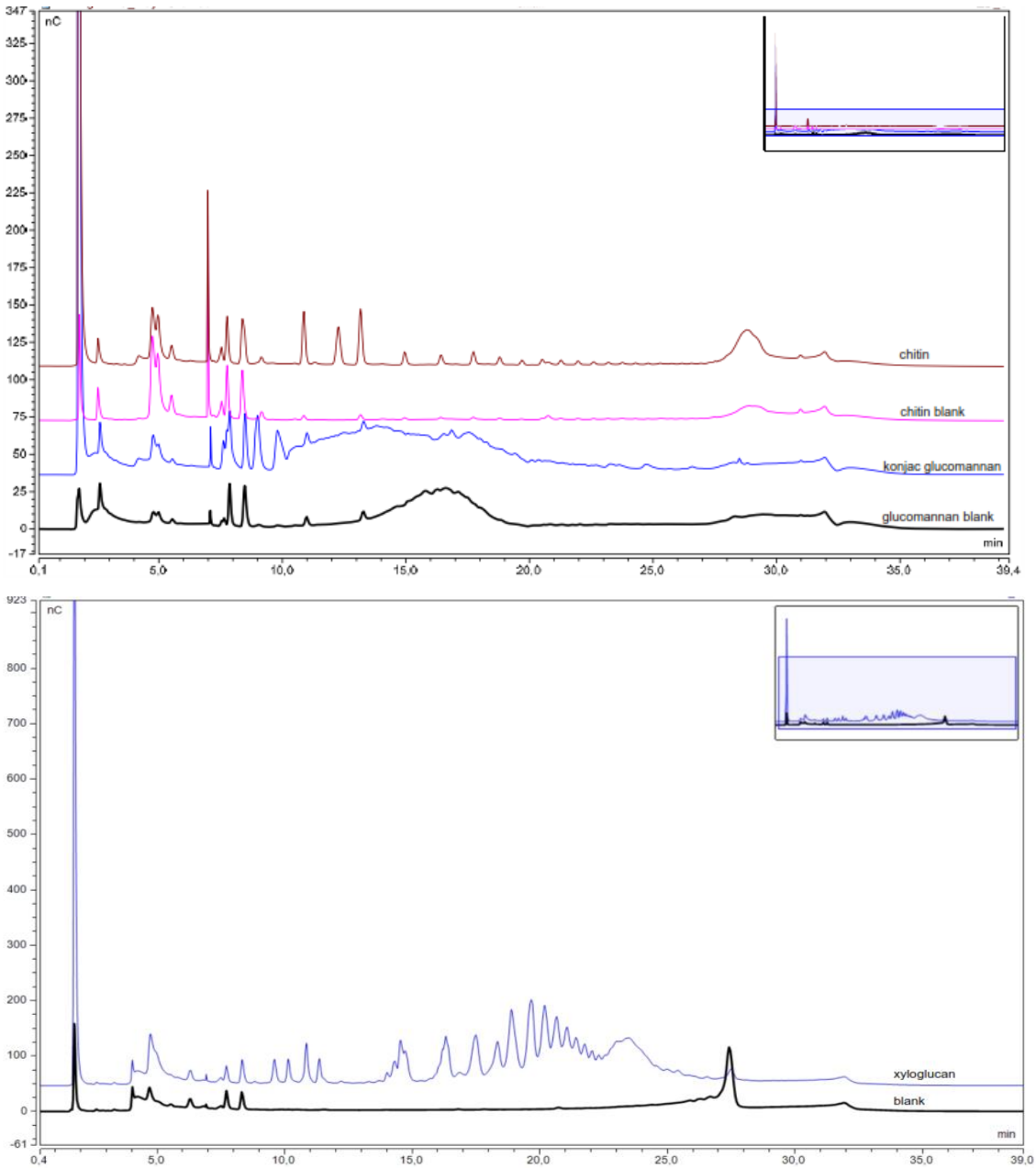
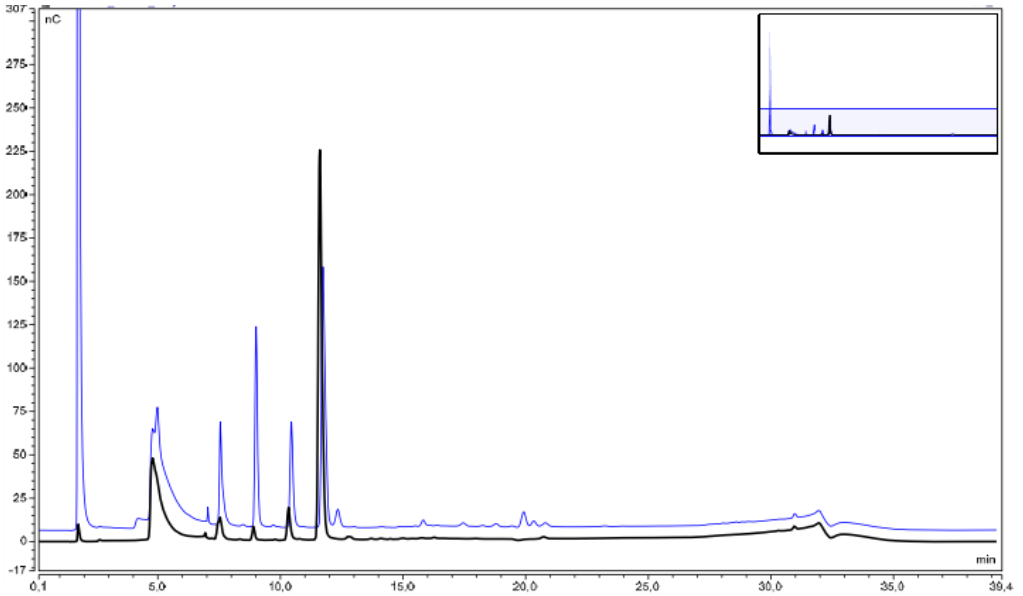


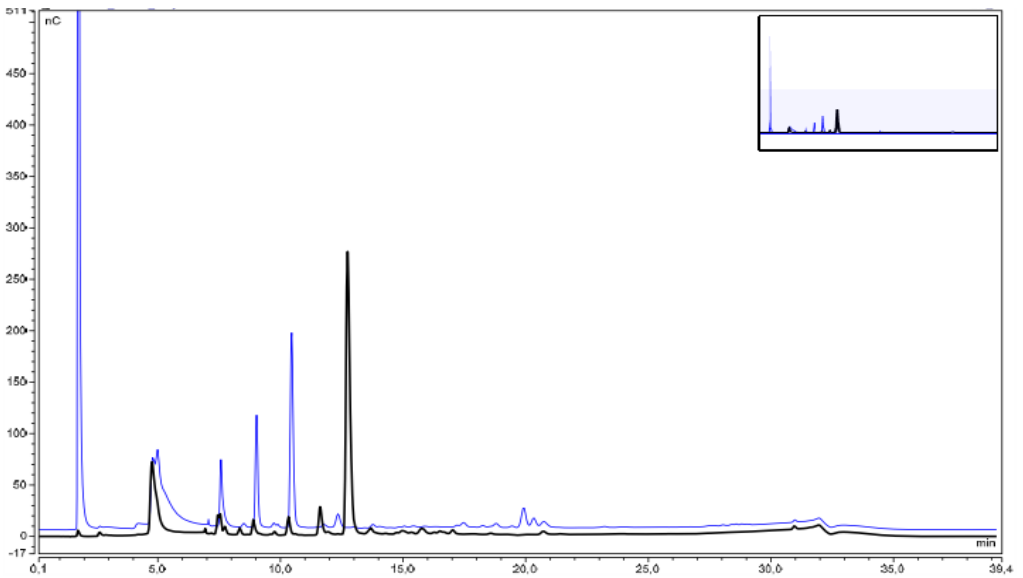
Figure 28. HPAEC analysis of *Tt*LPMO111088 reactions with different polysaccharides at a final concentration of 0.5 % (w/v).

According to Figure 28, *Tt*LPMO111088 is capable of oxidizing cellulosic compounds such as PASC and Avicel, but is not active on polysaccharides such as xylan, arabinoxylan or glucomannan. Furthermore, it exhibits the ability of oxidizing xyloglucan. It is therefore concluded that the enzyme recognizes and binds only β-1,4-linked glucose units, generating a typical C4-oxidizing pattern in HPAEC chromatograms, but not mannose or xylose. The presence of xylose decorations in the case of xyloglucan does not seem to inhibit the LPMO's ability to bind to the cellulosic chain, as well. The same substrate specificity has been exhibited by the enzymes *Nc*LPMO9C and *Pa*LPMO9H, which present the highest homology with *Tt*LPMO111088 (Table 13) [175,176]. The chromatogram of PASC reaction reveals a small amount of peaks in the area of 27-29 min, corresponding to possible double oxidized products. This phenomenon of minor cleavage of PASC using both oxidizing mechanisms has been referred previously also for *Ls*AA9, another C4-oxidizing, LPMO [177] and according to our observations cannot justify a broad C1-activity for *Tt*LPMO111088.

In the case of starch and chitin, the chromatograms reveal many peaks that could correspond to oxidized oligosaccharides, but, in contrast to products from reactions with the polysaccharides of β-1,4-cellulose, these are not detected in the specific area between 19-25 min, where C4- oxidized products are spotted. It is therefore arguable whether oxidation of these substrates has been a result of the enzyme's action. Also the exhibited activity on chitin and starch is surprising, given the fact that the LPMOs belonging to the AA9 family are not considered capable of oxidizing these substrates. Such an activity would be a novelty for *Tt*LPMO111088 and further analysis of reactions needs to be carried out to ensure their creditability.



a)



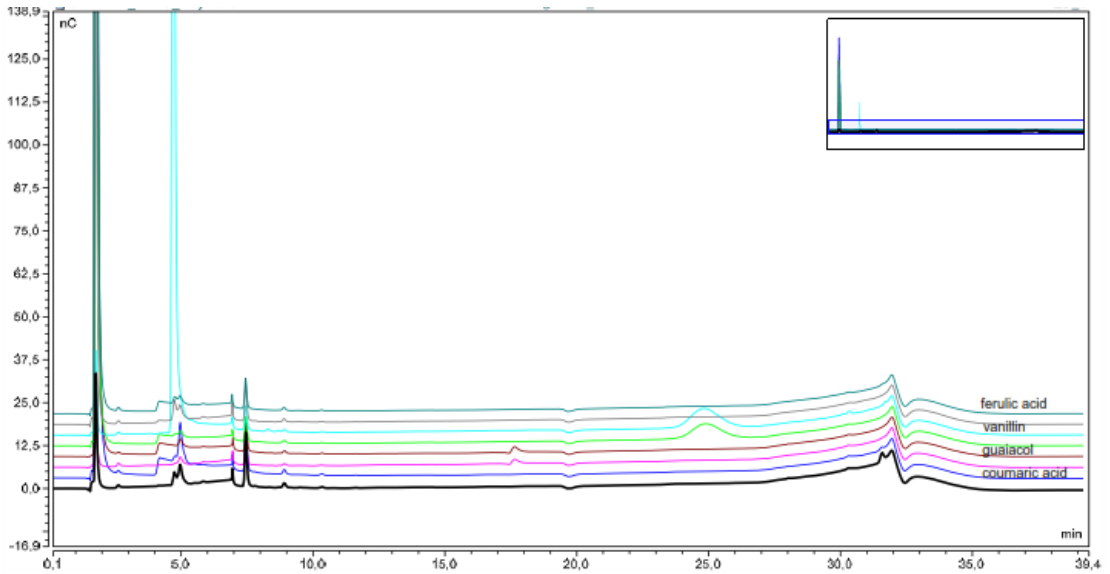
b)

Figure 29. HPAEC Chromatographs of *Tt*LPMO111088 reactions with 0.5 mg/mL a) cellopentaose, b) cellohexaose. Blank reactions are distinguished by black colour

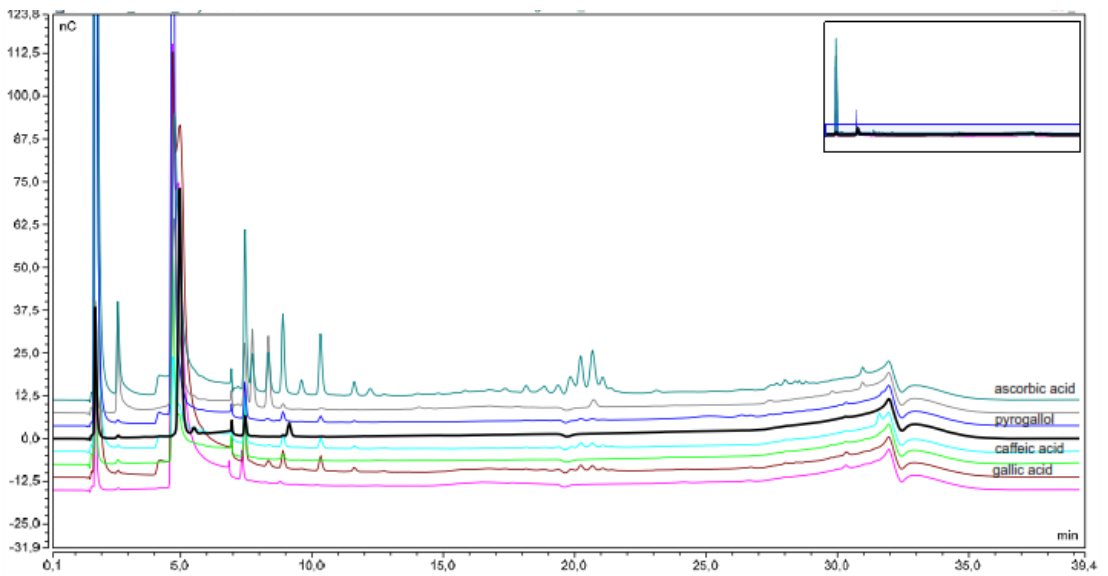
Based on the activity of LPMOs with a high degree of homology, the ability of the new enzyme to oxidize soluble oligosaccharides was also examined. Although not able to oxidize chito-, manno- and xylo-oligosaccharides-following the behavior on polysaccharide substrates-, *Tt*LPMO111088 could efficiently oxidize cello-oligosaccharides with a degree of polymerization (DP) 5 or 6 (Fig. 29). *Pa*LPMOH had a similar behavior but was also capable of oxidizing DP4 cello-oligosaccharides [173]. The reaction releases a neutral linear oligodextrin and a C4-oxidized product that is detected as a small peak around 20 min. According to the chromatograms the major non-oxidized products detected are cellotetraose and cellotriose from oxidation of cellohexaose and cellopentaose respectively. Thus it is speculated that the LPMO's active site binds cellohexaose both from -4 to +2 subsites and -3 to +3, while cellopentaose from -3 to +2. Similar behavior has been exhibited by other LPMO9s, active on soluble substrates, and proven through crystallographic evidence [178,179].

Effect of different electron donors in enzyme activity

Optimum conditions, in regard to the oxidation of PASC by the enzyme, were examined. The optimum temperature, pH and electron donor were recognized after HPAEC analysis.



a)



b)

Figure 30. HPAEC chromatograms of TtLPMO111008 reactions with PASC as substrate, with the addition of different external compounds as electron donors and the corresponding blank reactions below each.

As already mentioned, LPMOs require an external electron donor to provide a second electron for conducting the oxidation reaction. Apart from ascorbic acid that served as the electron donor in all other oxidation reactions of the enzyme, phenolic compounds with one, two or three substituted hydroxyl groups were examined for their ability to offer the electron, necessary for the completion of the polysaccharide oxidation. The efficiency of each molecule was estimated based on the amount of oxidized products released by the enzyme. According to Figure 30, phenolic compounds with two or three substituted hydroxyl-groups in the phenolic ring effectively provide an electron for LPMO activation. On the other hand, mono-substituted donors, such as guaiacol and vanillin, resulted in no visible oxidation of the substrate. This is not a novel observation. In general, monophenols tend to be less effective as reducing agents than phenolic compounds with more hydroxyl substitutions [180]. An efficiency index for LPMOs' external electron donors can be the reduction potential of the substance, measured by cyclic voltammetry. It is a general rule, which however exhibits some exceptions, that the lower the potential of a donor, the higher its effectiveness in an LPMO reaction. Owing, however, to the small exceptions observed, it is critical to define the best reducing agents for each enzyme.

Although not a phenolic compound, the ascorbic acid was the best candidate, based on the total area of the peaks corresponding to oxidized products in the chromatogram (Fig. 30). Ascorbic acid is a widely used external electron donor that usually yields good results [177]. However, it has recently been suggested that ascorbic acid can indirectly enhance the LPMO's reaction rate by reducing free Cu(II), present in the reaction mixture. The free copper reduction leads to formation of H_2O_2, thus speeding up the total reaction [181]. In order to avoid any biased conclusions, regarding the use of ascorbic acid, no copper saturation has been carried out in *Tt*LPMO111088, while the blank

reaction also contained the exact same substrate and ascorbic acid amount, to rule out any influence of copper contamination of the substrate. Therefore, it is safe to say that ascorbic acid is the preferred electron donor in the specific reaction.

Nevertheless, the clear primacy of an electron donor (Fig. 30) should not be taken for granted for all the reaction conditions of the specific LPMO. LPMOs' reducing agent dependency is a multifactor relation- yet not fully understood- that changes according to the pH, temperature and even substrate [177,182]. Therefore, the examination of the enzymatic reaction conditions is also needed.

Effect of reaction parameters T and pH in enzyme activity

In order to reach a conclusion regarding the optimum temperature and pH, *Tt*LPMO111088 reactions on PASC were incubated under different conditions and analysed using HPAEC. The total area corresponding to the peaks of the oxidized products was calculated and used to estimate the optimum incubation condition. According to Fig. 31, the temperature of 50 ^{o}C and pH 6 generated a higher total amount of oxidized products, answering for a higher LPMO activity.

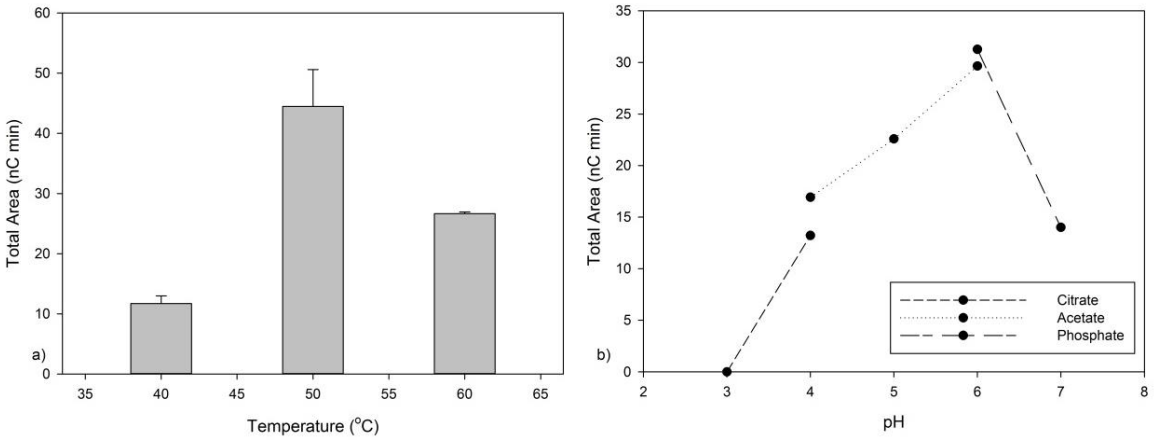


Figure 31. a) Temperature and b) pH optimum conditions of PASC oxidation by *Tt*LPMO111088, as a result of the total oxidized soluble products produced

Reaction conditions, such as pH and temperature, are not yet deeply studied in the case of LPMOs, in contrast to reducing agents. Yet, their effect is profound, not only on the protein itself, but also on the selected reducing agent. Frommhagen et al. have researched the behavior of two reducing agents, namely ascorbic acid and 3-methylcatechol, under different temperature and pH. They concluded that these conditions influence the stability and redox potential of the electron donors, reflecting the effect to the final LPMO catalytic performance [182]. More specifically, the reducing potential of the agents is decreasing when increasing the pH of the mixture, thus enhancing its ability to donate electrons to LPMO and leading to higher activity measurements. This behavior can justify the results in Fig. 31b, where pH increase is followed by an enhanced catalytic activity. The increase cannot go on forever, however, since very high pH values cause the ascorbic acid to become unstable. Moreover, a very high reducing efficiency of the electron donor can lead to sudden generation of reactive oxygen species (ROS) that quickly inactivate the enzyme. This might be the reason for the very low catalytic performance of *Tt*LPMO111088 at pH 7. Citrate buffer doesn't

yield good results in comparison to acetate. It is speculated that citrate, being a chelating factor, might interact with the copper ion, which is coordinated in the catalytic center, and therefore decrease the enzymatic performance.

Temperature on the other hand has a very low effect on the reducing agent's potential or stability [182]. It is therefore safe to assume that the amounts of oxidized products depicted in Figure 31a reflect the temperature's effect on the LPMO's activity, without much interference from the electron donor. According to the theoretical T_m of the enzyme, the temperature of 60 ^oC is constantly promoting thermal degradation of a part of the enzyme, thus rendering part of it unstable during the 24 h of the reaction. Therefore, the optimum temperature of 50 ^oC (Fig. 31a) might be a result of the denaturing conditions under higher heating rather than reflecting the best condition for enzymatic performance [183].

The reducing potentials of TtLPMO111088 (vs NHE.) at different temperatures (20-50 ^oC) were calculated between 236 ± 2.6 mV and 238 ± 2.2 mV in the Electrochemistry Laboratory of NTUA by Dimitrios Zouraris . The values corresponded to values around +250 mV (vs SHE.), which are characteristic for LPMOs [180]. It is also apparent that the reducing potential, relative to the reducing ability of the enzyme, is not greatly affected by the different temperatures.

Amino acid sequence comparison with known LPMOs

The sequence of the *Tt*LPMO111088 was compared with the sequences of previously characterized enzymes, using the tool of Clustal12.1 for multiple sequence alignment (Figure 32) [184]. The LPMOs selected were all C4- oxidizing enzymes, apart from one, being able to oxidize both C1- and C4- carbon; *Nc*LPMO9C, *Pa*LPMO9H, *Tt*LPMO9E

(also known as *Tt*LPMO9J) and *Ls*AA9. Their characteristics are summed up in Table 13.

Table 13. Characteristics of selected AA9 LPMOs

Enzyme	*Ls*AA9	*Tt*LPMO9E	*Nc*LPMO9C	*Tt*LPMO111088	*Pa*LPMO9H
Organism	*Lentinus similis*	*T. thermophila*	*Neurospora crassa*	*T. thermophila*	*Podospora anserina*
Expression system	*Aspergillus oryzae*	*Neurospora crassa*	*P. pastoris*	*P. pastoris*	*P. pastoris*
Regios/vity	C4	C4	C4	C4	C1/C4
CBM	No	No	Yes	Yes	Yes
Active on oligosac/des	Yes	Yes	Yes	Yes	Yes
Seq. Id % with Tt LPMO111088	40%	64%	67%	-	71%*
Reference	[185]	[186]	[174]	-	[175]

*The catalytic domains alone exhibit 78% sequence identity

All the examined enzymes were selected for being active on soluble cello-oligosaccharides, a characteristic shared by the newly discovered *Tt*LPMO111088. The sequences where examined for conserved residues that have been found to participate in substrate-enzyme interactions. According to the alignment, *Tt*LPMO111088 appears to have conserved the residue Tyr206, responsible for stacking of the substrate at subunit -3 by the formation of a strong CH-π bond (corresponding to Tyr203 of *Ls*AA9). The subunit nomenclature follows the rules applying for glycosyl hydrolases. Also residues of Asn28 and His66 present in *Ls*AA9, are conserved in *Tt*LPMO111088, possibly maintaining the ability to interact with the substrates in +2 subunit, through a network of hydrogen bonds and in +1 with the coordinating His1[177,185]. In contrast to the

positive subunits, the residues of *Ls*AA9, identified as responsible for interactions with negative subunits of the substrate, are not conserved in *Tt*LPMO111088, apart from the previously mentioned Tyr203 and Ser77. Surprisingly, these non-conserved residues are missing from all other four enzymes examined, highlighting the importance of previously mentioned conserved residues and indicating that other residues can as well interact efficiently with the rest of the substrate.

In the alignment Figure 32, the characteristic loops L2, L3,LS and LC of LPMO9s are indicated. Although these regions are supposed to vary significantly regarding their substrate-binding surfaces, only L2 and LC exhibit any substantial difference, in the case of these five enzymes. The loops are thought to affect further the morphology of the relatively flat surface, where the active site is located, thus interfering with the affinity of the protein for different substrates [174]. It might be well suspected that the aligned enzymes have a similar specificity, as it is also confirmed by the HPAEC analysis.

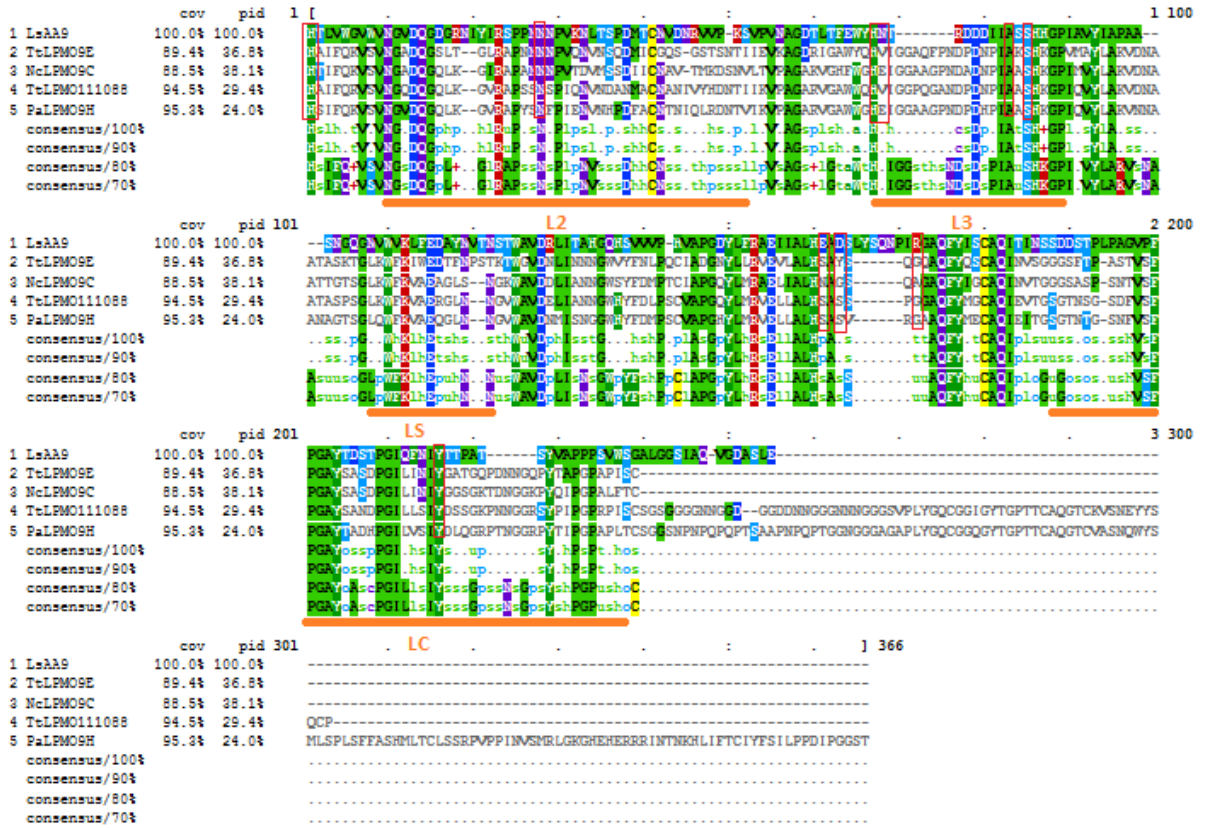


Figure 32. Sequence alignment of 5 LPMOs (together with the presently characterized one), L regions correspond to *Nc*LPMOAC, as they have been described by A. Borisova et al. With red are marked the residues participating in oligosaccharide binding, as they were p presented for the Cello6:*Ls*AA9 and Cello5:*Ls*AA9 crystal complex by K. Frandsen et al. [187] and T. Tandrup et al. [177], as well as those proven to shift for substate binding, according to G. Courtade et al. [176].

Application in an enzyme cocktail for the enhanced hydrolysis of pretreated biomass

In order for *Tt*LPMO111088 to have application in the bioconversion of lignocellulosic waste to omega-3, it should exhibit the ability to act synergistically with the enzymatic mixture used for hydrolysis of the biomasses, thus enhancing the final sugar recovery. To examine this possibility, the lignocellulosic samples No #6 (H_2O/THF (50/50%), O_2 16 bar, 175^{o}C, 120min) and #7 (H_2O/EtOH (50/50%), O_2 16 bar, 175^{o}C, 60min), which

exhibited higher performance as bioconversion substrates, were employed for synergistic hydrolysis with Cellic® CTec2 and *Tt*LPMO111088. Short hydrolysis time and dry matter loading that maintain enzyme stability and avoid product inhibition effects was selected, in order for the synergy results to represent initial rates [188]. The resulting glucose release after 24 h of hydrolysis is depicted in Figure 33.

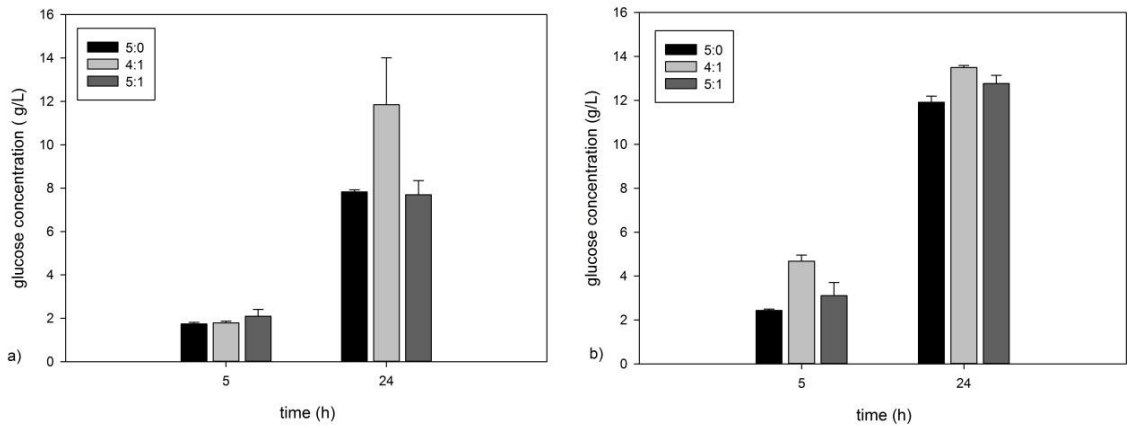


Figure 33. Hydrolysis performance of materials #6 (a) and #7 (b) with addition of a mixture of CellicCTec2:*Tt*LPMO111088 in different final protein loadings x:y. graphs depict the glucose concentration after 5 h and 24 h of hydrolysis.

In cases of reactions solely with *Tt*LPMO111088, no glucose release was observed. The degree of synergy (DS) was calculated as follows:

$$DS = \frac{Activity\ of\ mixture}{Activity\ of\ Cellic + Activity\ of\ LPMO} \qquad (6)$$

where the *Activity of Cellic* corresponded to the activity measured in the hydrolysis of 5:0 enzyme loading. Based on the measurement of the released glucose, the DS at 24 h for a 5:1 complementation of *Tt*LPMO111088 was 1.0 and 1.1 for the materials #6 and #7 accordingly, exhibiting a small synergy effect for the second substrates. In case of the substitution of the total protein loading with a 4:1 Cellic:LPMO analogy, DS was

substantially high-1.5- for material #6 but equal to 1.1 for the substrate #7, exhibiting a strong synergy for material #6, releasing 50% more glucose. An explanation regarding LPMO and cellulase mixture synergy takes into consideration the gradual changes occurring in the surface of the lignocellulosic material. It is supposed that the LPMO activity leads to the degradation of small cellulosic fibrils that are situated on the surface and are constantly revealed due to gradual removal of the amorphous region by EG and CBH II [189].

In order to function properly, the LPMOs require an external electron donor. In the above reactions, it was supposed that the enzymes would obtain the electrons needed from substances already present in the pretreated substrate, like residual lignin. Indeed the appearance of synergy- also higher for the material with the higher lignin content- is indicative of electron provision by lignocellulosic components. In the next set of experiments, the hydrolysis reactions were supplemented with 1 mM ascorbic acid, as reducing agent, for further enhancement of the oxidation efficiency. The results are depicted in Figure 34.

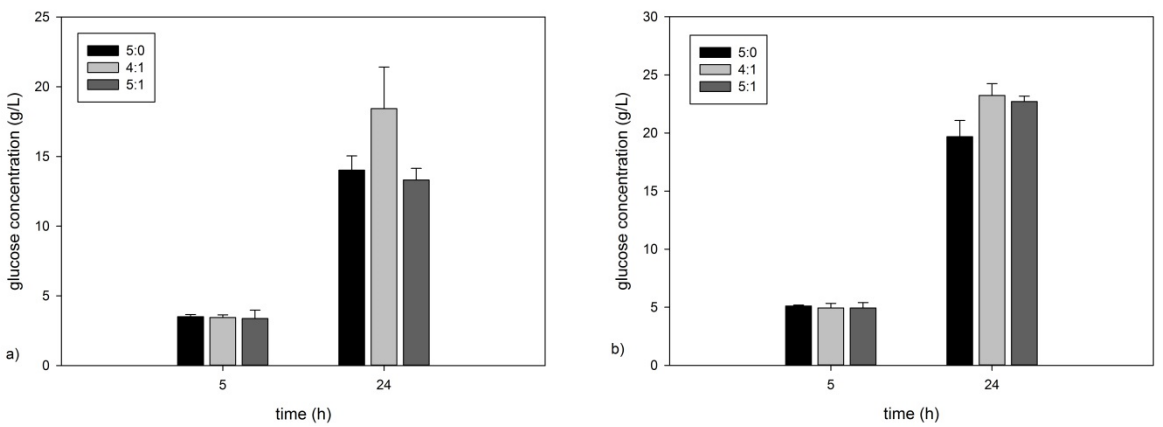


Figure 34. Hydrolysis performance of materials #6 (a) and #7 (b) with addition of a mixture of CellicCTec2:TtLPMO111088 in different final protein loadings x:y and 1 mM ascorbic acid. Graphs depict the glucose concentration after 5 h and 24 h of hydrolysis.

In the case of ascorbic acid addition, all hydrolysis reactions were more efficient, leading to the release of higher glucose concentration. The reason for that is that the commercial mixture of CellicCTec2 already includes enzymes of the LPMO category, whose activity is further enhanced with the external electron donor. Still, the synergistical effect of *Tt*LPMO111088 is more prominent. The DS with ascorbic acid for the material #7 was 1.2 both for 4:1 and 5:1 ratios, while that for substrate #6 had fallen to 1.3 for 4:1 ratio. The ratio of 5:1 exhibited no synergy with and without ascorbic.

As it has been already concluded in other studies, synergy effects are substrate-dependent [188]. It is interesting to observe that the material #6 is more prone to synergy of *Tt*LPMO111088 and CellicCTec 2, but only for the ratio of 4:1. What is more, ascorbic acid seems to affect negatively the DS of the mixture. It must be noted that according to Table 10, material #6 is rich in lignin, even after organosolv pretreatment. Supposing that lignin acts as an efficient electron donor for *Tt*LPMO111088, the DS of 1.5 is deciphered. It can be speculated that further addition of ascorbic acid is unnecessary, leading to sudden generation of reactive oxygen species (ROS) that possibly inactivate part of the enzyme, as mentioned previously.

On the contrary, in the case of biomass #7, the synergy was lower, and also increased after ascorbic addition. The lower DS may be a result of the pretreatment method of beechwood material #7 and its effect on the surface of the final material. Karnaouri et al. have discovered that organosolv pretreatment with a ratio of EtOH:H_2O of 50:50 (v/v) leads to the formation of lignin droplets, released from the inner biomass, on the outer surface of the solid fraction, which are deposited there after cooling [190]. Since it is expected that LPMOs act mostly on surface exposed crystalline regions of the cellulosic substrate [189], it may be speculated that lignin droplets inhibit access of the enzymes to

the surface regions, thus limiting the activity of *Tt*LPMO111088. The addition of ascorbic seems to enhance the DS, but only in small amounts.

Although the ratio of 5:1 translates to a higher final protein loading, it does not seem to favor the hydrolysis more than 4:1, while at the same time hydrolysis with 6 mg Cellic protein/ g substrate resulted in higher glucose release than all other reactions after 24 h (data not shown). Previous research has shown the possibility of competition between cellulases and LPMOs for access in crystalline regions of mixed amorphous-crystalline substrates, and more specifically CBH I that attack also crystalline cellulose [189]. Since CellicCTec 2 of Novozymes contains a mixture of CBH I and II and EG, it may be possible that a loading of 5:1 mg/g substrate is enough to cause enzyme traffic between TtLPMO111088 and CBH I unities, a development that doesn't happen in case of solely CellicCTec2 addition, even in higher loadings. If such a hypothesis is true, then we would expect the DS to be higher in case that the CellicCTec 2 didn't include any LPMOs.

Conclusions

Summarizing the above results, one can reach conclusions regarding the integration of waste-derived substances-specifically dark fermentation effluents and lignocellulosic hydrolysates- in biorefinery processes for the production of omega-3 fatty acids by the heterotrophic microalga *Crypthecodinium cohnii*. Dark fermentation effluents from biowaste are rich in volatile fatty acids (VFA) that may serve as carbon source for this oleaginous strain, which is capable of accumulating high amounts of the valuable omega-3 docosahexaenoic acid (DHA). Indeed data from batch fermentations of *C. cohnii* demonstrated a successful assimilation of the pure acids acetate, butyrate and propionate for concentrations 10 g/L and higher, thus ascertaining its value as a bioconversion tool. The ability to incorporate solely propionic and butyric acid, reported for the first time in this study, highlighted the potential of the selected strain for the utilization of the liquid fraction of an industrial dark fermentation effluent. Prolonged fed-batch cultures, with a feed of volatile acids, under pH-auxostat regulation were more successful in biomass and DHA accumulation and found to be operated under nitrogen starvation conditions that are known to induce lipid production. In relevance to the effect of each different VFA, results indicate that, although acetate induces a much higher biomass yield, propionate appears to provoke higher lipid accumulation. This observation can be useful for the proper design of the dark fermentation process, in order to enhance the effluent's biorefinery potential by regulating its composition.

More importantly than the fermentation of pure VFA, the work of this thesis included the use of real effluents from an operating waste treatment plant as a feed, thus demonstrating the utilization of them as main carbon source for omega-3 production. The strain was capable of consuming the total VFA of the liquid fraction and accumulating intracellular lipids and DHA. Therefore, longer-chain VFA present in the waste-derived liquid, such as valerate and caproate that haven't been examined

separately, are also assimilable by the cells. It must be noted that utilization of other VFA begins after acetate exhaustion, due to catabolite repression. The low acid concentration of the feed constitutes the main difference between pure, commercially available and waste-derived VFA that can hamper the application of the dark fermentation's liquid fraction. To this direction, efforts to increase the VFA final concentration of the effluent were proven successful in enhancing the total cultivation duration, as well as the final biomass production. That means that in order for such a process to be industrially exploitable, it is crucial to augment the acidic capacity of the feed, by concentrating the effluent. To the best of our knowledge, this is the first report of *C. cohnii* growing on an anaerobically treated biowaste feedstock and utilizing environmental pollutants for the production of high added-value products that can be incorporated into the food industry. In order to ensure a total utilization of the algal biomass, it has also been proposed that biomass residue, after the extraction of lipids, can be again added as a feedstock for dark fermentation process, therefore promoting a circular economy.

Although, the production of a high added-value metabolite, such as omega-3, justifies a higher production cost, it still needs to be limited by adopting proper practices in order to design a sustainable process. For that reason, the fermentation conditions that ensure maximum DHA recovery were examined. Although batch cultures performance indicated specific initial values, scale-up experiments following a fed-batch feeding protocol exhibited a different behavior. It seems that the constantly changing parameters affecting biomass growth and lipid production in the bioreactor need further clarification. Since the results clearly demonstrated the important influence of temperature and nitrogen to the final DHA productivity, these factors, together with the aeration of the system, were thoroughly examined. The best temperature for high DHA

recovery under each cultivation mode was found to be 23 ^{o}C, yeast extract was the optimum nitrogen source, while response surface methodology indicated that the aeration rate is more significant than the initial C/N ratio to final DHA control.

Regarding the utilization of agricultural waste, in the present thesis, biomass-derived sugars were used as a proof of concept to produce DHA from *C. cohnii*. Results demonstrated that microalgae can efficiently utilize both glucose and xylose from organosolv pretreated beechwood and accumulate up to 60 wt. % TFA with 26.6 wt. % DHA, thus offering the first example of DHA production from lignocellulosic biomass by heterotrophic microalgae, on behalf of the second generation biorefinery concept. Towards lignocellulosic utilization, it is of importance the favorable effect of lignin-possibly due to antioxidant properties-in microalga biomass production. Moving one step further, examination of a fed-batch strategy with a hydrolysate feed revealed the accumulation of inhibitors present in the feed, which slow down growth. The promising bioconversion yields of hydrolysate-derived sugars, coupled with the inability of the microalga cells to produce cellulolytic enzymes, indicated the use of more efficient hydrolytic enzymatic cocktails as the safest way to enhance the DHA production of the strain, consuming hydrolysates. For that reason, the thesis included the first attempts of characterization of a newly discovered lytic polysaccharide monooxygenase. The enzyme, which exhibited C4-regioselectivity, was able to oxidatively cleave the β-1,4-bond of cello-poly and oligo-saccharides. Most importantly, it exhibited significant synergy with the commercially available enzymatic mixture CellicCTec 2, thus highlighting its potential contribution in the production of a sugar-rich hydrolysate from lignocellulose, which, after proper dilution, can act as a microalga feed, with a lower inhibitor content.

To sum up, this thesis highlights the potential of *C. cohnii* as means of bioconversion of biowaste and agricultural residues, providing the reader with thorough experimental proofs. Most importantly however, it offers reliable solutions for the efficient and sustainable application of such processes in the industry, which include fermentation conditions regulation and concentration of the carbon content of the feed with physical or enzymatical methods.

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Appendix 1

Recipe of ATCC 460 Medium, used for *C. cohnii* cell stock maintenance

ATCC Medium: 460 A_2E_6 Medium

Base Medium

NaCl…….……..23.48 g
$MgCl_2$ x $6H_2O$…….10.63 g
Na_2SO_4……3.92 g
$CaCl_2$ (anhydrous)…….……1.11 g
KCl…….……0.66 g
$NaHCO_3$……...0.19 g
KBr……..……...0.1 g
H_3BO_3……..…….0.03 g
$SrCl_2$ x $6H_2O$……..……..0.04 g
Metal Mixture (see below)…….……..3.0 ml
$FeCl_3$ x $6H_2O$…..…….0.01 g
Na_2 Glycerophosphate……0.15 g
$(NH_4)_2SO_4$……...…….0.05 g
Tris Buffer……...3.0 g
Vitamin Solution (see below)…….…….1.0 ml
K_2HPO_4…….0.01 g
Glucose……..……3.0 g
Glutamic Acid……1.5 g
DI Water…….1000 ml

Adjust pH to 6.4-6.6. Dispense into appropriate vessel and autoclave at 121ºC.
*Dispense 5ml for 16 x 125 mm test tube.

Appendix 1

Metal Mixture

Na_2EDTA……1.0 g
$FeCl_3$ x $6H_2O$…….0.05 g
H_3BO_3……1.0 g
$MnCl_2$ x $4H_2O$……...0.15 g
$ZnCl_2$……..0.01g
$CoCl_2$ x $6H_2O$……0.005 g DI Water…….100 ml

Vitamin Solution

Biotin……..……..0.003 g Thiamine…….1.0 g
DI Water……..…….1000 ml

Appendix 2

Report of statistical analysis of the model describing the DHA production response (no transformation)

Diagnostics Case Statistics									
Standard Order	**Actual Value**	**Predicted Value**	**Residual**	**Leverage**	**Internally Studentized Residual**	**Externally Studentized Residual**	**Influence of Fitted Value DFFITS**	**Cook's Distance**	**Run Order**
1	57.00	61.50	-4.50	0.309	-0.529	-0.52	-0.347	0.016	3
2	57.00	61.50	-4.50	0.309	-0.529	-0.52	-0.347	0.016	1
3	71.00	61.50	9.50	0.309	1.117	1.124	0.751	0.07	16
4	47.00	46.50	0.50	0.309	0.059	0.057	0.038	0	7
5	49.00	46.50	2.50	0.309	0.294	0.287	0.192	0.005	6
6	44.00	46.50	-2.50	0.309	-0.294	-0.287	-0.192	0.005	13
7	105.00	100.83	4.17	0.309	0.49	0.481	0.321	0.013	21
8	92.00	100.83	-8.83	0.309	-1.038	-1.04	-0.695	0.06	9
9	106.00	100.83	5.17	0.309	0.607	0.598	0.4	0.021	19
10	44.00	36.17	7.83	0.309	0.921	0.917	0.613	0.047	26
11	48.00	36.17	11.83	0.309	1.391	1.425	0.952	0.108	15
12	17.00	36.17	-19.17	0.309	-2.253	-2.524	-1.688	0.284	17
13	61.00	71.24	-10.24	0.284	-1.182	-1.194	-0.753	0.069	2
14	80.00	71.24	8.76	0.284	1.012	1.013	0.639	0.051	8
15	72.00	71.24	0.76	0.284	0.088	0.086	0.054	0	27
16	79.00	66.24	12.76	0.284	1.474	1.519	0.958	0.108	5
17	67.00	66.24	0.76	0.284	0.088	0.086	0.054	0	11
18	52.00	66.24	-14.24	0.284	-1.645	-1.719	-1.084	0.134	14
19	77.00	80.90	-3.90	0.284	-0.451	-0.442	-0.279	0.01	29
20	87.00	80.90	6.10	0.284	0.704	0.695	0.439	0.025	18
21	78.00	80.90	-2.90	0.284	-0.335	-0.328	-0.207	0.006	4
22	13.00	19.57	-6.57	0.284	-0.759	-0.751	-0.474	0.029	20
23	19.00	19.57	-0.57	0.284	-0.066	-0.064	-0.041	0	25
24	26.00	19.57	6.43	0.284	0.743	0.734	0.463	0.027	23
25	49.00	55.23	-6.23	0.176	-0.671	-0.662	-0.306	0.012	22
26	48.00	55.23	-7.23	0.176	-0.779	-0.771	-0.356	0.016	10
27	76.00	55.23	20.77	0.176	2.235	2.499	1.154	0.133	12
28	60.00	55.23	4.77	0.176	0.513	0.504	0.233	0.007	24
29	44.00	55.23	-11.23	0.176	-1.209	-1.223	-0.565	0.039	28

Appendix 3

Codon optimized sequence of the gene encoding TtLPMO111088 for heterologous production in *P. pastoris*

```
AsuII
\
TTCGAAATGAAACCCTTTTCGTTGGTTGCTCTAGCAACTGCCGTTTCTGGTCATGCCATA
10        20        30        40        50        60
----:----|----:----|----:----|----:----|----:----|----:----|
AAGCTTTACTTTGGGAAAAGCAACCAACGAGATCGTTGACGGCAAAGACCAGTACGGTAT
/
AsuII

F  E  M  K  P  F  S  L  V  A  L  A  T  A  V  S  G  H  A  I
----:----|----:----|----:----|----:----|----:----|----:----|


TTTCAGCGAGTATCCGTTAATGGACAAGATCAGGGACAATTGAAGGGTGTAAGAGCACCT
70        80        90        100       110       120
----:----|----:----|----:----|----:----|----:----|----:----|
AAAGTCGCTCATAGGCAATTACCTGTTCTAGTCCCTGTTAACTTCCCACATTCTCGTGGA


F  Q  R  V  S  V  N  G  Q  D  Q  G  Q  L  K  G  V  R  A  P
----:----|----:----|----:----|----:----|----:----|----:----|


TCATCAAACAGCCCAATCCAGAACGTGAATGACGCTAACATGGCTTGTAACGCCAATATC
130       140       150       160       170       180
----:----|----:----|----:----|----:----|----:----|----:----|
AGTAGTTTGTCGGGTTAGGTCTTGCACTTACTGCGATTGTACCGAACATTGCGGTTATAG


S  S  N  S  P  I  Q  N  V  N  D  A  N  M  A  C  N  A  N  I
----:----|----:----|----:----|----:----|----:----|----:----|


GTGTACCATGACAACACGATCATCAAAGTGCCTGCTGGAGCTAGAGTTGGTGCTTGGTGG
190       200       210       220       230       240
----:----|----:----|----:----|----:----|----:----|----:----|
CACATGGTACTGTTGTGCTAGTAGTTTCACGGACGACCTCGATCTCAACCACGAACCACC


V  Y  H  D  N  T  I  I  K  V  P  A  G  A  R  V  G  A  W  W
----:----|----:----|----:----|----:----|----:----|----:----|


CAACACGTTATTGGTGGACCACAAGGAGCCAATGATCCCGATAACCCAATTGCTGCAAGT
250       260       270       280       290       300
----:----|----:----|----:----|----:----|----:----|----:----|
GTTGTGCAATAACCACCTGGTGTTCCTCGGTTACTAGGGCTATTGGGTTAACGACGTTCA


Q  H  V  I  G  G  P  Q  G  A  N  D  P  D  N  P  I  A  A  S
----:----|----:----|----:----|----:----|----:----|----:----|
```

Appendix 3

```
CACAAAGGCCCGATTCAGGTCTATTTGGCTAAGGTCGATAATGCTGCAACTGCAAGTCCA
310       320       330       340       350       360
----:----|----:----|----:----|----:----|----:----|----:----|
GTGTTTCCGGGCTAAGTCCAGATAAACCGATTCCAGCTATTACGACGTTGACGTTCAGGT


H  K  G  P  I  Q  V  Y  L  A  K  V  D  N  A  A  T  A  S  P
----:----|----:----|----:----|----:----|----:----|----:----|



TCTGGTTTGAAGTGGTTCAAAGTAGCTGAACGTGGGTTGAATAACGGAGTTTGGGCAGTT
370       380       390       400       410       420
----:----|----:----|----:----|----:----|----:----|----:----|
AGACCAAACTTCACCAAGTTTCATCGACTTGCACCCAACTTATTGCCTCAAACCCGTCAA


S  G  L  K  W  F  K  V  A  E  R  G  L  N  N  G  V  W  A  V
----:----|----:----|----:----|----:----|----:----|----:----|


GACGAACTGATTGCCAACAACGGTTGGCACTATTTCGATCTTCCGTCATGCGTCGCACCA
430       440       450       460       470       480
----:----|----:----|----:----|----:----|----:----|----:----|
CTGCTTGACTAACGGTTGTTGCCAACCGTGATAAAGCTAGAAGGCAGTACGCAGCGTGGT


D  E  L  I  A  N  N  G  W  H  Y  F  D  L  P  S  C  V  A  P
----:----|----:----|----:----|----:----|----:----|----:----|


GGACAGTATCTTATGAGGGTGGAATTACTTGCTCTACATTCCGCTTCTTCACCAGGAGGT
490       500       510       520       530       540
----:----|----:----|----:----|----:----|----:----|----:----|
CCTGTCATAGAATACTCCCACCTTAATGAACGAGATGTAAGGCGAAGAAGTGGTCCTCCA


G  Q  Y  L  M  R  V  E  L  L  A  L  H  S  A  S  S  P  G  G
----:----|----:----|----:----|----:----|----:----|----:----|


GCTCAATTTTACATGGGGTGTGCTCAAATAGAGGTTACTGGAAGTGGCACCAATAGCGGT
550       560       570       580       590       600
----:----|----:----|----:----|----:----|----:----|----:----|
CGAGTTAAAATGTACCCCACACGAGTTTATCTCCAATGACCTTCACCGTGGTTATCGCCA


A  Q  F  Y  M  G  C  A  Q  I  E  V  T  G  S  G  T  N  S  G
----:----|----:----|----:----|----:----|----:----|----:----|


TCTGATTTCGTCTCTTTTCCTGGGGCCTATAGTGCCAATGATCCAGGCATTCTGTTGAGC
610       620       630       640       650       660
----:----|----:----|----:----|----:----|----:----|----:----|
AGACTAAAGCAGAGAAAAGGACCCCGGATATCACGGTTACTAGGTCCGTAAGACAACTCG


S  D  F  V  S  F  P  G  A  Y  S  A  N  D  P  G  I  L  L  S
----:----|----:----|----:----|----:----|----:----|----:----|
```

Appendix 3

```
ATATACGACTCGTCAGGTAAGCCTAACAATGGTGGAAGATCCTATCCCATTCCTGGACCA
670       680       690       700       710       720
----:----|----:----|----:----|----:----|----:----|----:----|
TATATGCTGAGCAGTCCATTCGGATTGTTACCACCTTCTAGGATAGGGTAAGGACCTGGT


I  Y  D  S  S  G  K  P  N  N  G  G  R  S  Y  P  I  P  G  P
----:----|----:----|----:----|----:----|----:----|----:----|


AGACCTATCTCTTGTTCCGGGTCTGGAGGAGGTGGCAATAATGGTGGAGATGGTGGTGAC
             730       740       750       760       770       780
----:----|----:----|----:----|----:----|----:----|----:----|----:----|
TCTGGATAGAGAACAAGGCCCAGACCTCCTCCACCGTTATTACCACCTCTACCACCACTG


R  P  I  S  C  S  G  S  G  G  G  G  N  N  G  G  D  G  G  D
----:----|----:----|----:----|----:----|----:----|----:----|----:----|


GACAACAATGGTGGTGGCAATAACAACGGTGGTGGTTCCGTCCCTTTATACGGCCAATGT
             790       800       810       820       830       840
----:----|----:----|----:----|----:----|----:----|----:----|----:----|
CTGTTGTTACCACCACCGTTATTGTTGCCACCACCAAGGCAGGGAAATATGCCGGTTACA


D  N  N  G  G  G  N  N  N  G  G  G  S  V  P  L  Y  G  Q  C
----:----|----:----|----:----|----:----|----:----|----:----|----:----|


GGAGGTATTGGGTATACAGGACCAACTACCTGTGCTCAAGGTACATGCAAGGTTTCTAAC
             850       860       870       880       890       900
----:----|----:----|----:----|----:----|----:----|----:----|----:----|
CCTCCATAACCCATATGTCCTGGTTGATGGACACGAGTTCCATGTACGTTCCAAAGATTG


G  G  I  G  Y  T  G  P  T  T  C  A  Q  G  T  C  K  V  S  N
----:----|----:----|----:----|----:----|----:----|----:----|----:----|

                           XbaI
                           \
GAGTACTACTCCCAATGCTTACCTTAATCTAGA
             910       920       930
----:----|----:----|----:----|---
CTCATGATGAGGGTTACGAATGGAATTAGATCT
                              /
                           XbaI

E  Y  Y  S  Q  C  L  P  *  S  R
----:----|----:----|----:----|---
```