Master Thesis

Kinetic study of biomass growth and lipid synthesis of the microalga *Chlorella vulgaris* under heterotrophic conditions and energy valorization of the produced biomass.

by

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Abstract

The present study focuses on the kinetic study of biomass growth and lipid synthesis of the microalga *C. vulgaris* under heterotrophic conditions in addition to the energy valorization of the produced biomass. The main reason of this strain attracting so much attention is that it constitutes a versatile microorganism. It has the ability to grow fast, in various kinds of media and wastewaters, on extreme conditions such as extreme pH values and temperatures and tolerate the presence of toxic compounds. Moreover, it accumulates intracellular lipids resulting in high lipid content, the composition of which is suitable for biodiesel production.

The goal of the first set of experiments was to investigate the pH range that can support the growth of *C. vulgaris*, and, more specifically, to identify the optimal pH for the microalga’s growth, under heterotrophic conditions. Furthermore, the effect of pH on accumulation of intracellular lipids was studied. A wide range of pH values was tested using buffer solutions. The optimal pH for biomass growth and lipid accumulation under sulfur starvation was found to be 7.5, resulting in the maximum specific growth rate of 0.541 days$^{-1}$ and the maximum total lipid content of 53.43% g g$^{-1}$DW.

The aim of the second set of experiments was to determine the effect of starvation from different nutrients (S, P and N) on biomass growth and lipid accumulation of *C. vulgaris*. The potential differences in the effect of nutrient starvation could affect the selection of the most appropriate wastewater mixture for microalgal cultivation, as in many cases, in a realistic application of wastewater treatment using microalgae the mixture of various wastewaters is used as a substrate. The nutrient starvation that had the most significant effect on lipid accumulation was that of sulfur.

Moreover, the use of Volatile Fatty Acids as potential carbon sources for *C. vulgaris* was investigated. VFAs are metabolic products, intermediate or final, of many processes, such as anaerobic digestion, dark fermentation and acidogenesis. The goal was to identify the VFAs that can be used as carbon sources by *C. vulgaris*, individually or in a combination with glucose, grown without the presence of light. The VFAs tested were acetic, propionic, butyric and isobutyric acid. *C. vulgaris* failed to grow on propionic, butyric and isobutyric acid as sole carbon sources. However it was able to grow on acetic acid, resulting in a maximum specific growth rate of 0.429 days$^{-1}$. Moreover, the combination of the VFAs mentioned above in addition to glucose was used as carbon source, simulating the case in which the produced effluent streams of these processes contain VFAs in addition to other organic compounds. The microalga was again unable
to assimilate propionic, butyric and isobutyric acid. The maximum specific growth rate obtained from the assimilation of glucose and acetic acid was 0.534 days\(^{-1}\).

In the following part of the present study, phenolic compounds were tested as potential carbon sources for the microalga. Before carrying out the experiments with different phenols used individually as sole carbon source, an acclimation stage took place. The phenolic compounds tested as potential carbon sources for \textit{C. vulgaris} were vanillic, ferulic, syringic, gallic, p-hydroxybenzoic acid and catechol, in a concentration of 0.5 g L\(^{-1}\). Catechol inhibited the growth of the microalga from the second stage of the acclimation process, in a concentration of 100 mg L\(^{-1}\), verifying the highly toxic effect of the specific phenolic compound. \textit{C. vulgaris} failed to grow on vanillic, ferulic and p-hydroxybenzoic acid as sole carbon sources in a cultivation period of 15 days. However, gallic and syringic acid were degraded by \textit{C. vulgaris} in the respective experiments, resulting in low \(\mu_{\text{max}}\) (0.063 and 0.103 days\(^{-1}\) respectively).

The biotreatment of Olive Mill Wastewater using \textit{C. vulgaris} was subsequently investigated. OMW is a wastewater with complex composition with the most distinctive features being the high concentration of phenolic compounds, and the low nitrogen content, all present in the form of organic nitrogen, which make OMW a non-easily degradable substrate for microalgae. The microalga was unable to grow on OMW, even after the regulation of pH at the optimal value and the addition of a sulfur and nitrogen source. However, the growth of the microalga in a medium containing 10% v v\(^{-1}\) OMW supplemented with glucose and BG-11 broth showed that it can tolerate the phenols present (in a concentration of approximately 0.5 g L\(^{-1}\)), and that the inhibiting factor in OMW is the lack of nutrient bioavailability.

The next part of the present study concerns the energy valorization of the produced \textit{C. vulgaris} biomass. Biodiesel production from microalgal biomass apart from harvesting and drying also requires the additional steps of transesterification and purification. These steps augment the cost of biofuel production, thus making it an economically unviable solution. In an effort to explore the potentials of microalgal biomass exploitation, the energy valorization of \textit{C. vulgaris} biomass through direct combustion and anaerobic digestion were also investigated. The specific calorific value of \textit{C. vulgaris} dry biomass was found to be \(24,525 \pm 182 \text{ kJ kg}^{-1}\) or \(5,861 \pm 44 \text{ kcal kg}^{-1}\). The methane potential of \textit{C. vulgaris} biomass was evaluated by conducting BMP assays in mesophilic and thermophilic conditions, with and without pretreatment (ultrasonication). The maximum yield was obtained in mesophilic conditions without pretreatment, presenting the value of 389.07 ml gVS\(^{-1}\) added. Finally, by comparing the energy valorization methods, the highest energy productivity (20.331 kJ L\(_{\text{reactor}}^{-1}\) day\(^{-1}\)) was obtained from the direct combustion of \textit{C. vulgaris} biomass.

Last but not least, the modeling of the behavior of \textit{C. vulgaris} in pH 7.5 under sulfur stress took place, resulting in satisfying data fitting and parameter estimation.
Περίληψη

Η παρούσα μεταπτυχιακή εργασία εστιάζει στην κινητική μελέτη ανάπτυξης και λιπιδιακής σύνθεσης του μικροφύκους C. vulgaris, υπό ετερότροφες συνθήκες, και την ενεργειακή αξιοποίηση της παραγόμενης βιομάζας. Ο βασικός λόγος για τον οποίο το στέλεχος αυτό έχει κεντρίσει το επιστημονικό ενδιαφέρον είναι ότι πρόκειται για έναν ευπροσάρμοστο μικροοργανισμό. Έχει την ικανότητα να αναπτύσσεται με γρήγορο ρυθμό, σε διαφόρων ειδών θρεπτικά μέσα και υγρά απόβλητα, σε ακραίες συνθήκες pH και θερμοκρασίας καθώς επίσης μπορεί να ανεχτεί την παρουσία τοξικών συστατικών. Επιπρόσθετα, συσσωρεύει ενδοκυτταρικά λιπίδια κατάλληλα καταλαμβάνοντας σε υψηλό λιπιδιακό περιεχόμενο, η σύσταση του οποίου είναι κατάλληλη για παραγωγή βιοντιητέλοι.

Ο στόχος του πρώτου σετ πειραμάτων ήταν η διερεύνηση του εύρους pH στο οποίο μπορεί να αναπτυχθεί το μικροφύκος C. vulgaris, καθώς και ο προσδιορισμός της βέλτιστης τιμής pH για την ανάπτυξη του συγκεκριμένου στελέχους. Έχει την ικανότητα να αναπτύσσεται με γρήγορο ρυθμό, σε διαφορετικά κρεπτικά μέσα και υγρά απόβλητα, σε ακραίες συνθήκες pH και κερμοκρασίας καθώς επίσης μπορεί να ανεχτεί την παρουσία τοξικών συστατικών. Επιπρόσθετα, συσσωρεύει ενδοκυτταρικά λιπίδια καταλαμβάνοντας σε υψηλό λιπιδιακό περιεχόμενο, η σύσταση του οποίου είναι κατάλληλη για παραγωγή βιοντιητέλοι.

Ο στόχος του πρώτου σετ πειραμάτων ήταν η διερεύνηση της επίδρασης της στέρησης διαφόρων θρεπτικών συστατικών (S, P και N) στην ανάπτυξη της βιομάζας και τη λιπιδιακή σύνθεση του συγκεκριμένου μικροφύκους. Οι εν δυνάμει διαφορές στην επίδραση της στέρησης των συστατικών αυτών μπορούσαν να αποτελέσουν κριτήριο για την επιλογή του κατάλληλου μίγματος υγρών αποβλήτων που θα υποστεί επεξεργασία με τη χρήση του συγκεκριμένου μικροφύκους. Το συστατικό του οποίου η στέρηση είχε τη σημαντικότερη επίδραση στη συσσώρευση ενδοκυτταρικών λιπιδίων ήταν το θείο.

Στη συνέχεια μελετήθηκε η χρήση Πτητικών Λιπαρών Οξέων (VFAs) ως πιθανές πηγές άνθρακα. Τα VFAs είναι μεταβολικά προϊόντα, ενδιάμεσα ή τελικά, διαφόρων ζωμωτικών διεργασιών. Στη συγκεκριμένη περίπτωση ο στόχος ήταν η διερεύνηση των VFAs που μπορούν να χρησιμοποιηθούν ως πηγές άνθρακα από το συγκεκριμένο μικροφύκος υπό ετερότροφες συνθήκες. Τα VFAs που δοκιμάστηκαν ήταν τα οξέα, προπιονικό, βουτυρικό και ισοβουτυρικό οξύ. Παρόλο που το μικροφύκος C. vulgaris δεν κατάφερε να αναπτυχθεί σε μέσο με μόνη πηγή άνθρακα τα οξέα προπιονικό, βουτυρικό και ισοβουτυρικό, ήταν ικανό να αποδομήσει το οξέο οξύ όπου παρουσίασε ειδικό ρυθμό ανάπτυξης 0.429 days⁻¹. Επιπρόσθετα, χρησιμοποιήθηκε σαν πηγή άνθρακα ο συνδυασμός των οξέων που προαναφέρθηκαν με προσθήκη γλυκόζης, προσομοιώνοντας την περίπτωση όπου οι βιολογικές διεργασίες που έχουν ως αποτέλεσμα την παραγωγή VFAs να υπολειτουργούν και τα ρεύματα εξόδου να περιέχουν, εκτός από οξέα, και άλλα οργανικά συστατικά. Και στην περίπτωση αυτή, το συγκεκριμένο μικροφύκος δεν
αποδόμησε τα οξέα προπιονικό, διουρουκικό και ισοβουτυρικό. Ωστόσο, ο μέγιστος ειδικός ρυθμός ανάπτυξης που παρατηρήθηκε από την αποδόμηση του οξικού οξέος και της γλυκόζης ήταν 0.534 days⁻¹.

Στο επόμενο κομμάτι της παρούσας εργασίας, φαινολικές ενώσεις δοκιμάστηκαν ως πιθανές πηγές άνθρακα. Πριν από τη διεξαγωγή των συγκεκριμένων πειραμάτων έλαβε χώρα η διαδικασία του εγκλιματισμού. Οι φαινολικές ενώσεις που δοκιμάστηκαν ήταν τα βανιλλικό, συριγγικό, φερουλικό, γαλλικό, ρ-υδροξυβενζοϊκό οξύ και η κατεχόλη σε συγκέντρωση 0.5 g L⁻¹. Η κατεχόλη παρεμπόδισε την ανάπτυξη του μικροφύκους κατά τη διαδικασία του εγκλιματισμού, σε συγκέντρωση 100 mg L⁻¹, επιβεβαιώνοντας την ισχυρά τοξική επίδραση της φαινολικής αυτής ουσίας. Το μικροφύκος δεν ήταν ικανό να αναπτυχθεί σε θρητικό μέσο που είχε σαν μόνη πηγή άνθρακα τις φαινολικές ουσίες βανιλλικό, φερουλικό και ρ-υδροξυβενζοϊκό οξύ σε χρονικό διάστημα 15 ημερών. Τα οξέα γαλλικό και συριγγικό αποδομήθηκαν από το μικροφύκος, καταλήγοντας όμως σε μικρό ειδικό ρυθμό ανάπτυξης (0.063 και 0.103 days⁻¹).

Στη συνέχεια ερευνήθηκε η επεξεργασία υγρών αποβλήτων ελαιοτριβείου (OMW) με τη χρήση του μικροφύκους C. vulgaris. Το OMW είναι ένα πολυσύνθετο απόβλητο με τα πιο βασικά χαρακτηριστικά του να είναι η υψηλή συγκέντρωση φαινολικών ουσιών και το χαμηλό περιεχόμενο του σε άξιο, το οποίο βρίσκεται σε μορφή οργανικού αξιώματος, χαρακτηριστικά που το μετατρέπουν σε δύσκολα αποδομήσιμο υπόστρωμα για μικροφύκη. Το μικροφύκος δεν αναπτύχθηκε στο OMW, ακόμα και μετά τη ρύθμιση του pH στη βέλτιστη τιμή και την προσθήκη πηγής θείου και αξιών. Ωστόσο, η ανάπτυξη του μικροφύκους σε μέσο αποτελούμενο από 10% ν v⁻¹ OMW, γλυκόζη και BG-11 θρητικό μέσο υπέδειξε ότι μπορεί να ανεχθεί τις υπάρχουσες φαινόλες (σε συγκέντρωση 0.5 g L⁻¹), καθώς και ότι ο παρεμποδιστικός παράγοντας το OMW είναι η χαμηλή βιοδιακεςιμότητα τρεις υπόλοιποι συστατικών.

Το οπίσθιο κομμάτι της παρούσας εργασίας αφορά την ενεργειακή αξιοποίηση της παραγόμενης βιομάζας. Η παραγωγή βιοτίζει από μικροφύκη, εκτός από τη συλλογή και έξοδος της βιομάζας επίσης απαιτεί τα επιπρόσθετα βήματα της τρανσπροσποιήσης και του καθαρισμού του παραγόμενου βιοτίζελ. Οι διεργασίες αυτές αυξάνουν το κόστος της παραγωγής βιοτίζελ και το καθιστούν μη-βιώσιμη επιλογή. Στην προσπάθεια εύρεσης των τρόπων ενεργειακής αξιοποίησης της βιομάζας, έλαβαν χώρα η μέτρηση της θερμογόνου δύναμης μέσω της καύσης και η αξιολόγηση του Βιοχημικά Μεθανογόνου Ναυδικού. Η θερμογόνου δύναμη της παραγόμενης βιομάζας βρέθηκε ίση με 24,525 ± 182 kJ kg⁻¹ ή 5,861 ± 44 kcal kg⁻¹. Το μεθανογόνο δυναμικό της βιομάζας αξιολογήθηκε μέσω της διεξαγωγής BMP τεστ σε μεσόφιλες και θερμόφιλες συνθήκες, χωρίς και με προπεξεργασία (χρήση υπερχιλν). Η μέγιστη απόδοση παρατηρήθηκε στις μεσόφιλες συνθήκες χωρίς προπεξεργασία, παρουσιάζοντας την τιμή 389.07 ml gVs⁻¹ added. Συγκρίνοντας τις μεθόδους ενεργειακής αξιοποίησης, η μέγιστη ενεργειακή παραγωγικότητα (20.331 kJ L_1reactor⁻¹ day⁻¹) λήφθηκε μέσω της απευθείας καύσης της βιομάζας.

Τέλος, έλαβε χώρα η μαθηματική μοντέλοποιότητα της συμπεριφοράς του μικροφύκους σε pH 7.5 υπό συνθήκες περιορισμού θείου, η οποία είχε ως αποτέλεσμα την ικανοποιητική προσαρμογή του μοντέλου στις πειραματικές τιμές και τον υπολογισμό των κινητικών του παραμέτρων.
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1.1 Energy and Environment

Human life is highly dependent on a consistent supply of energy. In modern societies almost every human activity (domestic, medical, transportation, industries etc.) is based on the consumption of energy. Thus, there is an imperative need of energy security in order to sustain the growing world population. It is now accepted that energy resources will play an important role in the future of mankind. We cannot leave this matter to chance.

Till now, most of the energy needs were covered by conventional fuels such as oil, coal and natural gas (fossil fuels). Nowadays, where the world’s population is on the rise and the energy consumption per person is greater than it ever was, humanity is starting to face the challenge of energy sustainability. In addition, the consumption of fossil fuels has a major impact on the environment, as it results in the accumulation of carbon dioxide in the atmosphere (Figure 1.1) and as a consequence the rise of global temperature.

The dwindling natural resources, the global warming effect and the continuous rise in petroleum price are amongst the most important facts that dictate us to find alternative, reliable and cost-effective solutions for energy supply.

![Figure 1.1: Accumulation of Carbon Dioxide in the Atmosphere (Measured at Mauna Loa, Hawaii)](image)

Figure 1.1: Accumulation of Carbon Dioxide in the Atmosphere (Measured at Mauna Loa, Hawaii)
1.2 Renewable Energy Sources

The sum of energy resources has been split into three categories: fossil fuels, renewable resources, and nuclear resources [Demirbas, 2005]. Renewable energy resources, or - often called - alternative sources of energy, such as wind, solar, geothermal energy, hydropower and biomass are abundant, inexhaustible and environmentally friendly. Renewable energy technologies produce energy via exploiting natural phenomena (wind, falling water, heat of earth’s core etc.) and via converting wastes (household, industrial etc.) into biofuels. Nowadays, there is an increasing interest in renewable energy sources as they offer many advantages when compared to conventional sources of energy. The most important of them are the following:

- They are practically inexhaustible sources of energy and contribute to reducing dependence on dwindling conventional energy resources.
- They are an answer to the energy problem for the stabilization of carbon dioxide emissions and other greenhouse gases. In addition, the replacement of energy generation plants which use conventional resources, results to a reduction in the emission of other pollutants, such as sulfur and nitrogen oxides which cause acid rain.
- They are domestic sources of energy and contribute to increasing energy independence and security of energy supply at the national level.
- They are geographically dispersed, leading to the decentralization of the energy system, making it possible for energy needs to be met at a local and regional level, thus reducing losses from energy transmission.
- They usually have low operating costs which are not influenced by fluctuations in the international economy and especially in prices for conventional fuels.
- Investments in this domain create a significant number of new jobs, especially at the local level.
- They can renew economically and socially depressed areas and encourage local development through the promotion of relevant investments.

There are many strategies aiming the increase of renewable energy in the energy mix, but also some examples of places and countries which have achieved the goal of using only renewable energy resources. For instance, Iceland generates all its electricity using renewable energy already, for Norway 98% of energy comes from renewable sources. That is the case for 86% of energy generated in Brazil, 62% in Austria, 65% in New Zealand, and 54 % in Sweden [REN21, 2010]. In addition, in Denmark the government decided to switch the total energy supply (electricity, mobility and heating/cooling) to 100% renewable energy by 2050 [Mathiesen et al, 2015].
The European Commission (EC) aims at reducing greenhouse gas emissions, diversifying energy supply, reducing the dependence on unreliable fossil fuel markets, and creating new technologies and improving trade balances. For example, it also targets reaching a 20% share of energy from renewable sources by 2020 and at least 55% of renewable energy in gross final energy consumption in 2050 [EC, 2013]. According to renewable energy progress report, Greece should be able to produce 18% of primary energy using renewable sources by 2020 (in 2005, energy production from renewable sources was at 6.9%, and in 2010, it was 9.7%). These goals are headline targets of the European 2020 strategy for growth, since they contribute to Europe’s industrial innovation and technological leadership as well as reducing emissions, improving the security of our energy supply and reducing our energy import dependence [EC, 2013]. In the European Union, the RES installed capacity reached 325 GW, with a main contribution from hydropower (147 GW), wind (94 GW), solar (52 GW) and biomass (31 GW).

Greece is a country endowed with a plethora of renewable sources because of its climate and geography. It enjoys high solar radiation throughout the year and the sunshine lasts more than 2700 hours a year. Several also areas of mainland and island Greece have stable and strong winds on an ongoing basis. Because of the terrain, in several points of the interior, especially in the western part, there are suitable conditions, encouraging the creation of small and large dams, which means production of electricity through hydropower. Moreover, Greece as a primarily agricultural country has enough reserves of biomass suitable for energy production.

1.3 Agro-Industrial Wastes

Large amount of wastes is generated every year from the industrial processing of agricultural raw materials. The composition and quantity of agro-industrial wastes depends on the source of raw materials, as well as the nature of products, operations and processing steps. Food processing wastes consist of large amounts of organic material (carbohydrates, proteins, fats, oils etc.) with high values of Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), and suspended solids (SS). However, due to their high nutrient content, agro-industrial wastes have a high potential to cause severe pollution, if not properly treated. Unfortunately, in many cases these wastes end up deposited in the environment, without being subjected to any processing.

The over-exploitation of natural resources has resulted in an imbalance in ecological systems. Waste recycling is a concept widely accepted as an effective tool with which to overturn the imbalance we have created. The presence of carbon sources, nutrients and moisture in agro-industrial wastes provide conditions suitable for the
development of microorganisms, and this opens up great possibilities for their reuse, and conversion into valuable products and energy.

1.3.1 Olive Mill Wastewater (OMW)

Mediterranean people have been growing olive trees and extracting olive oil for thousands of years. Olive oil is produced from olive trees, each olive tree yielding between 15 and 40 kg of olives per year. Worldwide olive oil production for the year 2002 was $2.5 \times 10^6$ tons [Paraskeva et al., 2006]. According to recent statistics of the International Olive Oil Council (November 2015), the major world producers of olive oil are in the EU (69.9%), accounting for 62.7% in Spain, 19.4% in Italy, 14.0% in Greece, and 3.4% in Portugal. Outside the EU, yet in the Mediterranean area, the relevant olive oil producers are: Tunisia (6.2%), Syria and Turkey (each 5.7%), and Morocco (4.2%). The majority of the world consumption of olive oil is also in the Mediterranean area: Italy (35.2%), Spain (30.5%) and Greece (10.7%). The USA, with 9.5% of the total world olive oil consumption, plays also an important role in olive oil business [Justino et al., 2012].

Despite the health and economic benefits of the production and consumption of olive oil, producing countries face two serious environmental issues: the high water consumption, and the extremely high toxicity of resulting effluents. The traditional process of olive oil extraction in batches has been ceased for some time, and nowadays the extraction of olive oil can be obtained by one of two different continuous processes: the two-phase or the three-phase systems [Justino et al., 2012]. These extraction processes generate two main wastes: a brownish-black liquid effluent called olive oil mill wastewater (OMW), and a solid waste, generally called pomace or husk. The wastewater arising from the milling process amounts to 0.5–1.5 m$^3$ per 1000 kg of olives depending on the process. The discontinuous process produces less but more concentrated wastewater (0.5–1 m$^3$ per 1000 kg) than the centrifugation process (1–1.5 m$^3$ per 1000 kg) [Hamdi, 1996]. OMW is one of the strongest industrial effluents, with BOD$_5$: 35–110 g L$^{-1}$, COD: 45–170 g L$^{-1}$ and SS: 1–9 g L$^{-1}$ [Paraskeva et al., 2006].

Besides its strong organic content, OMW contains high concentrations of compounds such as lignin and tannins which give it a characteristic dark color (52 –180 g L$^{-1}$ Pt-Co units), but, most importantly, it contains phenolic compounds and long-chain fatty acids which are toxic to microorganisms and plants [Paraskeva et al., 2006]. The phenolic compounds can be either simple phenols and flavonoids, or polyphenols which result from polymerisation of the simple phenols. The concentration of phenolic compounds in OMW varies greatly from 0.5 to 24 g L$^{-1}$ [Borja et al., 2006]. The high organic load and the associated toxicity make the treatment of OMW
imperative. Problems also arise from the fact that olive oil production is seasonal and so the treatment process should be flexible enough to operate in a non-continuous mode, otherwise storage of the wastewater will be required.

OMWs have been spread, without any valorization treatment, into soil or nearby streams and rivers, for many years, being very harmful to soil microflora, plants and freshwater species [Aggelis et al., 2003; Roig et al., 2006]. In order to minimize these environmental impacts, olive mills have been obliged to treat or even reduce substantially their wastes. However, the complex physico-chemical composition of OMW results in technical difficulties to achieve efficient treatments, since its composition is highly variable, depending on many factors such as the type of olive oil extraction process, the local and seasonal nature of oil production, the climatic conditions and cultivation methods [Roig et al., 2006].

Consequently, the use of OMW as a substrate for microorganism growth may not always be possible without pre-treatment, given the complex composition which varies according to many factors and the high concentration of toxic compounds in this particular wastewater.

1.4 Microalgae

Microalgae are microscopic photosynthetic organisms that are found in both freshwater and marine environments. Their photosynthetic mechanism is similar to land based plants, but due to a simple cellular structure, and their presence in an aqueous environment where they have efficient access to water, CO₂ and other nutrients, they are generally more efficient in converting solar energy into biomass [Carlsson et al., 2007]. They constitute a highly diverse group of prokaryotic and eukaryotic organisms. The classification into divisions is based on various morphological features. The most common microalgae are Cyanophyta (blue-green algae), Chlorophyta (green algae), Bacillariophyta (diatoms), Euglenophyta (euglenoids) and Chrysophyta (golden algae) [Carlsson et al., 2007].

Microalgae have been studied for decades because of the large variety of metabolic products that can be obtained through their cultivation, such as pigments, enzymes, polymers, toxins and lipids that can be converted into biodiesel [Perez-Garcia et al., 2011]. They also proved to be a source of products such as proteins, carbohydrates, vitamins and minerals [Mata et al., 2010] and thus they are often consumed as food supplement [Fradique et al., 2010]. They can be grown on diverse media, synthetic or real wastewaters, provided that the latter contain all the necessary nutrients to support microalgal growth.
1.4.1 History

Microalgae have started leaving their footprint in the distant past. Studies have shown that the first prokaryotic phototrophs evolved around 3.5 billion years ago, when the oldest known cyanobacterium fossilized in rocks at Western Australia [Stanley, 1992]. However, other reports indicate that the evolution of cyanobacteria is estimated to be closer to 2.7 billion years ago [Brasier et al., 2002], and the appearance of eukaryotic cells is dated around 1.8 billion years ago [Knoll et al., 2006]. Cyanobacteria have the ability to produce gaseous oxygen as a byproduct of photosynthesis. They are thought to have gradually changed the composition of the “early atmosphere” of Earth, by filling it with oxygen [Schopf, 2012]. They have, thereby, dramatically changed the composition of life forms on the planet, stimulating biodiversity and created the conditions for the evolution of aerobic metabolism and eukaryotic photosynthesis [Stewart et al., 2008]. Many of these primitive microorganisms remained unchanged through time, and others are assumed to have evolved to other species, as biologists estimate that microalgae may be the ancestors of plants [Bhattacharya et al., 1998].

![Picture 1.1: A fossilized colonial chroococcalean form (cyanobacteria) dating to the Late Proterozoic era, about 850 million years old.](http://www.ucmp.berkeley.edu/precambrian/bittersprings.html)

Microalgae are very adaptable microorganisms. They can grow in a variety of environmental conditions, from the deserted areas of Sahara to the frozen lakes of Scandinavia [Lee, 2008]. In addition, they are characterized by their high nutritional value, as well as many other distinctive properties. These are the main reasons for which microalgae have been consumed as food by many civilizations though the passing of time. There are many proofs that Aztecs and other Mesoamericans used blue-green algae as food source before the 16th century [Diaz Del Castillo, 1928], and continue to do so till now (Picture 1.2).
Undoubtedly, microalgae play a major role in the flourishing of mankind. Not only have they been an important food source for some civilizations, they also have been the main source of biomass which was buried billion years ago under sedimentary rock and subjected to intense heat and pressure under anoxic conditions [Kvenvolden, 2006], forming petroleum, a fuel that nowadays drives human life.

### 1.4.2 Classification and Characteristics

There are numberless species of microalgae. It is not an exaggeration to say that they are infinite in numbers. An estimate of 200,000 – 800,000 species of microalgae is widely quoted, of which only about 35,000 are classified. Their classification is complex and sometimes controversial, especially concerning the Cyanobacteria (blue-green algae) which are sometimes known as blue-green bacteria or Cyanophyta and sometimes are included in Chlorophyta [Hasan et al., 2009]. However, one of the proposing taxonomical outlines of algae is presented in the following figure (Figure 1.2):
**Figure 1.2:** Phylogenetic tree highlighting the diversity and distribution of algae (boxed groups; colors indicate the diversity of pigmentation) across the domains of life (adapted from www.keweenawalgae.mtu.edu). For comparison animals and land plants are encircled in red and green, respectively.

[http://www.glycomar.com/marine_natural_products_microalgae.htm]

The main characteristics of each taxonomic group are the following [Janse van Vuuren et al., 2006]:

1. **Cyanophyta** (blue-green algae)

The name “cyanophyta” is derived from the Greek words “cyano” (κυανό) which means blue and “phyta” (φυτά) which means plants. They are often known as cyanobacteria, blue-green algae or blue-green bacteria. Being prokaryotic, they are considered to be more closely related to bacteria than to other algae. They may be in the form of single cells, colonies or filaments. The blue-green color is the result of photosynthetic pigments such as chlorophyll-a (green pigment) and phycocyanin (blue pigment), but some also contain phycoerythrin (a red pigment). When all three pigments are present, the cells may appear purplish. Most commonly their color is blue-green but rarely bright green. They include marine, freshwater as well as terrestrial species, which reproduce asexually. Under conditions of excessive nutrient availability, slow moving or stagnant water, and warmth, cyanobacteria may proliferate, producing a variety of problems such as surface scum, taste and odor...
problems, skin irritations and the release of toxic substances. When they decompose severe oxygen depletion may occur, resulting in fish kills.

![Image of various cyanobacteria](shigen.lab.nig.ac.jp)

**Picture 1.3**: Various kinds of cyanobacteria

2. **Chlorophyta** (green algae)

Chlorophyta comes from the Greek words “chloros” (χλωρός) that means green and “phyta” (φυτά) which means plants, and therefore representatives of this phylum are commonly referred to as green algae. They may be unicellular, colonial, filamentous or more complexly structured. The most conspicuous organelle in the cell is the chloroplast, which is mostly bright green in color due to the presence of chlorophylls a and b. Certain species may appear yellow-green or blackish-green due to the presence of carotenoid pigments or high concentrations of chlorophyll. The chloroplast morphology varies greatly and usually contains one or more pyrenoids that store starch as a food reserve. The protoplast is surrounded by a more or less firm cell wall, composed of cellulose, along with other polysaccharides and proteins. Chlorophyta constitute one of the major groups of algae when considering the abundance of genera and species, and the frequency of occurrence. They grow in waters of a great range of salinity, varying from oligotrophic freshwaters to those that are marine and supersaturated with solutes.

![Image of Chlorella protothecoides and Pediastrum boryanum](shigen.lab.nig.ac.jp)

**Picture 1.4**: *Chlorella protothecoides* (left), *Pediastrum boryanum* (right)

[Photograph: shigen.lab.nig.ac.jp]
3. **Bacillariophyta** (diatoms)

Bacillariophyta comes from the Latin word “bacillus” that means little stick and the Greek word “phyta” (φυτά) that means plants. They are commonly referred to as diatoms. The single cells, colonies or filaments are microscopic and usually yellow to light brown in color. Most diatoms are autotrophic but a few are obligatory heterotrophs as they completely lack chlorophyll. Their storage products are chrysolaminarin and oil droplets, and those with chloroplasts contain the photosynthetic pigments chlorophyll a and c and fucoxanthin. Most diatoms are classified within two major morphological groups, the centric and pennate diatoms. Centric diatoms exhibit radial symmetry, while pennate diatoms are bilaterally symmetrical. Except for male gametes, diatoms lack flagella. The primary means of reproduction is asexual, by cell division. Diatoms are extremely important components of phytoplankton. Besides being the largest contributors to global primary production, they are used as ecological tools to investigate past conditions (fossils) and monitor environmental changes over time. Diatoms also have many industrial and commercial applications in products such as foods, filters, paints, and cosmetics.

![Picture 1.5: Various kinds of diatoms][Photograph: Damián H. Zanette]

4. **Euglenophyta** (euglenoids)

The phylum Euglenophyta is named after the common genus Euglena, which, in turn, comes from Greek, “eu” (Eu) meaning well, good or true and “glene” (γλήνη), referring to eye. The unicellular organisms have bright green chloroplasts (although colorless forms also occur) and a conspicuous red eyespot at the front end. Their
chloroplasts contain the pigments chlorophyll a and b and carotenoids and the cytoplasm contains many paramylon storage granules and a contractile vacuole (in freshwater members). Even though they contain more than one flagellum, in most euglenoids only one is clearly visible. Euglena, and some other euglenoids, are best known for their characteristic undulating, shape-changing motion, called metaboly. Most species live in freshwater environments and reproduce asexually. Euglenoids live in hard or soft water habitats of varied pH and light levels. Populations thrive under high nutrient levels and are, therefore, useful bio-indicators of such conditions.

![Image of Euglena gracilis](The image shows Euglena gracilis, a representative species of Euglenoids.]

**Picture 1.6: Euglena gracilis**
[Photograph: Dr Ralf Wagner]

5. **Chrysophyta** (golden algae)

Their name is derived from the Greek words “chryso” (χρυσό), which means golden and “phyta” that means plants. They are commonly referred to as the golden-brown algae, and are mostly unicellular or colonial, but filaments may also occur. Chrysophytes are both photosynthetic and heterotrophic. Photosynthetic genera usually have yellow to golden-brown chloroplasts as a result of the presence of the pigment fucoxanthin, which conceals the green color of chlorophylls a and c. Chrysophyta possess two anterior implanted flagella of unequal length. The long flagellum is used as a feeding apparatus in some species by directing the water current and food particles towards the cell. Some chrysophytes are excellent bio-indicators, as they inhabit particular environmental niches in fresh and marine waters. They are usually most abundant and diverse in freshwaters of neutral or slightly acidic pH with low conductivity, alkalinity and nutrient levels and colder temperatures. Sexual and asexual reproduction can produce cysts, often in response to changes in environmental conditions or population density. Although common, they are often hard to examine as the cells tend to be fragile and they break up readily when mounted. It is, therefore, possible to underestimate or even completely overlook members of this group in a sample.
Microalgae are eukaryotic cells which have many structural elements similar to plants (Figure 1.3). Their cell walls, which are rigid and protect the cell against invaders and harsh environment, vary according to each growth phase. In newly formed cells, the cell wall is fragile, and gradually increases its thickness until maturation. However, in the mature stage the cell wall composition and thickness are not constant, as they can change according to different growth and environmental conditions [Safi et al., 2014].

Figure 1.3: Schematic ultrastructure of *C. vulgaris* representing different organelles [Safi et al., 2014].
The cytoplasm is the substance which is confined within the barrier of the cell membrane and is composed of water, soluble proteins and minerals. It hosts the internal organelles of the microalgal cell such as the nucleus, mitochondria, vacuoles, chloroplasts and the Golgi body [Safi et al., 2014].

The mitochondria contain genetic material, the respiratory system, and a double-layer membrane. The outer membrane surrounds the organelle and is composed of proteins and phospholipids in equal proportion. The inner membrane is composed of three times more proteins than phospholipids, and surrounds the internal space, which contains the majority of mitochondrial proteins [Solomon et al., 1999].

Some microalgae, like *C. vulgaris*, contain a single chloroplast. The chloroplast contains a double enveloping membrane composed of phospholipids; the outer membrane is permeable to metabolites and ions, but the internal membrane is more selective to the transportation of proteins. Starch granules, composed of amylose and amylopectin, can be found inside the chloroplast, especially during unfavorable growth conditions. The pyrenoid is the center of carbon dioxide fixation. In addition, the chloroplast stores thylakoids, where chlorophyll, the dominant pigment in green microalgae, is synthesized. During nitrogen stress, lipid droplets accumulate mainly in the cytoplasm and the chloroplast [Lee, 2008, Van den Hoek et al., 1995].

### 1.4.4 Modes of cultivation

There are many conditions of cultivating microalgae such as autotrophic, heterotrophic, photo-heterotrophic and mixotrophic mode. However, not every microalgal strain can grow without the presence of light. Table 1.1 summarizes the main features of each growth mode:

<table>
<thead>
<tr>
<th>Growth mode</th>
<th>Energy source</th>
<th>Carbon source</th>
<th>Light availability requirements</th>
<th>Metabolism variability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autotrophic</td>
<td>Light</td>
<td>Inorganic</td>
<td>Obligatory</td>
<td>No switch between sources</td>
</tr>
<tr>
<td>Heterotrophic</td>
<td>Organic</td>
<td>Organic</td>
<td>No requirements</td>
<td>Switch between sources</td>
</tr>
<tr>
<td>Photo-heterotrophic</td>
<td>Light</td>
<td>Organic</td>
<td>Obligatory</td>
<td>Switch between sources</td>
</tr>
<tr>
<td>Mixotrophic</td>
<td>Light and organic</td>
<td>Inorganic and organic</td>
<td>Not obligatory</td>
<td>Simultaneous utilization</td>
</tr>
</tbody>
</table>

Table 1.1: Growth modes of algae (microalgae) cultivation [Perez-Garcia et al., 2015].
The main characteristics of each cultivation tactic are more extensively presented below.

1.4.4.1 Autotrophic growth

Today, the most common procedure for cultivation of microalgae is autotrophic growth. Due to the fact that all microalgae are photosynthetic, and many are especially efficient solar energy convertors, microalgae are cultivated in illuminated environments naturally or artificially. Under autotrophic cultivation, the cells harvest light energy and use CO$_2$ as a carbon source. The introduction of sufficient natural or artificial light to allow massive growth and dense populations is the main objective and a limiting factor of the cultivation: the more light, up to a limit for the species, the better [Mandalam et al., 1998; Yang et al., 2000; Suh et al, 2003].

Therefore, open ponds that mimic natural environments of microalgae are the most common option for mass cultivation [Oswald, 1992; Tredici, 2004]. The most common shape of open ponds is the “raceway pond”, an oval form resembling a car-racing circuit [Lee, 2001; Pulz, 2001; Chisti, 2007]. These cultivation systems present relatively low construction and operating costs and the large ones can be constructed on degraded and nonagricultural lands that avoid use of high-value lands and crop producing areas [Chen, 1996; Tredici, 2004]. Nevertheless, open ponds have several inherent disadvantages such as (1) poor light diffusion inside the pond, decreasing with depth, (2) mono-cultivation of the desired microalgae is difficult to maintain (3) environmental growth parameters of cultivation rely primarily on local weather conditions (5) continuous and clean water is needed; and (6) production of pharmaceutical or food ingredients is not feasible or is very limited [Chen, 1996; Tredici, 1999; Lee, 2001; Pulz, 2001; Wen Z.Y. et al., 2003; Sansawa and Endo, 2004; Carvalho et al., 2006; Chen et al., 2006; Chisti, 2007; Patil et al., 2008].

To overcome inherent disadvantages of using open, less controlled environments, numerous closed photo-bioreactors (PBR) of various volumes and shapes have been designed [Tsygankov, 2000; Zhang et al., 2001; Barbosa, 2003; Suh et al, 2003; Zijffers et al., 2008]. The main goal of any PBR is reduction in biomass production costs. This has been done by shaping of the PBR, controlling environmental parameters during cultivation, aseptic designs, and operational approaches to overcome rate-limiting of growth, such as pH, temperature, and gas diffusion. Overcoming these limitations make monocultures and production of pharmaceutical and food goods possible [Chen, 1996; Pulz, 2001; Wen et al., 2003; Lebeau et al., 2006].

To sum up, the main advantages of autotrophic growth of microalgae are presented below:
Microalgae are photosynthetic microorganisms – most species are obligate autotrophs rather than facultative heterotrophs [Behrens, 2005]. They fixate the atmospheric carbon dioxide (CO\(_2\)), which they use as the sole carbon source, producing oxygen (O\(_2\)) via photosynthesis. They produce carotenoids, chlorophyll and other light-induced metabolites which have multiple industrial uses. There is lower risk of contamination, in contrast to heterotrophic growth where there is the competition of microorganisms for organic substrate.

However, there are some major disadvantages considering this tactic:

- Illumination increases the operation costs.
- Difficult scaling-up process.
- Poor light diffusion can be a limiting factor, especially in high cell densities where self-shading may occur. Consequently shallow depth and big surfaces are required.
- Lower cell densities can be achieved, when compared to other modes of cultivation.
- They have lower growth rates in comparison to heterotrophically or mixotrophically grown microalgae.

Low biomass density in a reactor also increases the biomass harvesting cost. As a result, producing valuable products, specifically biofuels, from autotroph microalgae needs a long development time and huge investments before it becomes commercially viable [Liang 2013]. For example, an insignificant amount of biodiesel is currently made from microalgae grown under autotrophic conditions and this is not considered a commercial source.

### 1.4.4.2 Heterotrophic growth

Heterotrophic growth is an aerobic process where assimilation of organic substrates generates energy through oxidative phosphorylation accompanied by oxygen consumption as the final electron acceptor [Perez-Garcia et al., 2015]. Heterotrophs are organisms whose substrate and energy needs are derived from organic compounds synthesized by other organisms. The basic culture medium composition for heterotrophic cultures is similar to the autotrophic culture with the only exception of replacing the fixation of atmospheric CO\(_2\) of autotrophic cultures with organic carbon sources dissolved in the culture media. In addition, they use oxygen in order to generate ATP, a process called respiration.
In a broad sense, all organisms, including microalgae, use the same metabolic pathways for respiration. During respiration, oxygen is consumed and CO$_2$ is produced. Dark respiration rates (mol O$_2$ mol carbon$^{-1}$d$^{-1}$), vary through the cell cycle and in general, increase with growth rates [Lloyd, 1974]. Under optimal conditions, respiration rates are about 20-30% of growth rates [Geider et al., 1989]. This dark respiration plays two major roles in microalgae: (1) It serves as the exclusive source of energy for maintenance and biosynthesis under dark environment and (2) It provides essential carbon skeletons for biosynthesis under any growth condition. Under heterotrophic growth conditions, values for CO$_2$ produced per carbon (C) incorporated into new biomass (CO$_2$/C) equaled 0.4-1.4 for several *Chlorella* species and diatoms. This indicates that biomass synthesis during heterotrophic growth conditions can proceed at nearly the maximal theoretical efficiency, since CO$_2$/C ratios for autotrophic growth are much lower than those for heterotrophic growth [Raven, 1976].

Independent of the supplied organic substrate or the microalgae species, growth rates are enhanced by higher levels of aeration [Griffiths et al., 1960]. Oxygen supply is a key factor in heterotrophic cultivation of microalgae. For example, the limitation of oxygen in a culture may reduce the specific growth rate of *Chlorella* spp. and thus lower the productivity of biomass when cell density is high.

Apart from cell metabolism, cell structure changes depend on growth regime. Under an autotrophic regime, transmission electron microscopy showed that chloroplasts were clearly visible in photosynthetic cells [Lebsky et al. 2001]. Membranes were abundantly accumulated in these chloroplasts and a number of starch granules could also be seen. In contrast, thylakoid membranes rapidly disappeared within 48 h after cells had undergone heterotrophic metabolism, suggesting degeneration of chloroplasts. Instead, the cytoplasm was almost totally filled with large lipid droplets [de-Bashan et al., 2002]. Biochemical and ultra-structural experiments suggested that chlorophyll breakdown and chloroplast degeneration was associated with lipogenesis during the heterotrophic growth of *C. protothecoides*. These cells were initially grown under autotrophic conditions and were rich of chloroplasts [Xiong et al. 2010].

The heterotrophic cultivation approach offers several advantages when compared to autotrophic growth. The most significant of them are presented below:

- It eliminates the cost of illumination.
- It allows the use of practically any fermentor as a bioreactor, such as those used for industrial production of medicines, beverages etc., resulting a significant reduction in costs for most processes.
- It offers relative simplicity of operations and daily maintenance.
- Easier scaling-up process.
• It is possible to obtain high densities of microalgae cells that provide an economically feasible method for large scale, mass production cultivation [Chen, 1996; Behrens, 2005].
• It favors higher growth rates [Raven, 1976].
• Thus, heterotrophically grown microalgae produce higher concentration of useful metabolic products.
• Higher lipid content per dry weight of cells (lipid productivity).
• Because of their achieving high cell densities, heterotrophically grown microalgae engulf larger quantities of organic and mineral substrates. Therefore, they constitute an efficient solution of treating different kinds of wastewaters, which can severely downgrade the environment if not properly handled.

Undoubtedly, one of the major advantages of heterotrophic cultivation is the high density of microalgal cells that can be obtained. Under some heterotrophic cultures, the growth rate, the dry biomass, lipid content and N content are significantly higher than under autotrophic cultures and are mainly dependent on the species and strain used [Yang et al., 2000; Behrens, 2005; Boyle et al., 2009]. Compared to the autotrophic condition, heterotrophic conditions have enhanced concentrations of Chlorella protothecoides up to 3.4 times and of C. vulgaris up to 4.8 times [Liang et al., 2009; Perez-Garcia et al., 2010]. A range of biomass between 4 and 20 g L⁻¹ day⁻¹ of microalgae are commonly produced by using heterotrophic cultivation [Xiong et al., 2010]. This compares with 0.06–0.1 g L⁻¹ day⁻¹ in open cultivation ponds and 0.36 g L⁻¹ day⁻¹ in closed photo-bioreactors [Pulz, 2001]. Under some heterotrophic growth conditions, the microagal biomass concentrations reach cells densities of 50-100 g of dry biomass per liter [Radmer et al., 1994], which is much higher than the maximum 30 g L⁻¹ of dry cell biomass in autotrophic cultures. In addition, these results are consistent and reproducible on a large scale. These large volumes and high productivity of cultures make the heterotrophic strategy far less expensive than the autotrophic approach [Radmer et al., 1994].

However, heterotrophic cultures have several major limitations [Chen, 1996]:
• There is a limited number of microalgal species that can grow heterotrophically.
• Increasing energy expenses and costs by adding an organic substrate.
• Contamination and competition with other microorganisms that grow faster than microalgae.
• Possible reduction of the quality of the desired metabolic products due to contamination [Perez-Garcia et al., 2015].
• Inhibition of growth by excess organic substrate.
- Inability to produce light-induced metabolites.
- Indirect use of arable land for carbon source production reduces the main advantage that microalgal cultivation systems have over land-based crops [Perez-Garcia et al., 2015].

Heterotrophic cultivation is inappropriate for most microalgal species. Yet, some are effectively grown in complete darkness and thus their cultures can be grown in conventional dark fermenters. A list with the required initial characteristics that a microalgae species must possess to be suitable for heterotrophic cultivation in large scale is presented below [Chen et al., 2006]:

- Faculty of cell division and active metabolisms in absence of light.
- Ability to grow in culture media with easy-to-sterile organic substrates where energy required for heterotrophic growth must be supplied by oxidation of part of the organic substrate [Droop, 1974].
- Ability to adapt to fast environmental changes.
- Capacity to resist hydromechanical stress inside the fermenters.
- Overall low cultivation costs, reflected as the ability of the strain to efficiently use inexpensive, common carbon sources, tolerate environmental changes, and generate economical worth in the quantity of the metabolite(s).

Consequently, these requirements reduce even further the microalgal strains that can be employed for large scale heterotrophic applications.

1.4.4.3 Mixotrophic growth

Mixotrophic cultivation is the growth mode where microalgae simultaneously use inorganic CO₂ and organic carbon sources in the presence of light. Therefore, autotrophy and heterotrophy occur concurrently [Wang et al. 2014]. CO₂ is fixed through photosynthesis, which is influenced by illumination, while organic compounds are assimilated through aerobic respiration, which is affected by the availability of organic carbon. Several species are able to switch between autotrophic and heterotrophic growth. This should not be confused with the mixotrophy regime, where both ways of uptake (organic and inorganic) occur at the same time. Mixotrophic microalgae use different sources of energy and carbon they may use organic or inorganic sources and light in different combinations. Mixotrophy makes microalgae more flexible because it may gather both carbon and energy demand by organic or inorganic sources and light simultaneously [Chen et al., 2011].
Mixotrophic cultivation appears to be a good strategy to obtain a large biomass and high growth rates, with the additional benefit of producing photosynthetic metabolites [Chen, 1996]. This cultivation mode offers several advantages [Chojnacka et al., 2004; Kröger et al., 2011; Wang et al. 2014]:

- Higher growth rates than heterotrophic or autotrophic regimes – production of higher concentration biomass.
- Prolonged exponential growth phase.
- Reduction of lost biomass from respiration during dark hours.
- Reduction or stopping of photo-inhibitory effect.
- Flexibility to switch the cultivation regime to heterotrophic or autotrophic regimes at will.
- Protection from photo-oxidative damage stimulated by accumulating oxygen in enclosed photo-bioreactors
- Production of light-induced metabolites.

An interesting observation is that the highest growth rate under mixotrophic regime ($\mu_{mixo}^{max}$) corresponds approximately to the sum of the maximum growth rates obtained under the autotrophic and heterotrophic modes ($\mu_{mixo}^{max} = \mu_{photo}^{max} + \mu_{hetero}^{max}$) [Girard et al. 2014]. Similar observations are known for other microalgae and cyanobacteria species, such as *Chlorella regularis, Chlorella vulgaris, Euglena gracilis, Haematococcus pluvialis*, and *Spirulina platensis* [Ogbonna et al. 2002]. Nevertheless, this is only a general rule; experimental verification of growth rate is required in each case.

The limitations that are encountered during this strategy are similar to those for heterotrophic mode (except of the inability to produce light-induced metabolites).

1.4.4.4 Photo-heterotrophic growth

In photo-heterotrophy, growth cells use light for energy, for fixation of nitrogen and organic matter as a carbon source without CO$_2$. Hence, because organic carbon and light are compulsory for photo-heterotrophic cultivation, it is rarely used as an approach to produce microalgal biomass to process valuable compounds [Wang et al., 2014]. Therefore, photo-heterotrophic cultivation will not be discussed further.

The following table (Table 1.2) summarizes the challenges met during large scale heterotrophic and mixotrophic cultivation of microalgae, and the opportunities for their solution [Perez-Garcia et al., 2015]:
**Table 1.2**: Challenges and opportunities for heterotrophic/mixotrophic cultivation of microalgae.

<table>
<thead>
<tr>
<th>Limitation</th>
<th>Opportunities</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Carbon sources cost</strong></td>
<td>Investigate new sources of cheap organic carbon, such as wastewaters, lignocellulosic material, and industrial processes waste</td>
</tr>
<tr>
<td></td>
<td>Bio-prospection of strains able to assimilate cheap carbon sources</td>
</tr>
<tr>
<td></td>
<td>Metabolic engineering of strains able to assimilate cheap carbon sources</td>
</tr>
<tr>
<td></td>
<td>Improve methods for breakdown of lignocellulose material</td>
</tr>
<tr>
<td><strong>Competition by fast-growing bacteria</strong></td>
<td>Development of mixotrophic cultivation strategies</td>
</tr>
<tr>
<td></td>
<td>Establishing cultures of microalgae able to thrive under bacteria-adverse environmental conditions</td>
</tr>
<tr>
<td></td>
<td>Bio-prospection of fast-growing strains</td>
</tr>
<tr>
<td></td>
<td>Metabolic engineering of fast-growing strains</td>
</tr>
<tr>
<td></td>
<td>Immobilization of microalgae in polymers</td>
</tr>
<tr>
<td><strong>Bioreactor implementation and operation costs</strong></td>
<td>Cheaper materials for bioreactor vessel</td>
</tr>
<tr>
<td></td>
<td>Implement alternative mixing strategies powered by a renewable energy source (hydraulic or wind)</td>
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<tr>
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<td>Implement cheap sterilization strategies</td>
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<td>Establish non-axenic microalgae cultures, such as open ponds</td>
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<td></td>
<td>Increase productivity of the metabolites of interest by optimizing bioreactor’s operation parameters</td>
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<td>Risk assessment studies and regulations of GMOs in large-scale facilities</td>
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<tr>
<td><strong>Downstream processes costs (biomass harvesting and raw product transformation)</strong></td>
<td>Enhance exo-polysaccharides production to promote biomass flocculation</td>
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<td>Develop immobilization technique for the algae in polymeric beads/sheets</td>
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<td>Promote spontaneous excretion of metabolite of interest</td>
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<tr>
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<td>Selection or design of strains that excrete products</td>
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<tr>
<td></td>
<td>Avoid compound extraction and separation by directly transform the biomass to products by pyrolysis, anaerobic digestion, gasification.</td>
</tr>
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</table>
1.4.5 Assimilation of Essential Nutrients

1.4.5.1 Carbon sources

It is important to consider that even though some species can grow on multiple carbon sources, not every microalgal species can be fed successfully with every organic nutrient for biomass production. For example, *C. vulgaris* yielded different biomass productivity in heterotrophic cultures from different carbon sources [Perez-Garcia et al. 2011].

1.4.5.1.1 Glucose

Glucose is the most commonly used carbon source for heterotrophic cultivation of microalgae, as is the case for many other microbial species. Far higher rates of growth and respiration are obtained with glucose than with any other substrate, such as other simple sugars, sugar alcohols, sugar phosphates, organic acids, and monohydric alcohols [Griffiths et al. 1960]. This happens because glucose possesses more energy content per mol, compared with other substrates. For example, glucose produces 2.8 kJ mol\(^{-1}\) of energy compared to 0.8 kJ mol\(^{-1}\) for acetate [Boyle et al., 2009].

Oxidative assimilation of glucose begins with phosphorylation of hexose, yielding glucose-6-phosphate, which is readily available for storage, cell synthesis, and respiration. An equivalent of a single phosphate bond is required per mole of glucose assimilated into glucose-6-phosphate. In that process, an additional 30 equivalents of phosphate bonds are generated by aerobic oxidation of a mole of glucose [Droop, 1974]. Of the several pathways used by microorganisms for aerobic glycolysis (breakdown of glucose), apparently only two, the Embden-Meyerhof pathway (EM pathway) and the Pentose Phosphate pathway (PP pathway) have been found in algae [Neilson et al., 1974]. Under complete darkness heterotrophic growth, glucose is mainly metabolized via the PP pathway (Figure 1.4), while the EM pathway is the main glycolytic process of cells in mixotrophic growth with light [Lloyd, 1974; Neilson et al., 1974; Yang et al. 2000].

Dark anaerobic cultivation of many microalgal strains has been used for fermentative ethanol production. Yet, algae cannot metabolize glucose under dark anaerobic conditions [Droop 1974; Neilson et al., 1974]. Although these studies did not report growth under dark anaerobic conditions, they reported anaerobic endogenous respiration of storage compounds.
Figure 1.4: Scheme of metabolic pathways for assimilation of carbon and production of energy in photoautotrophic, heterotrophic and mixotrophic microalgae.

Compound abbreviations are following specified. 2-OG 2-oxoglutarate, 2PG 2-phosphoglycerate, 3PG 3-phosphoglycerate, ACA acetaldehyde, R5P ribulose-5-phosphate, ACCoA acetyl-Coenzyme A, ADP adenosinediphosphate, ATP adenosine-triphosphate, BPG 1,3-bisphosphoglycerate, BPG 1,3-bisphosphoglycerate, CIT citrate, F6P fructose-6-phosphate, FDP Fructose 1,6-bisphosphate, FUM fumarate, G1P glucose-1 phosphate, G3P glyceraldehyde-3 phosphate, G6P glucose-6 phosphate, GLN glutamine, GLU glutamate, ICIT isocitrate, MAL malate, NAD+ nicotinamide adenine dinucleotide (oxidized), NADH nicotinamide adenine dinucleotide (reduced), NADPH nicotinamide adenine dinucleotide phosphate (reduced), OAA oxaloacetate, OXA oxalosuccinate, PEP phosphoenolpyruvate, PYR pyruvate, RBP ribulose-1,5 biphosphate, SUCC succinate, SUCCCoA succinyl-Coenzyme A [Perez-Garcia et al., 2015].

1.4.5.1.2 Acetic Acid

Uptake of dissolved carboxylic acids, such as acetic, citric, fumaric, glycolic, lactic, malic, pyruvic, and succinic, under microalgal heterotrophic cultivation, is well known for decades [Bollman et al., 1977]. The starting point for assimilating acetate is acetylation of coenzyme A by acetyl-CoA synthetase. This forms acetyl coenzyme A
(acetyl-CoA) in a single-step catalyzing reaction, using a single ATP molecule, (Figure 1.4) [Boyle et al., 2009; Droop 1974]. Acetate (carried by acetyl-CoA) is generally oxidized metabolically through two pathways: the glyoxylate cycle to form malate in glyoxysomes (specialized plastids in the glyoxylate cycle) and the TCA cycle to citrate in the mitochondria, which provides carbon skeletons, energy as ATP, and energy for reduction as NADH. By definition, microalgae that grow by assimilating acetate must possess a glyoxylate cycle pathway to efficiently incorporate acetyl groups of acetyl-CoA into carbon skeletons. However, acetate does not always promote growth. It is toxic for many microorganisms at high concentrations.

1.4.5.1.3 Phenolic compounds

Phenolic compounds are among the most frequently occurring pollutants of surface waters as they are the waste products of many industrial activities. Both cyanobacteria and green algae are sensitive to phenols whose toxicity is related to the number and to the polarity of the substituents on the aromatic ring [Della Greca et al., 1992]. However, several studies confirm that many microalgae can grow on phenols as the sole carbon source in the light [Semple et al., 1996; Papazi et al., 2007; Petroutsos et al., 2007] and/or in the dark [Semple et al., 1996]. The growth rates depend on the species, the phenolic compound and the growth conditions. The microalga Ochromonas danica, a nutritionally versatile mixotrophic chrysophyte, is able to metabolize phenol completely in the dark with 20% of the carbon being accounted for in the biomass [Semple et al., 1996]. These experiments confirmed that phenol was a genuine carbon source for O. danica for both assimilation and oxidation to CO₂. This alga is able to carry out the metacleavage of exogenous phenol [Semple et al., 1996]. In experiments using cultures of the unicellular green alga Scenedesmus obliquus grown in the light, different specific growth rates were found for different phenolic compounds (phenol, chloroplenols, bromoplenols, iodoplenols) [Papazi et al., 2007]. These experiments suggest that phenols biodegradation is a very demanding bioenergetic process.

In another study, catechol, the main anti-algal phenol occurring in olive oil mill wastewaters [Della Greca et al., 2001] was used to select algal strains resistant to phenols [Pinto et al., 2002]. Several microalgae species were incubated in plates under the presence of light. Catechol affected the growth of the majority of algae, and only five strains were found resistant to it, namely Nostoc commune, Scytonema hofmanni, Ankistrodesmus braunii, Chlorella saccharophila, and Scenedesmus quadricauda. Further tests were carried out by preparing liquid cultures of each resistant strain at 10⁻⁴ M catechol. After 5 days of catechol exposure the strains Ankistrodesmus braunii and Scenedesmus quadricauda removed more than 50% of
the initial amount of catechol from the medium, whereas strains *Chlorella saccharophila* and *Scytonema hofmannii* respectively gave 45% and 30% catechol removal. The strains *Ankistrodesmus braunii* and *Scendesmus quadricauda*, were subsequently exposed to each of the most abundant phenols isolated from olive mill wastewater (catechol, tyrosol, hydroxytyrosol, *p*-hydroxybenzoic acid, ferulic acid, *p*-coumaric acid, synapic acid, caffeic acid and vanillic acid) [Della Greca et al., 2001]. The presence of a high phenol concentration in the medium did not affect algal morphology for the first five days but, in the following days, some algal cells became larger, many oil droplets were observed in the cytoplasm and some cells showed anomalous shapes. Both strains degraded phenols within 5 days, achieving, in some cases, a removal greater than 70%, even though better results were obtained with *S. quadricauda*. These results indicate that green algae may represent an alternative to other biological treatment used for the biodegradation of phenol-containing wastewater.

There is also evidence that biodegradation of phenol increased considerably when light intensity increased and the culture was not supplied with external carbon. *Tetraselmis marina*, a green microalga, has the ability to remove chlorophenols under phototrophic conditions, showing a higher efficiency for *p*-chlorophenol but not the highest growth [Petroutsos et al., 2007]. Moreover, *o*-chlorophenol did not affect the growth rate, compared to the control, in contrast to *m* - and *p*-chlorophenol which decreased the biomass concentration, with the *m*-chlorophenol having the highest decrease. This could be explained by the higher bond dissociation energy requirements of the *meta*-position of the halogen compared with those of the *ortho* - and *para*-ones [Papazi et al., 2007]. Moreover, *p*-chlorophenol removal was enhanced by increasing the light period duration or the NaHCO$_3$ concentration [Petroutsos et al., 2007]. Both studies [Papazi et al., 2007; Petroutsos et al., 2007], support that biodegradation proceeds faster when oxygen supply increases.

Toxicity of 4-chlorophenol and 2,4-dichlorophenol on the growth of *C. vulgaris* was investigated in batch reactors under the presence of light [Sahinkaya et al., 2009]. Results showed that although addition of both 4-CP and 2,4-DCP at low concentrations caused a little increase in specific growth rates, while lower growth rates and longer lag periods were observed at high concentrations. With the supplementation of 4-CP and 2,4- DCP, chlorophyll-a content of the cell varied, which indicate physiological changes. Finally, no degradation of 4-CP and 2,4-DCP was observed by *C. vulgaris* over a 30-day incubation.

No direct experiments were performed both in light and dark to compare differences in growth rates. Nevertheless, some differences should be expected since in the light, in addition to biotransformation of the phenolic compound into biomass, there is phototrophic growth, the extent of which is depending on the CO$_2$ availability. The
difference in the growth rates can be positive or negative, depending on the species. Either in the light or in the dark, the presence of glucose increases the specific growth rate and thus the biomass concentration, but this increase does not enhance the specific biodegradation rate [Semple et al., 1996; Tikoo et al., 1997; Papazi et al., 2007]. The addition of glucose improved the removal of pentachlorophenol by *Chlorella VT-1*, but they suggest that this result may be related to the increased number of cells [Tikoo et al., 1997].

In a study, where a model for describing aerobic biodegradation of phenolic compounds by microalgae was proposed [Lika et al., 2009], the results are in accordance with experimental studies that confirm that many microalgae can grow on phenols as the sole carbon source. The authors propose that inhibition or enhancement of bioremoval of substrates should be studied in terms of specific rates. Their results clearly indicate that specific biodegradation rates decrease in the presence of glucose. Thus, they conclude inhibition of bioremoval of phenols in the presence of a nontoxic substrate, such as glucose. Moreover, they numerically observed that the phenolic compound affects the rate of removal of the non-toxic organic substrate. These results are in accordance to another study where a mathematical model is used [Wang et al., 1996]. The authors of this study suggest that there should be an uncompetitive cross-inhibitory interaction between phenol and glucose transformation by *Pseudomonas putida* and that, though the substrates are degraded by different enzymes, each one has some affinity for the enzymes involved in the degradation of the other. Additionally, glucose and phenols were utilized concurrently by *O. danica* in the dark and though the removal of phenols was slower, they had a negligible effect on the rate of glucose removal [Semple et al., 1996].

Several studies [Kar et al., 1996; Semple et al., 1996; Tsai et al., 2005] suggest that removal patterns of the two organic substrates, toxic and nontoxic, are influenced by the acclimation characteristics of the culture. Cultures not acclimated to a substrate show a lag period before initiation of its removal, mainly, because the presence of the substrate is required to initiate the biosynthesis of the enzymes involved in the biotransformation [Tsai et al., 2005]. Moreover, it is known that biodegradation mechanisms in microorganisms (bacteria, fungi and algae) require the presence of molecular oxygen to initiate enzymatic attack on the aromatic rings [Semple et al., 1996], which suggest that the dissolved oxygen may be a limiting factor of the treatment process.

1.4.5.4 Olive Mill Wastewater (OMW)

As previously stated, OMW constitutes a wastewater with a complex composition and a variety of compounds that are not easily biodegradable and, most of the
times, are toxic for microorganisms (§1.3.1). Thus, it is not always a suitable substrate for microorganism growth. Additionally, in reference to microalgae, this particular wastewater displays another limiting factor: it contains small amounts of nitrogen, and all of it is in the form of organic nitrogen. Although microalgae are able to assimilate some forms of organic nitrogen, the nitrogen form that is present in OMW seems to be unusable to the plethora of microalgal strains. Consequently, there are two tactics followed in order to use this wastewater as a substrate for microalgae. The first is the addition of a preferred nitrogen source, and the second is the combination of OMW with another, nitrogen-rich, wastewater. However, even then, the results seem to be less than exciting.

In a study where *Scenedesmus* sp. was selected for the biotreatment of unsterilized OMW in concentration 9% v/v, the addition of BG-11 medium or tap water supplemented with NaNO₃ and K₂HPO₄ was necessary in order to promote microalgal growth [Di Caprio et al., 2015]. The net biomass generation reached a concentration of 0.35 and 0.22 g L⁻¹ respectively, showing the positive effect of the mineral salts present in OMW. The positive effect of the supplementation of BG-11 medium was also exhibited in the degradation of phenols and COD, while it had no effect in the consumption of total carbohydrates. Finally, OMW had a negative effect on the specific growth rates, in both runs, compared to the controls. Both runs were labeled as heterotrophic, even though light was provided, as, because of the dark color of OMW, light cannot penetrate.

In another study, the mixture of olive mill wastewater and urban wastewater from secondary treatment (UWST) as culture medium for *Scenedesmus obliquus* was studied, under the presence of light [Hodaifa et al., 2013]. Two experimental series were prepared. In the first, the culture medium was formed by 0%, 1%, 2.5%, 5%, 10% (v/v) of OMW added to urban wastewater. In the second, a constant proportion (5% v/v) of bleached OMW was maintained and 0, 10, 25, and 50 (v/v) of UWST were added. In cultures where increasing amounts of untreated OMW (1–10% v/v) was added to UWST the biomass concentrations were increased from 0.011 to 0.040 g L⁻¹. However, in cultures where 0–50% (v/v) UWST added to 5% (v/v) bleached OMW the biomass concentrations had increased from 0.011 to 0.302 g L⁻¹. In other words, the color removal of OMW by active carbon, which was found to be the main inhibitor of growth in this case allows a net increase in biomass concentration equal to 10 times.

In a study where olive mill wastewater (OMW) was used for the mixotrophic cultivation of the cyanobacterium *Arthrospira (Spirulina) platensis*, the pretreatment of OMW was essential in order to make it suitable for microalgal growth [Markou et al., 2012]. Namely, the wastewater was treated with sodium hypochlorite (NaOCl), resulting in the decrease of phenol concentration and
turbidity. It was also supplemented with NaNO₃, as the lack of nitrogen was found to be a limiting factor. Maximum biomass production (1.696 g L⁻¹) was obtained when the concentration of OMW in the cultivation medium was 10%, while maximum removal of COD and carbohydrates was 73.18% and 91.19%, respectively. Phenols, phosphorus and nitrate were completely removed in some runs.

To sum up, in most cases, without any pretreatment or addition of nutrients, OMW cannot support algal growth. These results indicate that nutrient bioavailability to microalgae may be very low in this particular wastewater. Many techniques of pretreatment are also applied, so as to eliminate the distinctive color of OMW, and make it suitable substrate for photoheterotrophic and mixotrophic cultivation. Even after the pretreatment and the addition of the essential nutrients, OMW also needs to be diluted to a final concentration around 10%, as the concentration of the contained toxic substances needs to be lowered, in order to be tolerated and maybe degraded by microalgae.

1.4.5.2 Nitrogen

Nitrogen is an essential nutrient for microalgal growth, as it is required to build amino acids, proteins and chlorophyll. The most common forms of inorganic N utilized by photosynthetic organisms are NO₃⁻ and NH₄⁺ [Kotur et al., 2013], with NH₄⁺ being the preferred one as it can be used directly (Figure 1.5). While most algae can take up and assimilate nitrate (NO₃⁻), there are significant exceptions, such as some strains of the marine cyanobacterium Prochlorococcus [Raven, 1984]. In addition, NO₃⁻ assimilation by nitrate reductase requires the presence of iron and, thus, phytoplankton cannot exhaust NO₃⁻ in Fe limited Antarctic oceans. High concentrations of ammonium may inhibit growth of photosynthetic organisms; this has been attributed to various causes [Allen et al., 1986]. One possibility is the excretion of protons produced in ammonium assimilation into organic matter; this ratio is at least one proton per ammonium. If these protons are excreted into a spatially limited or poorly buffered medium, then acidity damage to the organism could occur.

Moreover, the most frequently used organic sources of nitrogen for microalgal cultivation are urea and yeast extract, with urea being the dominant as its cost is lower. It has been found that urea is the best nitrogen source for culturing Chlorella sp. [Becker, 1994]. Additionally, this organic nitrogen source showed positive effects on the growth of Spirulina.
1.4.5.3 Sulfur

Sulfur is an essential element for the growth of photosynthetic organisms. In microalgae sulfur is assimilated as sulfate (SO$_4^{2-}$). Sulfate, after uptake into the cytoplasm, is transported into the plastids, or, if present in excess, stored in vacuoles. At first it is reduced to sulfide (S$_2^-$) and then is incorporated as a thiol group in cysteine, which directly or indirectly, serves as the precursor for all compounds containing reduced sulfur (Figure 1.5). About 40% of the assimilated sulfur is allocated into low molecular weight compounds such as GSH and
dimethylsulfiniopropionate (DMSP), 35% into proteins, 21% into ester-sulfate, and 4% into sulfolipids [Bates et al., 1994].

The most widely known metabolic product of microalgae containing sulfur is undoubtedly dimethylsulfiniopropionate (DMSP). Although most marine microalgae produce DMSP, Chlorophyta and cyanobacteria produce very small amounts, if any. Once in the atmosphere, DMS reacts with NO$_3^-$ and OH$^-$ to generate SO$_2$, which is subsequently oxidized by OH$^-$ to sulfuric acid (H$_2$SO$_4$); H$_2$SO$_4$ is probably quickly converted to NH$_4$HSO$_4$. These products of DMS oxidation act as cloud condensation nuclei and thus affect the radiation balance of the Earth and the acid-base chemistry of the atmosphere. DMS also impacts the local acid-base balance in water and may determine local changes in the equilibria of dissolved inorganic carbon with some effect on photosynthesis, especially in poorly buffered waters [Giordano et al., 2008].

1.4.5.4 Phosphorus

Microalgae can take up inorganic phosphate (P$_i$) at a rate that allows them to successfully compete with other microorganisms in natural conditions and accumulate P up to 2–3% of their cell dry weight [Powell et al., 2009]. The P$_i$ concentrations inside cells are typically close to 5–10 mM [Rasala et al., 2015], independent of external concentrations that may vary. Polyphosphate is the main form of P stored in algal cells, and has a central role in many biological processes [Rasala et al., 2015].

Complex bioavailable P compounds are decomposed to P$_i$ by extracellular or cell-wall-bound phosphatase enzymes. The negative charge of the P$_i$ ions prevents its spontaneous diffusion across the hydrophobic lipid bilayer of the algal cell membrane, which is not only hydrophobic but also negatively charged on its internal side (Figure 1.6). Kinetic and biochemical studies of P$_i$ uptake in microalgae suggest at least two distinct mechanisms of P$_i$ transport across the plasmalemma: one is activated when the P$_i$ concentration in the environment is low and the other when P$_i$ is abundant.

P$_i$ uptake is an active process occurring at the expense of ATP hydrolysis and/or membrane potential energy and involving co-transport of cations (H$^+$ or Na$^+$) [Cembella et al., 1984]). In low-P environments, this energy intensive process can be facilitated by increasing the bioavailable P concentration in the environment through extracellular alkaline phosphatase or by enzymes excreted by other microorganisms. In P-rich environments, passive diffusion can also take place.
When $P_i$ first enters the cell, a considerable part of it is consumed for the biosynthesis of cellular functional building blocks or stored for a possible P shortage (Figure 1.6). Among the functional structures, nucleic acids serve primarily for containing and transferring biological information. Ribosomal RNAs are the most abundant nucleic acid molecules in the cell and play an essential role in protein biosynthesis [Blank, 2012]. A functionally important pool of $P_i$ is involved in phosphorylation and de-phosphorylation of proteins that serve a crucial regulatory role [Ruelland et al., 2015]. Phospholipids are essential structural elements of biological membranes [Khozin- Goldberg et al., 2011].

Phosphorus also plays an indispensable role in energy storage and transduction. The short-term energy storage and effective “energy currency” are the triphosphate bonds of ATP, which is a ubiquitous energy storage form. ATP plays a central role in algae because it is the primary product of photosynthesis. A considerable part of intracellular P is also associated with different P metabolites, primarily sugar phosphates formed with the participation of ATP and other energy-rich phosphorylated compounds. The typical energy storage compounds are carbohydrates or lipids, but significant free energy is also often contained in polyphosphate, which contains tens to hundreds of bonds similar to ATP.
The intracellular $P_i$ may be stored in polyphosphate pools deposited in vesicles or vacuoles. Polyphosphates serve as an internal $P$ depot that cells can use when needed [Nishikawa et al., 2006]. There are 4 different types of polyphosphates (polyphosphate A-D, A, C: acid-soluble; B, D: alkali-soluble), which are constructed according to light and $P_i$ availability. These reserves can be mobilized differentially into nucleic acids, $P$ lipids, or into $P_i$ [Miyachi et al., 1961].

### 1.4.6 Effect of pH

The pH is one of the most important parameters in microalgal growth. In most cases microalgae prefer pH values close to 7, but there are staggering exceptions. For example for the microalga *Dunaliella salina* the optimal pH value is close to 11.5, while for *Dunaliella acidophila* is between 0 and 3 [Varshney et al., 2014]. Of the first magnitude is the acclimation of the microalga to a certain environment. For instance, in a study two acidophilic algae, identified as strains of *Chlorella protothecoides* and *Euglena mutabilis*, were isolated from abandoned copper mines [Ñancucheo et al., 2012]. When the *Chlorella* isolate was cultivated in pH-controlled bioreactors, it grew optimally at pH 2.5. Therefore, each strain has a unique pH range in which it can grow and function properly. Consequently, in order to discuss about optimum pH, one has to focus on a particular strain.

In a study *Chlorella vulgaris* was cultivated under the presence of light, in order to find the optimal value for this strain [Rachlin et al., 1991]. Based on previous studies, the control pH was 6.9. Under acidic conditions, pH 3.0-5.0, the growth of *Chlorella vulgaris* was found to be less (27.3-55.2%) than the control value. At alkaline pH values of 8.3 to 9.0, growth was again reduced respectively to 46.1 - 34.2% of the control. However, at the alkaline pH values of 7.5 and 8.0 growth exceeded control values, indicating an optimization of growth conditions within this narrow pH range.

In another study, where the same strain was cultivated under illumination, the pH values tested were 7.0, 8.0, 9.0, and 10.0 [Gong et al., 2014]. Two series of experiments were conducted: one with adjustment of the initial value alone, and the other under pH-controlled conditions. The authors concluded that the initial pH adjustment, with no further control, does not have a major impact in cell densities, as they observed that regardless of the initial pH value, the pH rose quickly and remained stable at around 10.0. However, under pH-controlled conditions there were noticeable differences. The cell densities increased gradually with the increase of pH, with the optimal value being that of 10.0. Additionally, the cell density in the optimum pH value under controlled conditions was 1.32 times higher than that with the initial pH adjustment alone. Thus, they concluded that the pH adjustment method leads to better performance of microalgae.
In a similar study, the effect of pH on the growth and lipid accumulation of *C. vulgaris* was investigated, using 50% biogas slurry as a basic medium [Wang et al., 2010]. Again, there were two sets of experiments: daily adjustment of pH and adjustment of the initial value alone. The pH values tested were 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5. The interpretation of the results showed that in terms of biomass growth the best performance was received in pH 6.5 and 7.0, while the accumulation of lipids was higher at pH 7.0 - 8.5. Hence, the authors concluded that the optimal pH value for both growth and lipid accumulation of *C. vulgaris* was 7.0. However, little information about this particular study is available, so the cultivation mode is unknown.

In a study where the same strain was used for the treatment of sludge originated from a fermentation system producing VFAs, under the presence of light, again the effect of pH-controlled conditions was investigated at pH 7.0 [Cho et al., 2015]. In this case the pH in the experiments under pH-uncontrolled conditions rose quickly, inhibiting cell growth, mainly due to the consumption of the contained organic acids. The results of this study confirmed that the pH-controlled environment results in higher biomass productivity.

An interesting effect of pH on microalgae is that when it is increased above 9, flocculation of the cells occurs (Picture 1.8). It can happen deliberately with the addition of a strong base, or spontaneously in microalgal cultures as a result of a pH increase due to photosynthetic CO₂ depletion. The latter is known as autoflocculation. This process is associated with the formation of calcium or magnesium precipitates. Depending on the conditions, these precipitates carry positive surface charges and can induce flocculation through charge neutralization and/or sweeping flocculation [Vandamme et al., 2013]. After the cells are aggregated due to the pH shift, gravity forces them to settle on the bottom of the reactor, thus facilitating the harvest. The experimental results suggest that the method is effective, and allows the reuse of the flocculated medium, thereby contributing to the economic production of biodiesel from algae [Wu et al., 2012].

![Picture](image)

**Picture 1.8:** Flocculation of microalgal cells, caused by the increase of pH above 9.
To sum up, although pH is one of the parameters that determine the microalgal growth, it is impossible to establish an optimum value, as it is highly dependent on the species and the cultivation conditions. Even in reference to a particular strain, the literature is somewhat confusing. However, it is safe to say that when the pH of the medium is controlled, the microalgae’s performance is more satisfying, as is the case for every microorganism.

1.4.7 Uses of microalgae

Microalgae are one of the most promising feedstock for sustainable production of valuable chemicals and biofuels (Figure 1.7). Nowadays, food supplements from microalgae comprise an important market in which compounds, such as β-carotene, astaxanthin, and polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and polysaccharides, such as β-glucan, are dominant. Research on algae is not only focusing on improving production of these compounds, but also on the introduction of new algae products, such as biodiesel, bio-ethanol, and renewable chemicals, such as starch, sucrose and ethylene. In addition, microalgae can be used for animal nutrition and biofertilizers. In every case, the raw material for bio-refining these compounds is microalgae biomass, which is produced by culturing microalgae under controlled or semi-controlled conditions.

![Figure 1.7: Potential applications of microalgae.](image)

The following streams of valuable compounds and products are derived from refined microalgae biomass:

- Lipids (triacylglycerides and isoprenoids) for biodiesel.
- Lipids (ω-3 fatty acids, DHA and EPA) for nutritional supplements.
• Lipids, hydrocarbons in general, as feedstock to produce bulk chemicals and fuels.
• Proteins (soluble proteins) for nutritional supplements and personal-care products.
• Proteins (insoluble proteins) for animal feeds.
• Carbohydrates (starch, glycogen, and cellulose) as feedstock to produce bulk chemicals and fuels (i.e. bioethanol).
• Pigments (chlorophyll, β-carotene, lutein, astaxanthin) for nutrient supplements and drugs.
• Oxygen (from photosynthesis) for general use, such as aquaculture.
• Hydrogen (from photosynthesis) used as biofuel.

A more detailed description of the applications of microalgae is presented below.

1.4.7.1 Food supplements - Pharmaceuticals

As mentioned above, microalgae are a source of high value products. In addition they contain bioactive compounds, which are physiologically active substances with functional properties in the human body. They also constitute a protein source, containing all essential amino acids. They contain numerous nutrients, particularly B vitamins (such as thiamin and riboflavin) and dietary minerals such as iron and manganese. Among other ingredients, microalgae contain natural pigments such as chlorophylls and carotenoids in addition to polyphenols, fatty acids and polyunsaturated compounds. These compounds exhibit several beneficial biological activities like antioxidant, anti-carcinogenic, anti-inflammatory, antimicrobial, antiobesity, anti-angiogenic and neuroprotective [Guedes et al., 2011; Pangestuti et al., 2011; Plaza et al, 2010].

• Polyunsaturated fatty acids (PUFAs)

Microalgae contain polyunsaturated fatty acids (PUFAs), such as α-Linolenic acid (ALA 18:3 ω-3), γ-Linolenic acid (GLA, 18:3 ω-6), eicosapentaenoic acid (EPA, 20:5 ω-3), arachidonic acid (ARA, 20:6 ω-6) docosapentaenoic acid (22:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3) [Fraeye et al., 2012; Ryckeboch et al., 2012]. These essential fatty acids (ω-3 and ω-6) are important for the integrity of tissues. γ-Linolenic acid has therapeutic applications in cosmetics; it revitalizes the skin and thus slows aging. Linoleic and linolenic acids are essential nutrients for the immune system and other related tissue regeneration processes. Linoleic acid is also used for the treatment of hyperplasia of the skin [de Jesus Raposo et al., 2013]. Additionally,
microalgae exhibit antimicrobial activity against human pathogens, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*, which has been attributed to γ-Linolenic acid, eicosapentaenoic acid, docosahexaenoic acid, arachidonic acid, hexadecatrienoic acid, palmitoleic acid, lauric acid and oleic acid [Amaro et al., 2011; Smith et al., 2010].

According to Adarme-Vega and colleagues [Adarme-Vega et al., 2012], these long chain ω-3 PUFA provide significant health benefits particularly in reducing cardiac diseases such as arrhythmia, stroke and high blood pressure. As well, they have beneficial effects against depression, rheumatoid arthritis, asthma and can be used for treatment of inflammatory diseases such as rheumatoid arthritis, Crohn’s disease, ulcerative colitis, psoriasis, lupus and cystic fibrosis. Additionally, in pregnant women, the adequate intake of EPA and DHA is crucial for healthy development of the fetal brain. In addition, ARA and DHA are required for normal growth and brain functional development, while EPA is essential for the regulation of some biological functions and prevention of arrhythmia, atherosclerosis, cardiovascular disease and cancer [Pulz et al., 2004].

- **Pigments**

Natural pigments are valuable sources of bioactive compounds. These pigments have various beneficial biological activities such as antioxidant, anticancer, anti-inflammatory, antiobesity, antiangiogenic, and neuroprotective action and are indicated for the treatment or prevention of several chronic diseases [Pangestuti et al., 2011].

Among the carotenoid compounds, β-carotene and astaxanthin are conspicuous. In microalgal metabolism, they protect photosynthetic tissues against damage caused by light and oxygen [Cazzonelli, 2011]. β-Carotene has higher provitamin A activity, which is essential for vision and the correct functioning of the immune system. Scientific findings indicate astaxanthin for multimodal intervention for many forms of degenerative diseases, including cardiovascular diseases, cancer, metabolic syndrome, cognitive impairment, age-related immune dysfunction, stomach and ocular diseases and skin damage [Richardson, 2011].

Fucoxanthin is considered as a promising dietary and weight loss supplement and for the treatment of obesity. Clinical studies by Abidov et al. [Abidov et al, 2010] demonstrated the effect of “xanthigen,” a fucoxanthin based antiobesity supplement. Furthermore, fucoxanthin may be useful for the prevention of bone diseases such as osteoporosis and rheumatoid arthritis. It has also been reported to
be effective for the therapeutic treatment of diabetic diseases, suppressing insulin and hyperglycemia [Pangestuti et al., 2011].

- Polysaccharides

Microalgal polysaccharides contain sulphate esters, are referred to as sulfated polysaccharides, and possess unique medical applications. The biological activity of sulfur polysaccharides is linked to their sugar composition, position, and degree of sulfation [Kim et al., 2012]. Among the microalgae capable of producing these compounds are *Chlorella vulgaris*, and *Scenedesmus quadricauda* [Mohamed, 2008].

Microbial polysaccharides have antiviral and antimicrobial action. Microalgae produce extracellular sulfated polysaccharide (EPS) with acidic characteristics that has a potential as a therapeutic agent [de Jesus Raposo et al., 2014]. Some studies have reported that sulfated polysaccharides derived from microalgae inhibit viral infection [Amaro et al., 2011]. Moreover, sulfur polysaccharides with anti-inflammatory activity can be applied in skin treatments [Matsui et al., 2003].

- Proteins, peptides, and amino acids

Microalgal proteins are of great interest due to their therapeutic potential in the treatment of various diseases [Ibañez et al., 2013]. Proteins, peptides, and amino acids can help to replace damaged tissues, in addition to providing nutritional benefits. Microalgae, such as *Chlorella* and *Spirulina* (*Arthrospira*), may be used to prevent diseases and damage to cells and tissues due to their rich protein content and amino acid profile [de Jesus Raposo et al., 2013]. For instance, dried *Spirulina* contains about 60% (51-71%) proteins [Khan et al., 2005]. Dimethylsulfoniopropionate and mycosporine amino acids, which are antioxidant compounds, were isolated from microalgae and are potent chemical blockers of UV radiation [Mata et al., 2010]. Protein peptides from *Chlorella* have a potential as dietary supplements for the prevention of oxidative stress-related diseases, such as atherosclerosis, coronary heart disease, and cancer [Plaza et al., 2009].

All these features contribute to the high nutritional and pharmaceutical importance of microalgae, and thus, they are increasingly used as a food supplement (Picture 1.9), usually in the form of pellets or powder, otherwise the compounds of interest are extracted, purified and used for pharmaceutical purposes. The most common species used for human nutrition are *Spirulina sp.* and *Chlorella sp.*
1.4.7.2 Biofuels

In this section, the potential of microalgae-derived biofuels is discussed. A brief outline of each procedure is presented below.

1.4.7.2.1 Thermochemical Conversion

Thermochemical conversion is the thermal decomposition of organic components in biomass to yield fuel products, and is achievable by different processes such as direct combustion, gasification, thermochemical liquefaction, and pyrolysis.

- Gasification

Gasification involves the partial oxidation of biomass into a combustible gas mixture at high temperatures (800 – 1000°C). In the normal gasification process, the biomass reacts with oxygen and water (steam) to generate syngas, a mixture of CO, H₂, CO₂, N, and CH₄. Syngas is a low calorific gas (typical 4–6 MJ m⁻³) that can be burnt directly or used as a fuel for gas engines or gas turbines.

*Spirulina* biomass gasification at 1000°C produced the highest theoretical yield of 0.64 g methanol from 1 g of biomass [Hirano et al., 1998]. An energy balance (ratio of methanol produced to the total required energy) of 1.1 was estimated, which gives gasification a positive energy balance, with the low value being attributed to the use of centrifuge during biomass harvesting. Gasification of the microalgae *C. vulgaris* in a novel system with nitrogen cycling resulted in a methane-rich fuel with all the nitrogen component of the microalgae converted into fertilizer quality ammonia [Minowa et al., 1999].
Thermochemical liquefaction

Thermochemical liquefaction is a low-temperature (300–350°C), high pressure (5–20 MPa) process aided by a catalyst in the presence of hydrogen to produce bio-oil. Reactors for thermochemical liquefaction and fuel-feed systems are complex and therefore expensive, but have advantages in their ability to convert wet biomass into energy, and, thus, eliminating the cost of drying.

Thermochemical liquefaction at 300°C of B. braunii resulted in a maximum yield of 64% dry wt. basis of oil with a Higher Heating Value (HHV) of 45.9 MJ kg⁻¹ and also declared a positive energy balance for the process (output/input ratio of 6.67:1) [Dote et al., 1994]. These results indicate that thermochemical liquefaction is a viable option for the conversion of algal biomass-to-liquid fuel.

Pyrolysis

Pyrolysis is the conversion of biomass to bio-oil, syngas and charcoal at medium to high temperatures (350–700°C) in the absence of air. This process, it is believed to have the potential for large scale production of biofuels that could replace petroleum based liquid fuel. Flash pyrolysis (500°C, hot vapor residence time ~1s) is considered to be a viable technique for biomass derived liquid fuels mainly because of the high biomass-to liquid conversion ratio (95.5%) that can be achieved. However, there are technical challenges as pyrolysis oils are acidic, unstable, viscous, and contain solids and chemically dissolved water. Therefore, the produced oil will require upgrading in order to lower oxygen content and remove alkalis [Demirbas, 2001].

Fast pyrolysis of heterotrophically grown microalgae *Chlorella prothetecoides* recorded oil yield of 57.9% dry wt. basis (HHV of 41 MJ kg⁻¹) and was 3.4 times higher than achieved by phototrophic cultivation [Miao et al., 2004]. In another study, bio-oil yields of 18% (HHV of 30 MJ kg⁻¹) and 24% (HHV of 29 MJ kg⁻¹) were achieved with fast pyrolysis of *C. prothetecoides* and *Microcystis aeruginos*, respectively, grown phototrophically [Miao et al., 2004]. Experiments with *C. prothetecoides* [Demirbas, 2006] showed that bio-oil yield increased in line with temperature increase up to a point and then decreased at higher temperatures. For example, the yield rose from 5.7% to 55.3% with an increase from 254 to 502°C, and subsequently decreased to 51.8% at 602°C. Results indicate that bio-oils from microalgae are of a higher quality than those extracted from lignocellulosic materials [Miao et al., 2004, Demirbas, 2006], and this process has potential in algal biomass to liquid biofuel conversion.

Direct combustion

In a direct combustion process, biomass is burnt in the presence of air to convert the stored chemical energy in biomass into hot gases, usually in a furnace, boiler, or
steam turbine at temperatures above 800°C. It is possible to burn any type of biomass, provided that the moisture content is less than 50% dry weight. The heat produced must be used immediately as storage is not a viable option. Energy conversion by direct biomass combustion has the disadvantage of biomass generally requiring pre-treatment processes such as drying, chopping and grinding, processes which increase the expenses. Net energy conversion efficiencies for biomass combustion power plants range from 20% to 40% [Demirbas, 2001]. There is little evidence of technically viable utilisation of algal biomass in direct combustion in literature, but a life cycle assessment (LCA) of coal-algae co-firing [Kadam, 2002] suggested that it could lead to lower greenhouse gas emissions and air pollution.

1.4.7.2.2 Biochemical Conversion

The biochemical processes of converting biomass into biofuels include anaerobic digestion, alcoholic fermentation and photobiological hydrogen production.

- Anaerobic digestion

Anaerobic digestion (AD) is the conversion of organic wastes into a biogas, which consists of primarily methane (CH₄) and carbon dioxide, with traces of other gases. The process is described thoroughly in § 1.7.

Microalgae usually have a high proportion of proteins that result in low C/N ratios which can affect the performance of the anaerobic digester. This problem may be resolved by co-digestion with a high C/N ratio product. A significant increase in methane production was achieved with the addition of waste paper to algal biomass [Yen et al., 2007]. Double the methane production rate was obtained (1.17 mL L⁻¹ day⁻¹ vs. 0.57 mL L⁻¹ day⁻¹) from 50/50 waste paper/algal biomass blend compared to anaerobic digestion of pure algal biomass. High protein content in the algae can also result in increased ammonium production, which inhibits anaerobic microorganisms. There are many obstacles that need to be overcome so that AD of microalgae is a feasible solution. However, it has been estimated that the conversion of algal biomass into methane could recover as much energy as obtained from the extraction of cell lipids [Sialve et al., 2009], while leaving a nutrient rich waste product that can be recycled into a new algal growth medium.

- Alcoholic fermentation

Alcoholic fermentation is the conversion of biomass materials which contain sugars, starch or cellulose into ethanol. The biomass is ground down and the starch is converted to sugars which is then mixed with water and yeast and kept warm in
fermenters. The yeast breaks down the sugar and converts it to ethanol. A purification process (distillation) is required to remove the water and other impurities in the diluted alcohol product (10–15% ethanol). The solid residue from the process can be used for animal feed or for gasification. Starch based biomass like microalgae require additional processing before fermentation [Demirbas, 2001]. Microalgae such as C. vulgaris are a good source of ethanol due to the high starch content (~37% dry wt.), and for which up to 65% ethanol conversion efficiency has been recorded [Hirano et al., 1997]. From the outlined concepts it is arguable that ethanol production from microalgae is technically viable.

- **Hydrogen production**

Microalgae possess the necessary genetic, metabolic and enzymatic characteristics to produce H₂ gas [Ghirardi et al., 2000], which constitutes an efficient energy carrier. During photosynthesis, microalgae convert water molecules into hydrogen ions (H⁺) and oxygen; the hydrogen ions are subsequently converted into H₂ under anaerobic conditions [Cantrell et al., 2008]. Due to reversibility of the reaction, hydrogen is either produced or consumed by the simple conversion of protons to hydrogen [Clark et al., 2008]. Photosynthetic oxygen production causes rapid inhibition to the key enzyme, hydrogenase, and the photosynthetic hydrogen production process is impeded [Cantrell et al., 2008]. Consequently, microalgae cultures for hydrogen production must be subjected to anaerobic conditions.

There are two fundamental approaches for photosynthetic H₂ production. The first is a two stage photosynthesis process where photosynthetic oxygen production and H₂ gas generation are separated procedures [Ghirardi et al., 2000]. In the first stage, algae are grown photosynthetically in normal conditions. During the second stage, the algae are subjected to anaerobic conditions and, thus, stimulating consistent hydrogen production [Melis et al., 2001]. This production process becomes limited with time, as hydrogen yield will begin to level off after 60 h of production. The use of this production system does not generate toxic or environmentally harmful products but could give valuable products as a result of biomass cultivation.

The second approach involves the simultaneous production of photosynthetic oxygen and H₂ gas. The H₂ productivity is theoretically superior to the two-stage photosynthetic process, but the simultaneous production process suffers severe hydrogenase inhibition after a very short period due to the photosynthetic production of oxygen [Ghirardi et al., 2000]. It was found that using the two-stage photosynthesis process and H₂ production a theoretical maximum yield of hydrogen by green algae could be about 198 kg H₂ ha⁻¹ day⁻¹ [Melis et al., 2001]
1.4.7.2.3 Microalgal Biomass Conversion to Biodiesel

One of the most distinctive features of microalgae is that they accumulate intracellular lipids, which and can be drastically increased when at least one of the essential nutrients (such as nitrogen, phosphorus and sulfur) is deprived. These lipids in many cases are suitable for biodiesel, depending mainly on the type of the strain and the cultivation mode. They compete favorably with oil producing crops by their potential of producing 10–20 times more oil [Demirbas, 2011] within a shorter period of time. Accumulation of lipids depends on several factors, such as culture temperature, pH, quantity of nutrients and carbon source, growth mode and the type of strain [Perez-Garcia et al., 2011]. When converted to biodiesel, the lipids produced constitute an attractive source of energy.

Biodiesel is a mixture of monoalkyl esters of long chain fatty acids (FAME) derived from a renewable lipid feedstock, which can be used directly in conventional diesel engines. A detailed report of its composition, properties and standard specifications that should be followed is presented in §1.6. Microalgal biodiesel has similar physical and chemical properties to petroleum diesel. However, the former has several advantages over the latter: (1) it is derived from biomass and therefore is renewable and biodegradable; (2) it is non-toxic and contains reduced levels of particulates, carbon monoxide, soot, hydrocarbons and SOx; (3) it results in reduced CO₂ emissions of up to 78% compared to emissions from petroleum diesel.

The reality is that a remarkable number of obstacles need to be overcome in order to achieve the commercialization of algal biofuels on a large scale, as the high production costs make it a solution far from competitive with fossil fuel. Hence, it is a subject that needs further research so that microalgal biofuel production becomes an overall sustainable source of fuel.

1.4.7.3 Wastewater Treatment

Most domestic and industrial wastewaters contain organic carbon, nitrogen, phosphorus, and other nutrients. This composition makes wastewater suitable for growing microalgae. Besides growing microalgal biomass for valuable compound production, the wastewater is simultaneously treated. Because of this double benefit, this tactic has attracted extensive attention in recent years.

Microalgae can assimilate soluble organic compounds found in wastewater. Auxenochlorella protothecoides grown mixotrophically in municipal wastewater decreased the COD concentration from 2.5 to 0.5 g L⁻¹ [Hu et al. 2012]. Probably the remaining 0.5 g L⁻¹ belonged to the non-biodegradable COD fraction. Chlorella pyrenoidosa, growing in sterilized sewage, was able to use some of the organic
matter, as indicated by a decrease in soluble BOD and dissolved volatile solids (VSS). However, not all organic chemical substances can be used as a carbon source for microalgae since some are known to inhibit microalgal growth [Chen 1996; Perez-Garcia et al. 2011]. Various studies show that supplementing readily-biodegradable carbon sources, such as glucose or acetate, is necessary to achieve the dual goal of high nutrient removal efficiency and high biomass production [Perez-Garcia et al. 2011; Rawat et al. 2011]. Naturally, adding extra nutrients for the purpose of increasing biomass production can lead to a wastewater even more polluted than the original one, even after the cultivation of microalgae. Microalgae have been reported to achieve high nitrogen and phosphorus removal from various wastewaters. Auxenochlorella protothecoides and Chlorella sp. achieved ammonium removal efficiencies from 52 to 72% in mixotrophic cultures, where, total nitrogen removal efficiencies ranged from 64 to 74% [Hu et al. 2012, 2013]. In addition, A. protothecoides removed 75% of the initial phosphorus concentration of 125 mg PO₄ L⁻¹ in municipal wastewater. However, in this study, removing phosphorus from mixotrophic cultures was a consequence of microalgae uptake, together with chemical precipitation of dissolved phosphorus.

Under realistic conditions of cultivation, increasing the algae competitiveness against fast-growing native bacteria in the wastewater and, at the same time, produce high quantities of biomass and lipids is still a major research challenge. The use of a microalgae consortium may increase the robustness of a microalgae population in wastewater under any cultivation regime. Autotrophic mixed cultures, dominated by C. vulgaris populations, showed higher biomass and lipid production than pure C. vulgaris cultures. In addition, lipid production in autotrophic mixed cultures was higher than that in heterotrophic culture [Zhang et al. 2014]. A microalgal consortium, mostly formed by Chlorella, Chlamydomonas, Scenedesmus, and Gloeocystis species cultivated in wastewater from the carpet industry, using raceway ponds, showed higher biomass production and removal of nitrogen and phosphorus than 13 pure strains [Chinnasamy et al. 2010].

Another strategy is the use of competitive local strains from wastewater environments. For example, native microalgae isolated from carpet industry wastewaters, such as Chlamydomonas globosa, Scenedesmus bijuga, and Chlorella minutissima were able to grow mixotrophically and removed nitrogen and phosphorus far better in dark, colored, and opaque wastewaters rich in organic and inorganic nutrients than in the standard laboratory medium [Bhatnagar et al. 2011]. Three strains of microalgae (Scenedesmus sp. ZTY2, Scenedesmus sp. ZTY3, and Chlorella sp. ZTY4) isolated from a domestic wastewater treatment plant were heterotrophically cultivated in real domestic wastewater with no illumination. The isolated strains contained lipid content from 55 to 80% of cell dry weight, while efficiencies of removal of dissolved organic carbon ranged from 55 to 65% [Zhang et
It is important to emphasize that under mixotrophic cultivations, microalgal consortia achieved higher biomass production than heterotrophic cultivations. New kinds of wastewater are being tested as potential sources of organic substrate for heterotrophic microalgal growth. For example, dairy manure and food waste hydrolysate contain high levels of nutrients that can be assimilated from microalgae [Wang et al. 2010, Pleissner et al. 2013].

Some wastewaters need a pretreatment to make them available as carbon sources for microalgae cultivation, as it eliminates side effects of containing solid particles and indigenous bacteria. Large amounts of glucose, nitrogen, and phosphate were recovered from food waste by fungal hydrolysis. Schizochytrium mangrovei and Chlorella pyrenoidosa grew well on the recovered nutrients. Growth rate, biomass, and lipid production were higher in cultures cultivated on food waste hydrolysate than in control cultures growing on conventional medium with glucose. At the end of the cultivation, 10–20 g of biomass, out of the original 100 g food waste (dry weight) were produced and were rich in carbohydrates, lipids, proteins, and saturated and polyunsaturated fatty acids [Pleissner et al. 2013]. In another example, cultivation of microalgae on swine manure proved to be a practical and economical organic substrate for production of algae feedstock [Hu et al., 2013]. In their study, acidogenic anaerobic digestion was used as a pretreatment of fresh swine manure before it was used as the substrate in heterotrophic cultures of Chlorella sp. It yielded nutrient removal rates of 751.33 mg COD L⁻¹ day⁻¹, 20.21 mg PO₄-P L⁻¹ day⁻¹ and 60.39 mg NH₃-N L⁻¹ day⁻¹. At the same time, the experiment achieved lipid productivity of 3.63 g m² day⁻¹ [Hu et al., 2013]. In another experiment, wastewater produced from hydrothermal liquefaction of biomass was mixotrophically grown in mixed cultures of microalgae and bacteria. This wastewater had high concentrations of COD (50 - 130 g L⁻¹), total nitrogen (5 - 20 g L⁻¹), ammonia (3 to 12 g L⁻¹) and phosphorus (0.6 - 2 g L⁻¹) and consequently, it needed to be diluted so as to be used for microalgae cultivation [Zhou et al. 2013].

Because of their versatile metabolism and their capacity to switch rapidly from one growth mode to another, mixotrophic microalgae can be successfully employed for remediating polluted environments [Subashchandrabose et al. 2013]. The ecological advantage of employing mixotrophic microalgae for bioremediation is that decreasing concentrations of organic pollutants will have no adverse effect on their growth. In contrast, if a heterotrophic regime is employed, no further biomass production can be expected after degradation of the organic pollutant. The dual-purpose process of chemical production and wastewater treatment, as a general strategy for many microalgal systems, though it looks very attractive, has many challenges.

The main drawbacks of this approach are the following:
• Some wastewater may be too toxic to support microalgal growth
• Outdoor wastewater treatment is significantly affected by seasonal changes
• Competition among the microbial community in the wastewater may lead to very slow algal growth.

Consequently, using microalgae to treat wastewater is problematic for high biomass productivity. Therefore, with current knowledge, expectations should be cautious. With better strains and better technologies, we may be able to achieve the microalgal potential and make the process efficient and cost effective [Liang 2013].

1.4.7.4 Biofertilizers

Blue-green microalgae have been reported to benefit plants by producing growth promoting regulators, vitamins, amino acids, polypeptides, antibacterial and antifungal substances, and polymers such as exopolysaccharides that improve plant growth and productivity [de Mulé et al., 1999]. The bio-fertilisation effect using microalgae extract are recommended for increasing the growth parameters, as well as pigments content of many plants [Adam, 1999; Saffan, 2001]. This is due to the biochemical profile of algae extract rich in nitrogenase, nitrate reductase, and minerals, which are essential nutrients for plant growth.

1.4.7.5 Animal Nutrition

It is estimated that about 30% of microalgal production is used for animal feed [Becker, 2007]. This type of nutrition showed positive effects on the animal’s health. Feeding phototrophically grown microalgae C. vulgaris to animals such as fish and poultry showed interesting pigmentation potential for fish flesh and egg yolk in poultry, together with enhancing health and increasing life expectancy of animals [Yamaguchi, 1996; Becker, 2007]. This outcome was attributed to the accumulated pigments – a result of this cultivation mode. Moreover, C. vulgaris showed a protective effect against heavy metals and other harmful compounds (lead, cadmium, and naphtalene) by reducing significantly the oxidative stress induced by these harmful compounds, and increasing the antioxidant activity in the organisms of tested animals [Vijayavel et al., 2007; Yun et al., 2011].

1.4.8 Chlorella vulgaris

C. vulgaris is a freshwater, eukaryotic, unicellular green algae with spherical shape and a size varying from 2 to 10 μm in diameter [Yamamoto, 2005]. It multiplies by
autosporulation (Figure 1.8), which is the most common asexual reproduction in algae [Yamamoto, (2004, 2005)]. Four daughter cells are formed inside the cell wall of the mother cell, which, after maturation, are liberated when the mother cell wall ruptures (Picture 1.10). The debris of the mother cell will be consumed as feed from the newly formed cells. The main reason of this strain attracting much attention is that it constitutes a versatile microorganism. It has the ability to grow fast, as it has been reported that it has a doubling time close to 6.5h when cultured in fermenters [Harel et al., 2004]. In addition, it is able to grow on different kinds of media and wastewaters, on extreme conditions such as extreme pH values and temperatures [Converti et al., 2009] and tolerate the presence of toxic compounds such as phenols [Scragg, 2006, Gao et al., 2011, Sahinkaya et al., 2009], and heavy metals [Alam et al., 2015].

![Figure 1.8: Drawings showing the different phases of daughter cell-wall formation in C. vulgaris: (a) early cell-growth phase; (b) late cell-growth phase; (c) chloroplast dividing phase; (d) early protoplast dividing phase; (e) late protoplast dividing phase; (f) daughter cells maturation phase and (g) hatching phase [Yamamoto et al., 2005](image)](image)

Picture 1.10: Newly formed cells emerging outside the cell wall of the mother cell after hatching [Yamamoto et al., 2004].

Furthermore, *C. vulgaris* cells have an interesting and complex composition. This particular microalgal strain has high concentration of carbohydrates, proteins, and pigments, as well as contains a wide range of vitamins. A more detailed description of *C. vulgaris* cell content is presented below.
Starch, which is stored in microalgal cells mainly under unfavorable conditions, is the most abundant polysaccharide in C. vulgaris. It is generally located in the chloroplast and is composed of amylose and amyllopectin, and together with lipids they serve as energy storage for the cells. Cellulose is a structural polysaccharide with high resistance, which is located on the cell wall of C. vulgaris as a protective fibrous barrier. In addition, one of the most important polysaccharides present in C. vulgaris is the $\beta_{1\rightarrow3}$ glucan [Lordan, et al., 2011], which has multiple health and nutritional benefits. During nitrogen limitation, total carbohydrates can reach 12–55% dry weight [Branyikova et al., 2011; Choix et al., 2012]. Moreover, C. vulgaris has a remarkably robust cell wall, mainly composed of a chitosan like layer, cellulose, hemicellulose, proteins, lipids and minerals [Griffiths et al., 1969; Abo-Shady et al., 1993]. The sugar composition of the cell wall is a mixture of rhamnose, galactose, glucose, xylose, arabinose and mannose, with rhamnose being the dominant sugar [Takeda, 1991].

Proteins are involved in essential roles in microalgal cells, such as growth, repair and maintenance of the cell as well as serving as chemical messengers, regulators of cellular activities and defense against foreign invaders [Solomon et al., 1999]. Total proteins content in mature C. vulgaris represents 42–58% of biomass dry weight [Becker et al., 1994; Safi et al., 2014], and varies according to growth conditions. Almost 20% of the total proteins are bound to the cell wall, more than 50% are internal and 30% migrate in and out of the cell [Berliner, 1986]. Protein nutritional quality is determined by its amino acid profile, and like the majority of microalgae, the amino acid profile of C. vulgaris compares favorably and even better with the standard profile for human nutrition proposed by World Health Organization (WHO) and Food and Agricultural Organization (FAO). Therefore, proteins of C. vulgaris open the gate for additional valorization options of this microalga in the food market.

Additionally, as a photosynthetic microorganism, it contains pigments. The most abundant pigment in C. vulgaris is chlorophyll, which can reach 1–2% dry weight and is situated in the thylakoids. It also contains β-carotene, astaxanthin, cantaxanthin and lutein [Cha et al., 2010; Chacón-Lee et al., 2010]. These pigments have multiple therapeutic properties (see §1.5.7.1).

C. vulgaris also has an interesting vitamin profile, containing B vitamins (B$_1$, B$_2$, B$_3$, B$_5$, B$_6$, B$_7$, B$_9$, B$_{12}$), as well as vitamins A, C and E [Yeh et al., 2011; Panahi et al., 2012]. Vitamins profile is sensitive to growth conditions. During heterotrophic conditions vitamins content was higher than autotrophic due to the presence of glucose in the medium and used as carbon source to produce organic compounds. Another possible explanation for the high content of vitamins may be the alterations in the photosynthetic apparatus which were found to be associated with changes in cellular components.
Moreover, it has the ability to accumulate intracellular lipids. Under optimal conditions, the lipid content of *C. vulgaris* has been reported to be about 5–40 %, in percentage of dry matter [Becker, 1994], but it can be increased up to 58% under unfavorable growth conditions, where the lipids produced are mainly composed of triacylglycerols [Becker, 1994]. Unlike other lipids, triacylglycerols do not play a structural role, but instead accumulate as storage lipid droplets [Hu et al., 2008]. The fatty acid profile varies with respect to growth conditions. For instance, it has been reported by Yeh and Chang [Yeh et al., 2011] that when grown mixotrophically under nitrogen limitation, *C. vulgaris* can accumulate 60-68% saturated and monounsaturated fatty acids, mainly consisted of palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1) and oleic acid (C18:1) [Zheng et al., 2011]. This profile makes this strain an eligible candidate for biodiesel production, as these saturated and monounsaturated fatty acids constitute the desired source for biodiesel production [Hoekman et al., 2012]. It also has properties [Wang et al., 2013] that compare favorably with the US Standard (ASTM6751), European Standard (EN14214), Brazilian National Petroleum Agency (ANP255) and Australian Standard for biodiesel [Francisco et al., 2010]. On the contrary, when there is no limiting substrate and thus the conditions are favorable for its growth, its fatty acid profile is unsuitable biodiesel production [Yeh et al., 2011], but proper for nutritional uses, as it is mainly composed by polyunsaturated fatty acids such as linoleic acid (C18:2), linolenic acid (C18:3), and eicosapentaenoic acid (C20:5) [Chen et al., 2011].

After lipid extraction the remnant is rich in proteins, carbohydrates and minor amounts of lipids. Thus, Wang et al. [Wang et al, 2013] treated *C. vulgaris* residue with fast pyrolysis using an atmospheric-pressure fluidised bed reactor at 500°C and obtained bio-oil, biochar and a small amount of biogas with a 94% energy recovery from the remnant. However, the quality of bio-oil turned out to be poor due to the presence of nitrogen in significant amounts (12.8% dry weight). As mentioned above, many studies are focused on the production of various kinds of biofuels from this particular strain.

### 1.5 Biodiesel

Biodiesel is defined by ASTM (American Society for Testing and Material) as “a fuel comprised of monoalkyl esters of long-chain fatty acids derived from vegetable oils or animal fats, designated B100”. The feedstock used to produce biodiesel is known as triacylglycerides (TAGs), or more simply, triglycerides. Biodiesel is produced by a chemical reaction known as transesterification (Figure 1.9), in which the triglycerides are reacted with alcohols, in the presence of a catalyst (acid, base or enzyme), to produce fatty acid alkyl esters. Except of the catalyst, usually heat is used to speed
the reaction. Since the most common alcohol used to produce biodiesel is methanol, another name for biodiesel is Fatty Acid Methyl Esters (FAMEs). A byproduct of transesterification is glycerine, also known as glycerol. Unless otherwise indicated, the term “biodiesel” refers to 100% FAME (B100). Lower concentrations, such as B20, are properly referred to as “biodiesel blends”.

\[
\text{CH}_2\text{COO} - R_1 \\
\text{CHCOO} - R_2 \\
\text{CH}_2\text{COO} - R_3 \\
\text{Triglyceride}
\]

\[
+ 3\text{R'}\text{OH} \xrightarrow{\text{Catalyst}} \\
\text{R}_1\text{COO} - \text{R'} \\
\text{R}_2\text{COO} - \text{R'} \\
\text{R}_3\text{COO} - \text{R'} \\
\text{Esters}
\]

\[
\text{CH}_2\text{OH} \\
\text{CH}_2\text{OH} \\
\text{CH}_2\text{OH} \\
\text{Glycerol}
\]

**Figure 1.9:** Transesterification reaction

The term “1× Generation” is used for biodiesel produced via transesterification of triglycerides from edible feedstocks like vegetable oils and animal fats, while the term “2× Generation” is used for biodiesel produced from non-edible triglyceride sources, like yellow grease (used cooking oil), lignocellulose, jatropha and microalgae grown in wastewaters.

### 1.5.1 Biodiesel Composition

Biodiesel can be produced by transesterification of any triglyceride feedstock. Nowadays, however, the dominant feedstocks are soybean oil in the U.S., rapeseed oil in Europe, and palm oil in Southeast Asia [Hoekman et al., 2009; Biodiesel 2020]. Animal fats and used cooking oil (yellow grease) represent significant markets for biodiesel in many locations. Other vegetable oils having real or potential commercial interest as biodiesel feedstocks include camelina, canola, coconut, corn, jatropha, safflower, and sunflower. In addition, there is great interest in utilizing microalgal lipids as biodiesel feedstocks, as it appears that algae have the potential to produce significantly larger annual volumes of biodiesel per cultivation area as compared to other sources, due to the fact that large volumes of microalgal biomass can be produced in relatively small cultivation areas and small amounts of time.

Although biodiesel fuel produced from transesterification of triglycerides contains numerous individual FAME species, a particular fuel is generally dominated by only a few species. More specifically, 5 species typically dominate the composition of FAME derived from vegetable oils and animal fats: palmitic acid (C16:0), stearic acid
(C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). Some microalgal lipids are dominated by these same fatty acid groups, while other microalgae are more diverse in their composition, containing significant amounts of several other FA groups [Hoekman et al., 2012]. In addition, yellow grease is a material with heterogeneous nature [Hoekman et al., 2012]. That is not surprising, as it is composed of used cooking oil from various sources and, thus, its composition varies accordingly.

Four triglyceride sources are predicted by EPA (Environmental Protection Agency) to provide most of the total volume of biodiesel needed to satisfy the RFS2 (Renewable Fuel Standard) requirements for biomass-based diesel by the year 2022. Those are: soy oil (660 million gallons/year), corn oil (680 mg/y), and yellow grease (230 mg/y), with the remainder predicted to come from microalgae (100 mg/y) [U.S., EPA, 2010].

Most microalgae that have been investigated as potential biodiesel feedstocks are green (Chlorophyta). Their lipid content was found to contain, except for triglycerides which are the desired component, small amounts of wax esters, sterols, tocopherols, hydrocarbons, and others impurities [Hoekman et al., 2012]. Just as with the vegetable oil feedstocks, triglyceride production within algae varies considerably from one species to the next. Additionally, it is known that for microalgae the FA compositional profiles are highly influenced by specific growth conditions such as nutrient levels, temperatures, and light intensities [Hu et al., 2008]. This makes it more difficult to define a single compositional profile for algal-based biodiesel, as compared to vegetable oil-based biodiesel.

Comparison of microalgal oil with the vegetable oil FA profiles reveals several interesting features. First, although most of these algal species have considerable amounts of C16 and C18 species, they are not as dominated by these species as are most vegetable oils. Second, some microalgal FA profiles are broader than those of vegetable oils, containing significant amounts of both lighter species (C12–C14) and heavier species (C20–C22). Third, many of the algal profiles contain substantial amounts of highly unsaturated species, including FAs with 3–6 double bonds [Hoekman et al., 2012].

1.5.2 Biodiesel Properties

Because of its considerable oxygen content (typically 11%), biodiesel has lower carbon and hydrogen contents compared to diesel fuel, resulting in about a 10% lower mass energy content. However, due to biodiesel’s higher fuel density, its volumetric energy content is only about 5–6% lower than petroleum diesel. Typically, biodiesel has higher molecular weight than petroleum diesel. Consisting
mainly of straight chain esters, most biodiesel fuels have excellent cetane content. The viscosity of most biodiesel fuels is significantly higher than petroleum diesel, often by a factor of 2. The following table (Table 1.3) compares the properties of biodiesel, to those of petroleum diesel:

Table 1.3: Typical properties of biodiesel compared to petroleum diesel [Hoekman et al., 2012].

<table>
<thead>
<tr>
<th>Property</th>
<th>No. 2 Petroleum Diesel</th>
<th>Biodiesel (FAMEs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon, wt.%</td>
<td>86.8</td>
<td>76.2</td>
</tr>
<tr>
<td>Hydrogen, wt.%</td>
<td>13.2</td>
<td>12.6</td>
</tr>
<tr>
<td>Oxygen, wt.%</td>
<td>0.0</td>
<td>11.2</td>
</tr>
<tr>
<td>Specific Gravity</td>
<td>0.85</td>
<td>0.88</td>
</tr>
<tr>
<td>Cetane no.</td>
<td>40-45</td>
<td>45-55</td>
</tr>
<tr>
<td>T90, °C</td>
<td>300-330</td>
<td>330-360</td>
</tr>
<tr>
<td>Viscosity, mm²/s at 40°C</td>
<td>2-3</td>
<td>4-5</td>
</tr>
<tr>
<td>Energy content (Lower Heating Value)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mass Bassis, Mj/kg</td>
<td>43</td>
<td>39</td>
</tr>
<tr>
<td>Mass Bassis, BTU/lb</td>
<td>18,500</td>
<td>16,600</td>
</tr>
<tr>
<td>Volume Bassis, 1000 BTU/gal</td>
<td>130</td>
<td>121</td>
</tr>
</tbody>
</table>

In order to compare the properties of biodiesel derived from different oil sources, it is essential to take into consideration the standard specifications that have been established by various fuel standard-setting organizations, particularly ASTM (U.S.) and the European Committee for Standardization (CEN). Table 1.4 summarizes the established specifications for biodiesel.

Two properties that greatly influence the overall behavior and suitability of FAME as a diesel blendstock are: (1) the size distribution of the fatty acid (FA) chains and (2) the degree of unsaturation within these FA chains. Some of the specifications comprising the regulatory standards for biodiesel are directly related to the chemical composition of the FAME – such as viscosity, cetane number, cloud point, distillation, and iodine value. Other specifications relate to the purity of the FAME product, and address issues pertaining to production processes, transport, and storage – such as flash point, methanol content, metals content, sulfur level, acid number, and cold soak filterability. Oxidative stability is an important property of biodiesel that is influenced by both FAME chemical composition and by storage and handling conditions [Hoekman, 2012].
**Table 1.4**: U.S. and European specifications for biodiesel (B100) and biodiesel blends [Hoekman et al., 2012].

<table>
<thead>
<tr>
<th>Property</th>
<th>Biodiesel B100</th>
<th>B6-B20 Blends</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water and sediment (vol.%, max)</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Total contamination (mg/kg, max.)</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Kinematic viscosity @ 40 °C (mm2/s)</td>
<td>1.9-6.0</td>
<td>3.5-5.0</td>
</tr>
<tr>
<td>Flash point, closed cup (°C, min)</td>
<td>93</td>
<td>101</td>
</tr>
<tr>
<td>Methanol (wt.%, max.)</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Cetane no. (min)</td>
<td>47</td>
<td>51</td>
</tr>
<tr>
<td>Sulfated ash (wt.%, max.)</td>
<td>0.020</td>
<td>0.020</td>
</tr>
<tr>
<td>Total ash (wt.%, max.)</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>Gp I metals Na + K (mg/kg, max.)</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Gp II Metals Ca + Mg (mg/kg, max.)</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Total Sulfur (ppm, max.)</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>Phosphorous (ppm, max.)</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Acid no. (mg KOH/g, max.)</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Carbon residue (wt.%, max)</td>
<td>0.05</td>
<td>0.30</td>
</tr>
<tr>
<td>Free glycerin (wt.%, max.)</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Total glycerin (wt.%, max.)</td>
<td>0.24</td>
<td>0.25</td>
</tr>
<tr>
<td>Mono glyceride (wt.%, max)</td>
<td></td>
<td>0.80</td>
</tr>
<tr>
<td>Diglyceride (wt.%, max)</td>
<td></td>
<td>0.20</td>
</tr>
<tr>
<td>Triglyceride (wt.%, max)</td>
<td></td>
<td>0.20</td>
</tr>
<tr>
<td>Distillation (T90-°C, max.)</td>
<td>36</td>
<td>343</td>
</tr>
<tr>
<td>Copper strip corrosion (3h at 50-°C, max.)</td>
<td>No. 3</td>
<td>No. 1</td>
</tr>
<tr>
<td>Oxidation Stability (h @ 110 °C, min)</td>
<td>3.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Linolenic acid methyl ester (wt.%, max)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyunsaturated acid methyl esters (wt.%, max)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ester Content (wt.%, min)</td>
<td>96.5</td>
<td>6-20 vol.%</td>
</tr>
<tr>
<td>Iodine Value (g I2/100 g, max.)</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>Density (kg/m3)</td>
<td>860-900</td>
<td></td>
</tr>
<tr>
<td>Lubricity at 60 °C, WSD, microns (max.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cold Soak Filterability (seconds, max.)</td>
<td>360</td>
<td></td>
</tr>
</tbody>
</table>
An increasingly recognized problem with some biodiesel fuels is their propensity to form insoluble precipitates upon storage at low temperature. In large part, this problem is believed to be due to the presence of trace impurities – particularly sterol glucosides and saturated monoglycerides. These same impurities impart favorable lubricity performance to biodiesel; thus efforts to eliminate them could have an unintended consequence of worsening lubricity [Hoekman et al., 2012].

Due to the conflicting effects certain FAME compositional features upon different fuel properties, it is not possible to define a single composition that is optimum with respect to all important properties. However, useful guidelines can be offered with respect to two critical biodiesel fuel properties: (1) low temperature performance and (2) oxidative stability. For good low temperature performance, biodiesel should have low concentrations of long-chain saturated FAME. For good oxidative stability, biodiesel should have high concentrations of saturated and monounsaturated FAME, but low concentrations of multi-unsaturated FAME. For this reason, the oxidative stability of some algal-based FAME may be of concern [Hoekman et al., 2012].

1.6 Anaerobic Digestion

Anaerobic digestion (AD) is the biological process during which organic matter, under the absence of free oxygen, is converted to biogas by anaerobic methanogens – a group of archaea [Mata-Alvarez, 2003]. Biogas is a gas mixture mainly composed of methane (CH₄) and carbon dioxide (CO₂). It is a process found in many naturally occurring anoxic environments including watercourses, sediments, waterlogged soils and the mammalian gut. Anaerobic digestion is the recommendable procedure for treating wastes with high organic load, and thus, it can be applied to a wide range of feedstocks including industrial and municipal waste waters, agricultural, municipal, food industry wastes, and plant residues [Ward et al., 2008]. The interest in the process is mainly due to the following two reasons [Angelidaki et al., 2003]:

- A high degree of reduction of organic matter is achieved with a small increase -in comparison to the aerobic process - in the bacterial biomass.
- The production of biogas, which can be utilized to generate different forms of energy (heat and electricity) or be processed for automotive fuel.

This technique is widely used at wastewater treatment plants (WWTPs) for reducing the waste volume and stabilizing the sludge produced from waste water treatment. The process can also be applied to treat e.g. household and agricultural waste, turning them into valuable resources and solving a waste handling problem. Greenhouse gas reduction is not only attained by the replacement of fossil fuels, but digesting household waste, sludge and manure prevents direct emission of the
strong greenhouse gases methane and nitrous oxide to the atmosphere. The residues from the digestion process can be used as fertilizer on farmland, and the carbon dioxide produced from the methane combustion is thus taken up by plants. Plants which once again can be used for biogas production, thus the carbon cycle is closed.

The evaluation of biogas and methane production through anaerobic digestion from agricultural wastes is not new and is being prompted in recent years, when the number of anaerobic digesters in the EU has increased dramatically. In early 2010, about 5900 biogas plants with an installed electrical capacity of 2300 MW_{el} were operational. Within the next five years, more than 3000 biogas plants with an electrical capacity of more than 1700 MW_{el} will be constructed [Zuber, 2010].

1.6.1 Advantages and Disadvantages of Anaerobic Digestion

Anaerobic digestion offers significant benefits over other forms of waste treatment. The main advantages are presented below:

- Energy is produced in the form of biogas.
- Wastes with high organic load can be treated.
- Small amounts of nutrients such as nitrogen (N) and phosphorus (P) are required.
- There is no need for aeration.
- Anaerobic methanogens can survive for a long period of time without substrate input.
- Biomass sources containing high water levels (even containing less than 40% dry matter) can be processed without any pretreatment [Ward et al., 2008].
- The produced slurry (digestate) is nitrogen rich and can in most cases (depending on the nature of the biomass) be utilized in agriculture as a nutrient fertilizer and/or organic amendment [Tambone et al., 2009].
- Small amounts of energy are consumed, mainly for temperature regulation.

However, the process of anaerobic digestion has some drawbacks, some of which are the following:

- High retention time is needed because of the small growth rate of anaerobic methanogens. Thus, the need for big bioreactors occurs and the expenses are augmenting.
- The startup is a slow process and so, a great period of time is needed to achieve steady state. However, this problem is solved when activated microorganisms from working bioreactors are used [Gavala et al., 1996].
- Systems are sensible to variations of organic loads.
- Dependence of systems to temperature causes the consumption of energy.
The effluent of anaerobic digestion is rich in nutrients such as nitrogen (N) and phosphorus (P) and needs further treatment before its deposition to the environment. Nevertheless, there is promising evidence that further treatment of Anaerobically Digested Effluents (ADE) using microalgae can be a viable solution [Abdel-Raouf et al., 2012].

1.6.2 Biochemical Process

Anaerobic degradation of organic material to methane and carbon dioxide is a complex system of biochemical reactions. The reactions are divided into four stages of processes; hydrolysis, acidogenesis, acetogenesis and methanogenesis:

![Figure 1.10: Schematic representation of the biochemical processes occurring during Anaerobic Digestion](image)

All these stages need to function in order to obtain a stable and efficient process. The individual degradation steps are carried out by different consortia of microorganisms, which partly stand in syntrophic interrelation and place different requirements on the environment [Angelidaki et al. 1993].

As shown in Figure 1.10, complex biopolymers (such as proteins and polysaccharides) are hydrolyzed to monomers by fermentative bacteria, which
subsequently ferment the monomers to a mixture of low-molecular-weight organic acids and alcohols. These fermentation products are further oxidized to acetic acid and hydrogen by obligatory hydrogen-producing acetogenic bacteria through a process called acetogenesis. Acetogenesis also includes acetate production from hydrogen and carbon. Hydrogen-producing acetogenic bacteria grow in syntrophic associations with hydrogenotrophic methanogens, which keep the hydrogen partial pressure low enough to allow acetogenesis to become thermodynamically favorable (this process is referred to as interspecies hydrogen transfer) [Thauer et al., 1977]. Finally, acetoclastic methanogens convert the acetate to methane and carbon dioxide (methanogenesis).

The general equation which describes the conversion of organic material into methane is the following [Buswell et al., 2005]:

$$C_aH_bO_cN_d + \left(\frac{4a - b - 2c + 3d}{4}\right)H_2O \rightarrow \left(\frac{4a + b - 2c - 3d}{8}\right)CH_4 + \left(\frac{4a - b + 2c + 3d}{8}\right)CO_2 + dNH_3$$

The formula $C_aH_bO_cN_d$ represents the general formula for substrate composition. The theoretical methane yield can be calculated using the following equation:

$$Y_{\text{methane}} \left(\frac{mL}{g \text{ Vs destroyed}}\right) = \left(\frac{4a + b - 2c - 3d}{8}\right) \cdot \frac{12a + b + 16c + 14d}{V_m} \cdot 1000$$

where $V_m$ is the molar volume of methane.

The ratio of methane to carbon dioxide can be computed as follows:

$$r = \frac{4 - n}{4 + n}$$

where $n = \frac{-b + 2c + 3d}{a}$ represents the average carbon oxidation state in the substrate.

The ammonium production yield can be evaluated using the following equation:

$$Y_{NH_3} = \frac{d \cdot 17 \cdot 1000}{12a + b + 16c + 14d}$$

Finally, the biodegradability is calculated by dividing the experimental methane yield with the theoretical methane yield:

$$\text{Biodegradability} = \frac{\text{experimental methane yield}}{\text{theoretical methane yield}}$$
1.6.3 BMP assays

Because of the increasing demand for renewable energy production, anaerobic digestion of various kinds of biomasses is becoming more and more attractive. Thus, a key parameter for the full scale implementation of anaerobic digestion processes is to determine the biogas potential for each substrate. Biochemical methane potential (BMP) is an experimental procedure developed to determine the methane production of a given organic substrate during its anaerobic decomposition. The BMP assay has proved to be a relatively simple and reliable method to obtain the extent and rate of organic matter conversion to methane [Owen et al., 1979; Chynoweth et al., 1993]. Methane potential of wastes is defined as the ultimate specific methane production, for indefinite degradation time. In practice the degradation time is definite and the methane potential is estimated by extrapolation of a methane time degradation curve. Methane potential can be expressed specifically per amount of waste (mL CH\(_4\)/g waste), volume of waste (mL CH\(_4\)/mL waste), per mass volatile solids added (mL CH\(_4\)/g VS) or COD added (mL CH\(_4\)/g COD). The volume is usually expressed in standard pressure (1 atm) and temperature (0°C) conditions (STP conditions).

1.7 Ultrasonication

Ultrasonication is a technique used to facilitate various processes, including the disintegration of cell walls in order to extract valuable intracellular products. It has the advantage of being able to disrupt the cells at relatively low temperatures when compared to other pretreatment techniques, leading to less thermal protein denaturation [Bussemaker et al., 2013]. In addition, sonication does not require the addition of beads or chemicals, which may need to be removed later in the process, thus increasing processing cost. Ultrasonic devices can be scaled-up and operated continuously.

When ultrasound is applied in liquid, there are two main mechanisms with which the cells or other structures may be altered: cavitation and acoustic streaming. Cavitation is the production of microbubbles because of the applied ultrasound. As the microbubbles continue to expand and contract, they eventually become unstable and implode violently, sending shock waves that disrupt surrounding materials such as cells [Bussemaker et al., 2013]. Acoustic streaming is the mechanism that facilitates the mixing of the solution.
Figure 1.11: (a) Formation of microjets near a solid boundary. The asymmetrical liquid motion near the boundary distorts the normally spherical cavitation. (b) Immiscible liquids before and after high power ultrasound treatments. The ultrasonic effects of cavitation and acoustic streaming enhance mixing of the two liquids [Bussemaker et al., 2013].

Moreover, ultrasonication may produce free radicals from the breakdown of the water molecule when applied for long periods of time, which may be detrimental to the quality of the sample undergoing that process. The series of reactions that take place during ultrasonication process is presented below [Bussemaker et al., 2013]:

\[
\begin{align*}
H_2O + \cdot \cdot \cdot \cdot & \rightarrow H\cdot + OH \quad (1) \\
\cdot OH + \cdot OH & \rightarrow O + H_2O \quad (2) \\
\cdot OH + H_2O & \rightarrow H_2O_2 + O \quad (3) \\
H\cdot + OH & \rightarrow H_2O \quad (4) \\
H + H\cdot & \rightarrow H_2 \quad (5) \\
2O & \rightarrow O_2 \quad (6) \\
\cdot OH + \cdot OH & \rightarrow H_2 + O_2 \quad (7) \\
\cdot OH_{(aq)} + \cdot OH_{(aq)} & \rightarrow H_2O_2_{(aq)} \quad (8) \\
HO_2\cdot + H\cdot & \rightarrow H_2O_2 \quad (9) \\
HO_2\cdot + HO_2\cdot & \rightarrow H_2O_2 + O_2 \quad (10) \\
H + O_2 & \rightarrow HO_2\cdot \quad (11) \\
O_2 & \rightarrow 2O \quad (12) \\
O_2 + O & \rightarrow O_3 \quad (13)
\end{align*}
\]

Thus, it is crucial to establish the optimal conditions for the effective pretreatment of biomass through ultrasonication. According to literature, the optimal energy input for microalgal cell disruption is approximately 80 J mL\(^{-1}\) [Gerde et al., 2012].
Chapter 2.

MATERIALS AND METHODS

2.1 Materials and methods

2.1.1 Microorganism and growth conditions

The microalgal strain *C. vulgaris* SAG 211-11b used in this study was obtained from SAG (Sammlung von Algenkulturen der Universität Göttingen), and was grown on BG-11 medium (Table 2.1). The pH of the medium was adjusted at 7.1, after being filter-sterilized, as instructed by the supplier. Pre-cultures were maintained at ambient temperature, in 250 ml Erlenmeyer flasks containing 125 ml of medium, under continuous light intensity of 20-25 mmol m\(^{-2}\) s\(^{-1}\). The cultures were subcultured every 3 to 4 weeks, using 1 ml of inoculum from the previous culture. All subcultures were performed inside a laminar flow-hood (BIOAIR TOP SAFE 1.8) in order to maintain axenic conditions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO(_3)</td>
<td>1500</td>
</tr>
<tr>
<td>K(_2)HPO(_4)</td>
<td>40</td>
</tr>
<tr>
<td>MgSO(_4)·7H(_2)O</td>
<td>75</td>
</tr>
<tr>
<td>CaCl(_2)·2H(_2)O</td>
<td>36</td>
</tr>
<tr>
<td>Citric acid</td>
<td>6</td>
</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>6</td>
</tr>
<tr>
<td>EDTA (disodium salt)</td>
<td>1</td>
</tr>
<tr>
<td>Na(_2)CO(_3)</td>
<td>20</td>
</tr>
<tr>
<td>H(_3)BO(_3)</td>
<td>2.86</td>
</tr>
<tr>
<td>MnCl(_2)·4H(_2)O</td>
<td>1.81</td>
</tr>
<tr>
<td>ZnSO(_4)·7H(_2)O</td>
<td>0.222</td>
</tr>
<tr>
<td>NaMoO(_4)·2H(_2)O</td>
<td>0.39</td>
</tr>
<tr>
<td>CuSO(_4)·5H(_2)O</td>
<td>0.079</td>
</tr>
<tr>
<td>Co(NO(_3))(_2)·6H(_2)O</td>
<td>0.0494</td>
</tr>
</tbody>
</table>

Heterotrophic inoculums used in the present study were supplemented with glucose and incubated in the dark using aluminum foil. All inoculums were used when they reached exponential phase. Glucose was sterilized via autoclaving at 121°C for 20
min, and when cooled to room temperature, it was supplemented with BG-11 broth and the pH was adjusted.

**2.1.2 Olive Mill Wastewater**

The raw wastewater used in all experiments was obtained from a local olive oil-mill (Panitsas N. & Co.) using a three-phase decanter centrifugation process for extraction of olive oil. Because of the fact that OMW is characterized by seasonal availability and high tendency for fermentation, all wastewater samples were collected fresh and stored immediately in the freezer at −18°C until subsequent use. Before its use as a substrate for microalgal growth, OMW was filtered using Whatman® glass microfiber filters, Grade GF/F, in order to remove all the solids that would interfere with the cultivation of microalgae, and would lead to an overestimation of biomass.

**2.2 Experimental set-up**

Two kinds of experiments were conducted in the present study: batch experiments of microalgal growth and Biochemical Methane Potential essays. The experimental set-up is described below:

**2.2.1 Batch experiments of microalgal growth**

All batch experiments of microalgal growth were conducted in 1L Erlenmeyer flasks with a working volume of 0.7 L (Picture 2.1 (a)). The reactors were covered with black garbage bags, which turned out to be more serviceable than aluminum foil, in order to avoid light penetration. The aeration was adjusted at 1 vvm, and the temperature was controlled at 25±1°C using a bath with an orbital shaker adjusted at 90 rpm (Grant OLS200) (Picture 2.1 (b)). The evaporation of water from the interior of each reactor caused by the aeration was monitored by weighting each reactor before and after a sample was extracted, and was incorporated in the calculations.

The synthetic medium used in each experiment was BG-11 supplemented with glucose, and the pH was adjusted using the corresponding buffer solution. For pH 3 and 4 the buffer solution used was 0.1M potassium hydrogen phthalate (C₈H₅KO₄) adjusted with 1M HCl, for pH range 5 to 8 phosphate buffer (KH₂PO₄) was used at concentration 0.1M, adjusted with 1M NaOH, and for pH values of 9.5 and 11, 0.1M borax (Na₂B₄O₇·10H₂O) and 0.1M disodium phosphate (Na₂PO₄) were used respectively, adjusted with 1M NaOH. Finally, for the experiment where the effect of
phosphorus stress at pH=7.5 was studied, tris(hydroxymethyl)aminomethane supplemented with 1M HCl was used as a buffer. For pH values greater than 5.5, the buffer solution and carbon source were sterilized separately, in order to avoid the caramelization of glucose. After cooling down, they were mixed and prepared for the respective experiment. Each experiment was carried out twice and the mean values are presented.

![Erlenmeyer flasks](image1.png) ![Orbital shaking water bath](image2.png)

**Picture 2.1:** (a) Erlenmeyer flasks used as reactors for the cultivation of microalgae (b) Orbital shaking water bath containing the reactors

### 2.2.2 Biochemical Methane Potential assays (BMPs)

In the present study, *C. vulgaris* biomass was subjected to BMP assay under various conditions. The procedure was conducted according to Angelidaki et al. [Angelidaki et al., 2009]. After degasing the inoculums for 5 days, a known amount of substrate and active anaerobic inoculum (10% v/v) were added to 160 mL serum bottles. They were subsequently supplemented with the proposed defined media containing nutrients and vitamins, to a final working volume of 100 mL. The serum bottles were flushed for 5 min with a mixture of N\textsubscript{2}:CO\textsubscript{2} (80%:20%) to maintain a neutral pH and sealed immediately using butyl rubber septum and aluminum crimp caps (**Picture 2.2 (b)**). Once sealed, the bottles were placed in an orbital shaking water bath (Grant OLS200) at 90 rpm (**Picture 2.2 (a)**) and maintained at a constant mesophilic (37°C) and thermophilic (55°C) temperature respectively. Each experiment was carried out twice, and the mean values are presented.
During the process of measuring the produced gas, water is lost from the serum vials through evaporation. The amount of water should be calculated in order to obtain valid results about the biogas production and the nutrient removal. The following corrections were carried out according to Richards et al. [Richards et al., 1991]:

Calculation of water loss due to evaporation:

\[ W = V_{TOTAL} \cdot D_W \]

where \( V_{TOTAL} \) is the total volume of produced biogas \([=] \) \( L_{biogas} \),

\( D_W \) is the vapor density \([=] \) \( g_{water} \cdot L_{biogas}^{-1} \)

It should be noted that the vapor density is calculated at the temperature of the respective experiment (37°C and 55°C). The water loss is incorporated in the results of nutrient removal.

In order to convert the measured biogas to dry biogas at STP conditions, the following equation was used:

\[ V_{STP} = DBF \cdot V_T \]

where DBF is the dry biogas factor,

\( V_T \) is the measured biogas volume at temperature \( T \)

In this case, the temperature is not the one of the experiment. However, it is assumed that the temperature \( T \) of the measured biogas is the average temperature of the respective experiment and the ambient temperature.
2.3 Analytical Methods

Physicochemical characterization was performed in raw Olive Mile Wastewater and all samples. For the measurement of soluble compounds (carbohydrates, VFAs, ions etc), the insoluble residue was separated from the supernatant via Whatman® glass microfiber filters, Grade GF/F. The determination procedure for each characteristic was conducted as followed.

2.3.1 pH

The pH measurements were carried out using a sterilizable electrode (Mettler Toledo).

2.3.2 Solids

Total solids (TS) and volatile solids (VS) were determined according to Standard Methods for the Examination of Water and Wastewater [APHA, 1998]. Total suspended solids (TSS) and volatile suspended solids (VSS) were determined by using Whatman® glass microfiber filters, Grade GF/F. Total solids were measured after sample drying at 105 °C (Section 2540 B, D), while volatile solids after sample ignition at 550 °C (Section 2540 E).

2.3.3 Biomass concentration

For the experiments where synthetic medium was used, the determination of algal biomass concentration was carried out by measuring the Optical Density of the sample at 550 nm, after a calibration curve was created (Figure 2.2). In order to create the calibration curve, the dry weight of the algal biomass was measured by using Whatman® glass microfiber filters, Grade GF/F, which were oven dried at 60°C after the biomass was separated from the supernatant. The samples were properly diluted so that the measured Optical Density is between the limits of the calibration curve.
The resulting equation was the following ($R^2 = 0.999$):

$$ y = 0.2944 \cdot x + 0.0302 $$

For the experiments where Olive Mill Wastewater was used as a medium, the biomass concentration was measured via filtration of the sample, as described above.

Additionally, the volumetric biomass productivity was calculated based on the following equation:

$$ P_{Biomass}(gL^{-1}day^{-1}) = (X_2 - X_1) \cdot (t_2 - t_1)^{-1} $$

where $X_1$ and $X_2$ are the biomass dry weight concentrations (g L$^{-1}$), on $t_1$ (start of cultivation) and $t_2$ (day of stationary phase achievement), respectively.

2.3.4 COD

Total and soluble COD (TCOD and SCOD) were determined according to Standard Methods for the Examination of Water and Wastewater [APHA, 1998], using “Closed Reflux, Colorimetric Method” (Section 5220 D). The oxidation of organic material is performed by heating of the diluted sample in extremely acidic conditions under the presence of excessive amounts of potassium dichromate ($K_2Cr_2O_7$), with silver sulfate (AgSO$_4$) used as a catalyst. In order to avoid the reaction between silver ions and
halogens that may be found in the sample, the addition of mercury ions in the form of mercury sulfate (HgSO\textsubscript{4}) is necessary, as it creates complexes with the halogens and transforms them into sediment.

The method is based on the colorimetric measurement of Cr\textsuperscript{3+} cations at 600 nm, produced by the oxidation of organic materials, as described by the following equation [Sawyer and McCarty, 1978a]:

\[
C_nH_{2a}O_b + cCr_2O_7^{2-} + 8cH^+ \rightarrow nCO_2 + \left(a + \frac{8c}{2}\right)H_2O + 2Cr^{3+}
\]

where: \(c = \frac{2}{3}n + \frac{a}{6} - \frac{b}{3}\)

### 2.3.5 Carbohydrates

The determination of carbohydrates was carried out according to the method proposed by Josefsson [Josefsson, 1983]. A colored sugar derivative was produced through the addition of L-tryptophan, sulfuric and boric acid, which was placed in a boiling bath for 20 min and subsequently measured colorimetrically in a Cary 50 UV/VIS spectrophotometer (Varian) at 520 nm. Calculations were based on a calibration curve with D-glucose as a standard.

### 2.3.6 Phenolic compounds

Total phenolic compounds were determined spectrophotometrically in centrifuged and filtered samples according to the Folin–Ciocalteu method [Waterman et al., 1994] using syringic acid as standard solution.

### 2.3.7 Ions

Ions in the culture medium were measured with a DIONEX ICS3000 ion chromatography system using a thermostated (30°C) Dionex IonPac analytical column (AS19 length 4 x 250 mm and 7.5 mm I.D) and a guard column (4x50 mm length and 12 mm I.D) and an electron conductivity detector (Dionex). Analysis was performed by applying an elution gradient with KOH solution, as mobile phase, at a flow rate of 1 mL/min. The eluent gradient was programmed to result in a 18 mM KOH solution during equilibration and analysis and a 50 mM KOH solution during column regeneration. The total running time of analysis was 30 min and the gradient
profile as follows: 18 mM KOH for 21 min, 50 mM KOH maintained for 4 min and
18 mM KOH until the end of run (30 min). The injection volume was 10 μL.

2.3.8 Phosphorus

Phosphorus measurement was achieved according to Standard Methods for the
Examination of Water and Wastewater [APHA, 1998] after using two general
procedural steps: conversion of the phosphorus form of interest to dissolved
orthophosphate, and colorimetric determination of dissolved orthophosphate with
the “Ascorbic Acid Method” (Section 4500-P, E).

2.3.9 Nitrogen

Ammonia and total Kjeldahl nitrogen (TKN) were analyzed by Kjeldahl methods
(Section 4500-N$_{\text{org}}$ B, C), according to Standard Methods for the Examination of
Water and Wastewater [APHA, 1998].

2.3.10 Volatile Fatty Acids (VFAs)

Composition analysis of volatile fatty acids (VFAs) was performed in a gas
chromatograph (Agilent Technologies 7890A). For quantification of volatile fatty
acids (VFAs) liquid samples were removed from the reactor, transferred immediately
to 2-mL vials where 1 mL of sample was acidified with 30 μL of 20% H$_2$SO$_4$ and then
centrifuged (5000 rpm) for 10 min to remove biomass. The supernatant was filtered
and transferred to 2-mL septum-capped vials. The gas chromatograph was equipped
with flame ionization detector (FID) using helium as carrier gas. A capillary column
(DB–FFAP, 30 m in length, 0.25 mm I.D. and 0.25 μm film) was used for determining
the concentration of the individual volatile fatty acids, i.e. acetic, propionic,
isobutyric, butyric, isovaleric and valeric acid. The oven was programmed at 110°C
(held for 5 min) to 250°C (held for 6 min) at a rate of 15°C min$^{-1}$. The operating
temperature of the injector and detector was set at 250 and 300°C, respectively.

2.3.11 Oil and Grease

Oil and Grease were determined according to Standard Methods for the Examination
of Water and Wastewater [APHA, 1998] using a “Soxhlet Extraction Method”
(Section 5520 D) with n-Hexane as an extraction solvent.
2.3.12 Intracellular unsaturated lipids via Sulfo-Phospho-Vanillin method

The estimation of intracellular unsaturated lipids using the sulfo-phospho-vanillin method was carried out as described by Sanjiv et al. [Sanjiv et al., 2014]. Phospho-vanillin reagent was prepared by dissolving 0.6 g vanillin in 10 mL absolute ethanol and 90 mL deionized water. Then 400 mL of concentrated phosphoric acid was added to the mixture and the resulting reagent was stored in the dark. In order to conduct this measurement cells were harvested by centrifuge at 5000 rpm for 10 min, and washed with 3D-water. The procedure was repeated at least 3 times in order to eliminate any organic compound as it interferes with the measurement, and the biomass was re-suspended in water. The method instructs that a volume of 100 μL of sample with known concentration of biomass is needed for each tube. After the addition of 2 mL concentrated (98%) sulfuric acid was added in every tube and the samples were heated for 10 min at 100°C, and then cooled for 5 min to room temperature. Subsequently, 5 mL of phospho-vanillin reagent were added and the samples were placed in a bath at 37°C for 15 min, and were periodically agitated. Finally, absorbance reading at 530 nm was taken in order to quantify the lipid content, based on calibration curves with oleic acid and canola oil as standards ($R^2=0.9995$ and 0.9997 respectively).

![Figure 2.4: Colored derivatives from Sulfo-Phospho-Vanillin method.](image)

2.3.13 Fatty Acid Methyl Esters (FAMEs)

The Fatty Acid Methyl Ester (FAME) content of algal biomass was measured via Gas Chromatography after performing in situ transesterification of microalgal lipids. The procedure was conducted as described by Levine et al. [Levine R.B. et al., 2011]. It dictates that an amount of 10 – 50 mg of microalgal biomass is needed, and so the volume of sample extracted was calculated based on the concentration of biomass at the time. The sample with the algal suspension was centrifuged and the supernatant was extracted carefully and used for further analyses. The biomass was rinsed with water as previously stated (§2.3.12), and was oven-dried overnight at
60°C. The samples were incubated with Argon in order to avoid oxidation caused by contact with oxygen and stored at -4°C for further analyses.

FAME analysis was performed using a GC with Flame Ionization Detector (Agilent 7890A) with a DB-WAX™ 127-7012 fused silica capillary column (10m × 0.10 mm × 0.10 μm). Automated splitless injection (1 μl; 250°C inlet temperature) was made with an initial oven temperature of 150°C. After a 2 min hold, the temperature was ramped at 4°C min⁻¹ to 220 °C, held for 8 min, and then increased at 10°C min⁻¹ to 260°C. Helium was used as the carrier gas at a constant flow rate of 1.0 mL min⁻¹. The inclusion of a known amount (50 mg L⁻¹) of internal standard was used in order to correct the FAME quantification towards analytical instrument variability and solvent evaporation during the analysis. The internal standard used in the present study was margaric acid (C₁₇:0), after verifying that it is not present in the intracellular lipid content of C. vulgaris SAG 211-11b.

The amount of microalgal lipids was calculated using the following equation:

\[
Total \text{FAME}_{C_{17:0}} \text{normalized} = \sum \frac{\text{FAME } C_i \text{ measured}}{\text{FAME } C_{17:0} \text{ measured}} \cdot \text{FAME } C_{17:0} \text{ added}
\]

The yield of microalgal lipids was calculated as followed:

\[
Total \text{FAME} (%) = \frac{Total \text{FAME}_{C_{17:0}} \text{normalized} \ (\%)}{\text{Concentration of Biomass} \ (\%)}
\]

### 2.3.14 Gaseous products

Biogas composition (methane and carbon dioxide) was analyzed via gas chromatography (Agilent Technologies 7890A) equipped with a capillary column (HP-POLOT/Q, 30 m in length, 0.53 mm i.D. and 40 μm packing film) and a thermal conductivity detector (TCD) using nitrogen as carrier gas. The temperature of injector and detector was kept at 250°C and the oven temperature was gradually increased from 80°C (held for 6 min) to 200°C (held for 2 min) at a rate of 50°C min⁻¹.

The biogas was sampled with a 1-mL gas-tight syringe. Total biogas and methane volumes, produced during each experiment, were converted at standard temperature and pressure conditions (i.e. STP = 0°C and 1 atm).

### 2.3.15 Lyophilization

The lyophilization of samples was conducted using a laboratory freeze dryer (Telstar, LyoQuest), until the water content was eliminated.
2.3.16 Elemental Composition analysis

Elemental composition analysis was performed on freeze-dried algal biomass in order to quantify the content of certain elements (C, O, H, N and S). The elemental analyzer contains a combustion furnace, which was maintained during the analysis at 1020°C. In the combustion furnace there was a quartz column for oxidation and reduction of the solid sample. The carrier gas was helium (constant flow of 100 mL/min), which was enriched with pure oxygen in order to achieve a strong oxidizing environment for solid material combustion. The solid samples were oxidized to gases CO₂, H₂O, NOₓ and SO₃ and then after reduction a gas mixture consisted of N₂, CO₂, H₂O and SO₂ was obtained. Finally, the gas mixture was analyzed in gas chromatography equipped with a packed column (Porapack Q) and a thermal conductivity detector (TCD).

2.3.17 Specific Calorific Value

The specific calorific value of microalgal biomass was determined using a calorimeter (IKA®, Calorimeter C200). Combustion is carried out in a calorimeter under specific conditions. The decomposition vessel is filled with a weighed fuel sample, the fuel sample is ignited and the temperature increase in the calorimeter system measured. The specific calorific value of the sample is calculated as follows:

\[ H_0 = \frac{C * \Delta T - Q_{\text{EXTERNAL1}} - Q_{\text{EXTERNAL2}}}{m} \]

where: m is the weight of the fuel sample

C is the heat capacity (C-value) of calorimeter system

\( \Delta T \) is the calculated temperature increase of water in inner vessel of measuring cell

\( Q_{\text{EXTERNAL1}} \) is the correction value for the heat energy generated by the cotton thread as ignition aid

\( Q_{\text{EXTERNAL2}} \) is the correction value for the heat energy from other burning aids

The decomposition vessel is filled with pure oxygen (99.95 %) to optimize the combustion process. The pressure of the oxygen atmosphere in the decomposition vessel is max 30 bar. Formula (2.6) for the calorific value of a material requires that combustion takes place under specifically defined conditions. The relevant standards are based on the following assumptions:

- The temperature of the fuel and its combustion products is 25°C.
• The water contained in the fuel before combustion and the water formed whilst combusting the hydrogenous compounds of the fuel is in fluid form after combustion.

• The atmospheric nitrogen has not oxidized.

• The gaseous products after combustion consist of oxygen, nitrogen, carbon dioxide and sulfur dioxide.

• Solid materials may form (e.g. ashes).

The calorimeter system must be calibrated before the use so that accurate measurements are obtained. This is done by combusting tablets made of certified benzoic acid with a known calorific value. The heat quantity required to raise the temperature of the calorimeter system by one Kelvin is used to determine the heat capacity of the so called "C-value" of the system. For this calculation the formula (2.6) is adapted.

2.3.18 Sterilization

Sterilization of media and glassware used was performed via autoclaving at 121°C for 20 min (RAYPA, STEAM STERILIZER).

2.3.19 HPLC

High Pressure Liquid Chromatography (HPLC, Agilent 1200) was used in order to verify the presence of glucose in OMW. More specifically the system was equipped with an ELSD detector (ELS-1200) and a Rezex RPM column (300 mm × 7.8 mm). An isocratic method was used with water as the mobile phase. The flow rate was adjusted at 0.6 mL min⁻¹ and the temperature at 80°C. The duration of each run was 30 min.

2.3.20 Ultrasonication

Sonication of the samples was carried out using an ultrasonic bath (Branson, Bransonics CPX3800H-E), working at 240V and 40kHz. The microalgal biomass was diluted in 10 mL 3D water and subsequently subjected to sonication energy of 80 J mL⁻¹ [Gerde et al., 2012].
Chapter 3.

RESULTS AND DISCUSSION

3.1 Effect of pH

The goal of this set of experiments was to investigate the pH range that can support the growth of *C. vulgaris*, in addition to the optimal pH for the microalga, under heterotrophic conditions. Furthermore, the effect of pH on accumulation of intracellular lipids was studied. A wide range of pH values was tested; namely 3, 4, 5, 6, 6.5, 7, 7.5, 8, 9.5 and 11. The culture medium was BG-11 broth supplemented with approximately 10 g L\(^{-1}\) glucose, and the respective buffer solution (§2.3.1).

Initially, this series of experiments was carried out by regulating the pH with the addition of HCl 1M and NaOH 1M, without the use of buffer solution. However, substantial changes in the pH value occurred during the cultivation period and the pH could not be considered stable. In order to draw a conclusion about the optimal pH and the pH range in which *C. vulgaris* can grow, the utilization of a buffer solution was necessary, so as to obtain a culture medium with resistance in pH changes. The buffer solutions offer the advantage of simulating a pH-controlled environment. It should be noted that in many studies where the effect of pH is investigated, the pH is regulated using strong acid or base after a sample is extracted, without the use of a buffer solution. This tactic results in questionable results due to the variation of pH during the experimental period, as usually a sample is extracted after every 2-3 days.

Moreover, in most studies the comparison of the growth behavior of a microorganism under different conditions is carried out in terms of maximum specific growth rates (\(\mu_{\text{max}}\)). However, this approach often results in over- or underestimated growth rates. In order to avoid the inference of misleading conclusions, in the present study the analysis of the data relating to growth was conducted in terms of \(\mu_{\text{max}}\) in addition to maximum biomass productivity (\(P_{\text{Biomass}}\)). It should be noted that the calculations of \(P_{\text{Biomass}}\) were carried out for the period until stationary phase. For the calculation of \(\mu_{\text{max}}\) the linear part of the figures representing the natural logarithm of biomass (\(\ln X\)) in relation to time was considered. More specifically, the differential equation representing the exponential growth of a microorganism is the following:

\[
\frac{dX}{dt} = \mu X \Rightarrow \frac{1}{X} \frac{dX}{dt} = \mu \Rightarrow \frac{d\ln X}{dt} = \mu \Rightarrow d\ln X = \mu \cdot dt \Rightarrow \ln X = \mu \cdot t + c
\]
And by applying the initial conditions (for t=0, X=X_0) we obtain the following equation:

\[ \ln X = \mu \cdot t + \ln X_0 \]

Where \( \mu \) is the specific growth rate:

\[ \mu = \frac{\mu_{\text{max}} \cdot S}{K_S + S} \]

However, in the exponential growth phase the substrate concentration is far greater than the half-velocity constant (S>>K_s) and thus \( \mu \cong \mu_{\text{max}} \). The equation now becomes:

\[ \ln X = \mu_{\text{max}} \cdot t + \ln X_0 \]

Consequently, by applying the above equation to the experimental data, an estimation of the maximum specific growth rate is obtained.

- **Biomass growth**

\( C. \) vulgaris was able to grow on the majority of pH values tested (5, 6, 6.5, 7, 7.5 and 8). However, pH values 3, 4 and 11 were found to be extreme for this stain, as lysis of the cells was observed within the first two days of the respective experiments. Moreover, in pH equal to 9.5, the microalga did not grow during a cultivation period of 15 days, and flocculation of the cells was observed.

Although in different pH values \( C. \) vulgaris displayed differences in its growth behavior, a feature was exhibited in all the experiments. Independent from the pH value, the microorganism presented a lag phase of two days. The following figure (Figure 3.1) depicts the growth behavior of \( C. \) vulgaris in the pH values tested. It should be noted that all the other parameters remained constant (aeration, agitation etc.), in order to obtain direct comparison.
By observing Figure 3.1 one can see that the growth behavior of *C. vulgaris* improves with the increase of pH. More specifically, for the values between 5 and 7 the microalga reached stationary phase in the 10th day of cultivation, while for 7.5 and 8 the growth stopped in the 8th day. However, the comparison of biomass productivities offers a better understanding. The following figure (Figure 3.2) presents the maximum biomass productivities for all the pH values tested.

**Figure 3.1**: Growth of *C. vulgaris* in various pH.

**Figure 3.2**: Effect of pH on Maximum Biomass Productivities.
As shown in the figure above, for pH 5, 6 and 6.5 the P\textsubscript{Biomass} varies between 0.2138 and 0.2297 g L\textsuperscript{-1} day\textsuperscript{-1}. For pH 7 to 8 an increase is observed as P\textsubscript{Biomass} is ranging between 0.2706 and 0.2976 g L\textsuperscript{-1} day\textsuperscript{-1}, with the highest value corresponding to pH 7.5.

When comparing the maximum specific growth rates (\(\mu\text{max}\)), similar conclusions are drawn. More specifically, the rise in pH caused the rise of \(\mu\text{max}\) which ranged between 0.4230 and 0.5626 days\textsuperscript{-1}, for pH 6 and 8, respectively. The highest value of \(\mu\text{max}\) was obtained in pH 8 and is followed by 0.5409 for pH 7.5. The effect of pH in \(\mu\text{max}\) is illustrated in the following figure (Figure 3.3).

![Figure 3.3: Effect of pH on Maximum Specific Growth Rate.](image)

As mentioned above, in pH 9.5 the microalga was not able to grow under heterotrophic conditions, and flocculation of cells was observed. In many cases flocculation is used as a cell harvesting technique. More specifically, after the microalga reaches stationary phase and obtains the desired characteristics (high content of lipids, starch or another high value product), the pH is changed to a relatively extreme value with the addition of a strong acid or base. This pH-shift strategy results in the aggregation of cells while gravity forces them to settle on the bottom of the reactor, thus, facilitating their harvest. In the present study, pH 9.5 was found to be a value which promotes the aggregation of *C. vulgaris* cells, and thus could be used for the harvest of *C. vulgaris* cells under pilot or full-scale conditions.

The combination of the data obtained leads to the conclusion that the optimum pH values for *C. vulgaris* growth are 7.5 and 8.

The following table summarizes the features obtained for the pH values tested:
Table 3.1: Main features obtained under various pH values

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationary Phase</td>
<td>day</td>
<td>5</td>
</tr>
<tr>
<td>Max Specific Growth Rate</td>
<td>days⁻¹</td>
<td>6</td>
</tr>
<tr>
<td>Maximum Biomass Productivity</td>
<td>g L⁻¹day⁻¹</td>
<td>6.5</td>
</tr>
<tr>
<td>Maximum Biomass Generation</td>
<td>g L⁻¹</td>
<td>7</td>
</tr>
<tr>
<td>7.5</td>
<td>8</td>
<td>7.5</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>8</td>
</tr>
</tbody>
</table>

a day of achievement; b calculated from the mean biomass concentration during stationary phase.

The following table provides the comparison of the data obtained in the present study, with the relevant data found in literature:

Table 3.2: Comparison of data obtained concerning the optimal pH for the growth of *C. vulgaris* with those found in literature.

<table>
<thead>
<tr>
<th>pH-controlled conditions</th>
<th>light</th>
<th>Optimal pH</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>Yes</td>
<td>7.5-8</td>
<td>[Rachlin et al., 1991]</td>
</tr>
<tr>
<td>Yes</td>
<td>Yes</td>
<td>10</td>
<td>[Gong et al., 2014]</td>
</tr>
<tr>
<td>Not provided</td>
<td>Not provided</td>
<td>6.5-7</td>
<td>[Wang et al., 2010]</td>
</tr>
<tr>
<td>Yes</td>
<td>No</td>
<td>7.5-8</td>
<td>Present study</td>
</tr>
</tbody>
</table>

As shown in the table, the data obtained in the present study agree with the findings of Rachlin et al., even though the mode of cultivation is not the same.

- Nutrient uptake

For this particular strain, the limiting nutrient in BG-11 was found to be sulfur. Phosphorus was not monitored, as it was contained in high concentrations inside the culture medium due to the fact that phosphate buffer was used (§2.3.1). The following table summarizes the values obtained for the consumption of nutrients in the pH tested:
Table 3.3: Nutrient consumption for the pH tested.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>pH 5</th>
<th>pH 6</th>
<th>pH 6.5</th>
<th>pH 7</th>
<th>pH 7.5</th>
<th>pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Y_{X/C}$</td>
<td>0.6148</td>
<td>0.5043</td>
<td>0.5009</td>
<td>0.4197</td>
<td>0.4016</td>
<td>0.5158</td>
</tr>
<tr>
<td>$Y_{X/N}$</td>
<td>29.91</td>
<td>28.24</td>
<td>28.30</td>
<td>29.73</td>
<td>28.93</td>
<td>32.01</td>
</tr>
<tr>
<td>$Y_{X/S}$</td>
<td>419.3</td>
<td>452.1</td>
<td>500.3</td>
<td>594.5</td>
<td>577.7</td>
<td>541.7</td>
</tr>
</tbody>
</table>

By observing the above table one can see that as the pH increases, the value for $Y_{X/S}$ has a declining course until pH 7.5 and then for pH 8 it increases again. These results indicate that with the increase of pH the portion of carbon consumed that is transformed in intracellular lipids increases until pH 8. These results are thoroughly discussed below.

The following figure (Figure 3.4) illustrates individually the growth of *C. vulgaris* in each pH tested. The assimilation of glucose and sulfur are also presented. By observing the following figure one can see that even though the growth ceased after the depletion of sulfur, glucose was still consumed. This consumption is ascribed to the fact that, after the achievement of stationary phase, carbon is accumulated in the form of intracellular storage compounds such as lipids, starch etc. Another observation concerning this figure is that sulfur is not completely depleted from the medium. This fact is attributed to the emergence of compounds from the lysis of dead cells.

It is a fact that after the depletion of basic nutrients such as nitrogen, phosphorus or sulfur, the microalgae are unable to form active biomass. However, in the present study, after the depletion of sulfur an increase in cell concentration was observed. This result can be attributed to a possible delay in the utilization of the engulfed from the microalga sulfur, and its bioconversion to active biomass.
**Lipid accumulation**

The lipid content was monitored by FAME analysis and by Sulfo-Phospho-Vanillin method (SPV). In SPV method, oleic acid and canola oil were used as standards for the calibration curves, as oleic acid is the dominant fatty acid contained in *C. vulgaris* intracellular lipids, and canola is an oleaginous crop with a fatty acid profile similar to those obtained by microalgae.
The main fatty acids detected in *C. vulgaris*, arranged in declining order, were oleic (C18:1), linoleic (C18:2), palmitic (C16:0), palmitoleic (C16:1) and stearic acid (C18:0). Other fatty acids were also contained in traces. The lipid content did not present significant variations in the pH values tested, and was mainly composed of monounsaturated fatty acids (MUFAs). The fatty acid composition of *C. vulgaris* seemed not to be associated with the pH. Moreover, the lipid content (%) remained in low levels during the first 4 days of cultivation, and was rapidly increased during the following days. This fact indicates that after the first days of cultivation where the microorganism gets acclimated in the new conditions, the metabolism is activated and apart from the reproduction, the microalga also consumes carbon sources in order to accumulate lipids. Thus, in the period after the acclimation until the stationary phase, the processes of reproduction and lipid accumulation occur concurrently.

In all experiments, the dominant fatty acid was oleic acid (C18:1). Moreover, the C18:1 content in total lipids presented the same pattern through time, in all pH values tested. The following figure (**Figure 3.5**) illustrates the oleic acid content in total lipids during the experimental period for pH 7.5 (all experiments presented the same pattern).

![Figure 3.5](image.png)

**Figure 3.5:** Content of Oleic acid (C18:1) in Total Lipids for pH 7.5.

As shown in **Figure 3.5**, the oleic acid content presented an increasing trend until it reached the value of 60% of total lipids, and remained constant for the rest of the experimental period. This fact indicates that oleic acid is not accumulated in the same rate during the cell cycle, but remains in its highest rate during the stationary phase.
The following figure (Figure 3.6) depicts the lipid content of *C. vulgaris* for the pH tested. It should be noted that the lipid content is presented in g per g dry weight (g g<sup>-1</sup> DW).

![Figure 3.6: Lipid content of *C. vulgaris* for pH values tested.](image)

The increase of pH, until a certain point, had positive effects on lipid content (%). More specifically, in pH 5 the lipid content slowly increased and reached its highest
value of 32.80%, 31.48% and 21.76% g g\textsubscript{DW}\textsuperscript{-1} in total lipids, unsaturated lipids and oleic acid respectively, during the 16\textsuperscript{th} day of cultivation. In pH 6, the rate of increase was a little higher, but the final lipid content is comparable (30.70%, 29.46% and 20.02% respectively during 16\textsuperscript{th} day). In pH 6.5, although the highest lipid content was again comparable (31.12%, 27.94% and 18.71%), the rate of increase was higher than those in lowest pH values and tended to stabilize after the 10\textsuperscript{th} day of cultivation. In pH 7 the trend was similar, while the highest lipid content was slightly increased (34.26%, 33.20% and 21.38%). The pH 7.5 presented the highest lipid content values of 53.43%, 48.17% and 32.70% in total lipids, unsaturated lipids and oleic acid, while the accumulation of lipids presented an increasing tendency during the experimental period. Finally, in pH 8, the highest lipid content was again lowered (34.78%, 33.91% and 22.50% respectively), and presented an increase when the pH shifted from 8 to 7.5.

These findings indicate that the optimal pH for the lipid accumulation of \textit{C. vulgaris} is 7.5. Moreover, when the pH value deviates from the initial one, an inhibiting effect on lipid accumulation is presented.

As shown in \textbf{Figure 3.6}, the analysis of intracellular lipids via SPV method underestimates the results, when compared to FAME analysis. FAME analysis via GC is a high resolution technique in which the inclusion of a compound as internal standard provides the validity of the results. On the other hand, SPV is a colorimetric method in which the colored derivatives are the result of the reaction of fatty acids with at least one double bond, with phospho-vanillin reagent. Saturated lipids fail to react in this method, and the absorbance increases in proportion to the amount of unsaturated fatty acids contained [\textit{Knight et al., 1972}]. The presence of saturated lipids results in lower absorbance, as they interfere with this particular method. Thus, in the lipid analysis of microalgal biomass where in addition to unsaturated, saturated lipids are also contained, the underestimation of the lipid content should be expected. This fact is shown in the figure above, where at pH 7.5 saturated fatty acids are contained in greater amounts compared to the other pH values, and the deviation between oleic acid measured by GC and by SPV method is greater than in the other cases. Consequently, the deviation of these two methods is strongly related to the amount of saturated compounds present. Additionally, the measurement of unsaturated lipids with SPV method using canola oil as standard should be expected to greatly deviate from the same measurement via GC.

The following table summarizes the main features obtained concerning the lipid accumulation, for the pH values tested.
**Table 3.4:** Main features obtained in relation to lipid accumulation, under various pH values

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>pH 5</th>
<th>6</th>
<th>6.5</th>
<th>7</th>
<th>7.5</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleic acid</td>
<td>% g $\text{g dry mass}^{-1}$</td>
<td>21.76</td>
<td>20.02</td>
<td>18.71</td>
<td>21.38</td>
<td>32.70</td>
<td>22.50</td>
</tr>
<tr>
<td>Total Lipids</td>
<td>% g $\text{g dry mass}^{-1}$</td>
<td>32.80</td>
<td>30.70</td>
<td>31.12</td>
<td>34.26</td>
<td>53.43</td>
<td>34.78</td>
</tr>
<tr>
<td>Unsaturated Lipids</td>
<td>% g $\text{g dry mass}^{-1}$</td>
<td>31.48</td>
<td>29.46</td>
<td>27.94</td>
<td>33.20</td>
<td>48.17</td>
<td>33.91</td>
</tr>
</tbody>
</table>

The following table provides the comparison of the data obtained in the current study in relation to lipid content, with the relevant data found in literature:

**Table 3.5:** Comparison of data obtained concerning the lipid content of *C. vulgaris* with those found in literature

<table>
<thead>
<tr>
<th>Light</th>
<th>pH</th>
<th>Medium</th>
<th>Lipid content (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>6.2</td>
<td>Basal medium</td>
<td>55.9 ± 2.1</td>
<td>[Yeh et al., 2011]</td>
</tr>
<tr>
<td>Yes</td>
<td>Not provided</td>
<td>Described by [Fan et al., 2008]</td>
<td>29\textsuperscript{a}</td>
<td>[Zheng et al., 2011]</td>
</tr>
<tr>
<td>Yes</td>
<td>7.0-8.0</td>
<td>Artificial wastewater</td>
<td>40.1</td>
<td>[Qiao-Hui et al., 2008]</td>
</tr>
<tr>
<td>Yes</td>
<td>Not provided</td>
<td>f\textsuperscript{2} medium</td>
<td>~25</td>
<td>[Josephine et al., 2015]</td>
</tr>
<tr>
<td>No</td>
<td>7.5</td>
<td>BG-11 supplemented with glucose</td>
<td>53.43</td>
<td>Present study</td>
</tr>
</tbody>
</table>

\textsuperscript{a} using grinding in liquid nitrogen for extraction

As shown in the table, the data obtained in the present study agree with the findings of Yeh et al., even though the mode of cultivation and the pH are not the same.
3.2 Effect of nutrient stress

The goal of this set of experiments was to determine the effect of starvation from different nutrients (S, P and N) on biomass growth and lipid accumulation of *C. vulgaris*, under heterotrophic conditions. This is of significant importance, as in many cases, in a realistic application of wastewater treatment using microalgae the mixture of various wastewaters is used as a substrate. Thus, the potential differences in the effect of nutrient starvation could affect the selection of the most appropriate wastewater mixture for microalgal cultivation.

All experiments were carried out in the optimal pH (7.5) for this strain. For this set of experiments BG-11 supplemented with glucose was used as a substrate. As mentioned above, the limiting nutrient for *C. vulgaris* in BG-11 broth was sulfur. Thus, in order to determine the effect of other nutrient starvation the addition of a sulfur source was essential. More specifically, for the investigation of the effect of nitrogen and phosphorus stress, the medium was supplemented with 350 mg L⁻¹ MgSO₄·7H₂O. However, the addition of a sulfur source would result in higher cell density culture, thus the medium was supplemented with greater amounts of glucose. Being impossible to achieve phosphorus stress while using phosphate buffer, in this case the regulation of pH was realized using tris(hydroxymethyl)aminomethane as a buffer solution (§2.3.1). The substrate composition of each experiment is stated in the following table:

**Table 3.3**: Substances added to BG-11 broth for the investigation of the effect of nutrient starvation.

<table>
<thead>
<tr>
<th></th>
<th>S stress</th>
<th>P stress</th>
<th>N stress</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial glucose</strong></td>
<td>10 g L⁻¹</td>
<td>27 g L⁻¹</td>
<td>40 g L⁻¹</td>
</tr>
<tr>
<td><strong>MgSO₄·7H₂O addition</strong></td>
<td>-</td>
<td>350 mg L⁻¹</td>
<td>350 mg L⁻¹</td>
</tr>
<tr>
<td><strong>Buffer solution</strong></td>
<td>Phosphate buffer</td>
<td>Tris(hydroxymethyl) aminomethane</td>
<td>Phosphate buffer</td>
</tr>
</tbody>
</table>

The results are thoroughly discussed below.

- **Biomass**

In the study of the effect of sulfur stress, the stationary phase was achieved during the 8th day of cultivation, after a lag phase of 2 days. Moreover, the maximum
biomass generation was 2.69 g L\(^{-1}\) and the biomass productivity was 0.2976 g L\(^{-1}\) day\(^{-1}\). The exponential growth phase lasted 6 days.

The addition of a sulfur source had a significantly positive effect on biomass growth, thus verifying that the limiting substrate for \(C.\ vulgaris\) in BG-11 medium was sulfur. In the experiment of phosphorus starvation, after 10 days of cultivation the microorganism reached stationary phase. The maximum biomass generation was 9.81 g L\(^{-1}\), and the biomass productivity 0.8289 g L\(^{-1}\) day\(^{-1}\), indicating that biomass productivity is related to nutrient bioavailability. The lag phase occurring under these conditions was insignificant (2 days) and the exponential phase lasted 8 days.

The supplementation of 350 mg L\(^{-1}\) MgSO\(_4\)-7H\(_2\)O combined with the overabundance of phosphorus resulted in nitrogen stress. \(C.\ vulgaris\) reached stationary phase in the 20\(^{th}\) day of cultivation, presenting an extended lag phase of 6 days attributed mainly to the nutrient overload and possibly to the low initial biomass (0.065 g L\(^{-1}\)). Additionally the maximum biomass generation was 11.12 g L\(^{-1}\), and the biomass productivity 0.5025 g L\(^{-1}\) day\(^{-1}\). The exponential growth phase lasted 12 days.

The following figure (Figure 3.7) depicts the growth behavior of \(C.\ vulgaris\), under the conditions previously stated.

![Figure 3.7: Biomass growth of \(C.\ vulgaris\) in BG-11 medium supplemented with glucose (and sulfur for P, N stress).](image)

The aeration supply in the experiment where S starvation was studied was sufficient (1vvm), and the pH had small variations attributed to experimental error. In the end
of the cultivation period, a slight drop in pH (final value approximately 7.22) is ascribed to compounds emerging from lysis of dead cells.

In the study of phosphorus stress the aeration, provided in a rate of 1 vvm, turned out to be inadequate for a culture with such a high biomass density. During the last days of cultivation, fermentation took place inside the reactors, resulting in the production of Volatile Fatty Acids (VFA’s). More specifically, there was a variety of organic acids in the medium, with acetic acid being the dominant, in concentration of 0.495 g L\(^{-1}\). The decline of the pH value was observed, reaching a value of approximately 4.67, even though the culture medium was resistant to pH changes.

In the case of N starvation the aeration rate of 1vvm was undoubtedly inadequate, a conclusion easily drawn while observing the figure illustrating the pH through time (Figure 3.8). After the microorganism reached stationary phase, the pH had a declining course, which reached the value of approximately 6.30 during the 32\(^{th}\) day of cultivation and was the result of fermentation.

![Figure 3.8: pH during the experiments of S, P, and N starvation, respectively.](image)

At this point, it should be noted that each time a sample was extracted, the pH was regulated at the initial value of 7.5, using 0.1 M NaOH, if a big change was observed.
The oxygen supply in the case of N starvation was not sufficient, and thus, a large portion of the microorganisms was subjected to anaerobic conditions, resulting to the production of organic acids. The analysis of the samples showed that in day 32 the accumulation of acetic acid was 1.643 g L⁻¹, in addition to many other organic acids in lower concentrations. In the end of the 32th day of cultivation, the aeration rate was elevated at 2 vvm. This resulted in the rise of the pH, due to the cease of the fermentation processes and consumption of organic acids, and the metabolism of C. vulgaris was restored. The consumption of organic acids caused the elevation of pH.

The results obtained, concerning the biomass growth, are summarized in the following table:

**Table 3.4**: Main features occurring during different kinds of nutrient starvation at pH=7.5.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Unit</th>
<th>S stress</th>
<th>P stress</th>
<th>N stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific Growth Rate</td>
<td>μ_max</td>
<td>days⁻¹</td>
<td>0.5409</td>
<td>0.5348</td>
<td>0.2953</td>
</tr>
<tr>
<td>Lag phase</td>
<td>-</td>
<td>days</td>
<td>2</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Stationary phase</td>
<td>-</td>
<td>day</td>
<td>8</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Initial biomass Concentration</td>
<td>X₀</td>
<td>g L⁻¹</td>
<td>0.084</td>
<td>0.081</td>
<td>0.065</td>
</tr>
<tr>
<td>Maximum Biomass Generation</td>
<td>X_gen</td>
<td>g L⁻¹</td>
<td>2.689</td>
<td>9.809</td>
<td>11.12</td>
</tr>
<tr>
<td>Maximum Biomass Productivity</td>
<td>P</td>
<td>g L⁻¹ day⁻¹</td>
<td>0.2967</td>
<td>0.8289</td>
<td>0.5025</td>
</tr>
</tbody>
</table>

- day of achievement;  
- during stationary phase;  
- calculated from the mean biomass concentration during stationary phase

- Nutrient uptake

In general, the increase of available nutrients has positive effects on the growth of a microorganism. However, after a certain point the increase of nutrient concentration may present an inhibiting effect due to nutrient overload. In the present study, the specific growth rate was found to be related to the initial glucose concentration. As mentioned above, in the experiment where nitrogen starvation was investigated the μ_max was significantly lower than the other two (S and P starvation). Hence, substrate inhibition was observed, which was triggered somewhere between 27 and 40 g L⁻¹
glucose. The following figure depicts the specific substrate consumption \(\left(\frac{1}{X} \cdot \frac{dc}{dt}\right)\) of *C. vulgaris* in the experiments where S, P and N starvation was investigated:

*Figure 3.9: Specific Glucose Consumption of *C. vulgaris* during the experimental period, under S, P and N stress respectively.*

By observing the figure above one can see that during S and P stress where the initial concentration of glucose was 10 and 27 g L\(^{-1}\) respectively, the specific substrate consumption presented a declining course from the beginning of the experimental period, in which the rate was lowered through time. This result indicates the normal function of the metabolism of the microalga, from the first days of each experiment. Nevertheless, during nitrogen stress, the specific glucose consumption presented an increasing course during the first days of cultivation, verifying the inhibiting effect of the initial glucose concentration, which in this case was approximately 40 g L\(^{-1}\).

The relation between the maximum specific growth rate and the initial glucose concentration is illustrated in the following figure (*Figure 3.10*). All the parameters (S, P content) were found to be unrelated to the specific growth rate.
In the experiments where S and N starvation was studied, phosphorus was not monitored because of the use of phosphate buffer. Due to the high concentration of phosphate in the culture medium, no conclusion could be drawn about its consumption.

In the study of S stress, the nitrogen consumption reached a value of 93.05 mg L\(^{-1}\) corresponding to 412.1 mg L\(^{-1}\) (NO\(_3^-\)) and in reference to sulfur, the uptake was 4.66 mg L\(^{-1}\), which corresponds to 13.98 mg L\(^{-1}\) sulfate (SO\(_4^{2-}\)).

During the investigation of the effect of P stress, the overall glucose consumption was 24.64 g L\(^{-1}\). Sulfur and nitrogen were not monitored, as additional amounts were added in order to ensure that they were in excess. The phosphorus uptake was 13.89 mg L\(^{-1}\) corresponding to 42.62 mg L\(^{-1}\) PO\(_4^{3-}\).

Due to the higher biomass density in the case of nitrogen stress, the nutrient uptake was significantly larger than the other two experiments. More specifically, in order to reach that biomass concentration, the microorganism consumed 53.66 mg L\(^{-1}\) of sulfur, which corresponds to 160.9 mg L\(^{-1}\) sulfate (SO\(_4^{2-}\)) and 291.8 mg L\(^{-1}\) of nitrogen, corresponding to 1292.2 mg L\(^{-1}\) (NO\(_3^-\)). Finally, the overall glucose consumption was 37.63 g L\(^{-1}\).

The following figure (Figure 3.11) illustrates the biomass growth and nutrient consumption of *C. vulgaris* in the conditions mentioned above:
Lipid accumulation

The effect of different nutrient starvation on lipid accumulation under heterotrophic growth was also studied. In all experiments the dominant fatty acid was oleic acid, and the fatty acid profile was unrelated to the different nutrient starvation. Moreover the oleic acid content in total lipids presented the same pattern as in the previous set of experiments (Figure 3.5). The intracellular lipid content was highest under sulfur stress (53.43%, 48.17% and 32.70% g g$_{DW}^{-1}$ in total lipids, unsaturated lipids and oleic acid respectively), followed by nitrogen stress (25.80%, 24.89% and 14.39% respectively) and phosphorus stress (17.42%, 13.66% and 10.04%). These findings indicate that the abundance of phosphorus has positive effects on lipid accumulation.

The following figure (Figure 3.12) depicts the lipid content of C. vulgaris under different nutrient starvation, during the cultivation period:
The lipid content increases rapidly during the growth period, and after a nutrient is depleted, it varies between relatively small ranges. Moreover, the lipid productivity was calculated, and the main features are discussed below.

During S and N starvation, the lipid productivity presented its highest value two days after the microalga reached stationary phase, while for P starvation it happened 4 days after the achievement of stationary phase. After this point, the lipid productivity had a declining course. The lipid productivity can be divided in two parts, the rising part and the declining part. The rising part presents a steep incline, due to the fact that two processes take place, the increase in cell concentration and the increase in lipid content. In this part the metabolism gets activated and the dual goal of biomass growth and lipid accumulation begins in a high rate. In the peak, the biomass has reached the highest cell density, but the lipid content still increases. In the declining part, the microalga does not increase its cell density as it has reached stationary phase and the death of cells is observed, while the main process taking place in the microalgal cell is the accumulation of intracellular lipids.
During sulfur stress, after the productivity reached a peak it remained relatively constant at 83.02 mg L$^{-1}$ day$^{-1}$ (total lipids) until the 18$^{th}$ day, where the death phase began and resulted in the drop of lipid productivity. In the study of nitrogen stress, a declining course was presented after the peak which indicates that due to the lack of oxygen supply (see above) the metabolism shifted, and the process of fermentation took place. The result is the retardation of lipid accumulation and the production of organic acids. After the aeration was restored (30$^{th}$ day) the total lipid accumulation rate was stabilized at 73.91 mg L$^{-1}$ day$^{-1}$. Finally, during phosphorus stress, the mean lipid productivity during stationary phase presented the lowest value of 66.78 mg L$^{-1}$ day$^{-1}$.

Furthermore, the concentration of lipids was the highest under nitrogen starvation, with the values of 2692.4, 2597.8, and 1501.1 mg L$^{-1}$ for total lipids, unsaturated lipids and oleic acid respectively, presented during the 36$^{th}$ day of cultivation. Although, the lipid content of *C. vulgaris* under these conditions was not the highest, the high concentration of biomass resulted in high concentration of lipids. As discussed above, the rise in lipid concentration after the 30$^{th}$ day was due to the rise in oxygen supply. However, the concentration of lipids in the other two experiments was comparable, even though the density of *C. vulgaris* cells was significantly larger during P starvation. More specifically, under P stress the highest concentration was 1469.1, 1329.6 and 1023.5 mg L$^{-1}$, while under S stress it was 1422.3, 1283.1 and 870.8 mg L$^{-1}$ for total lipids, unsaturated lipids and oleic acid respectively.

Another conclusion drawn is related to the optimal time of harvest of the cells. More specifically, when the microalga has achieved stationary phase, it possesses the most desirable characteristics for biofuel production. At this time, the intracellular lipids (and the most suitable for biodiesel production, oleic acid) are accumulated at the highest rate and the concentration has reached a value that is comparable to the maximum one. More specifically, the lipid content increases approximately 5% during the period between early stationary phase and the end of each experiment. If the microalga remains further in the medium, without external nutrient supply, the lipid content is not going to be substantially increased and the assimilation of storage lipids could be triggered if there is not enough carbon in the medium. Additionally, in terms of nutrient uptake viewed as a wastewater post-treatment process, the highest rate is observed during the exponential phase of growth. Hence, in order to harness the desired characteristics of *C. vulgaris* at the greatest extent, both from biofuel production and wastewater treatment efficiency point of view, the harvest should be performed at the point of early stationary phase. In continuous systems, the microalgal specific growth rate is related to the hydraulic retention time (HRT). HRT is a parameter strongly related to the energy requirements and operational costs of a unit. Thus, HRT should be adjusted so as to achieve the dual
goal of nutrient uptake and biofuel production, without increasing the operational costs.

The following table summarizes the results obtained relating to lipid accumulation.

**Table 3.5:** Main features relating to lipid accumulation, occurring during different kinds of nutrient starvation at pH=7.5 (maximum values).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>S stress</th>
<th>P stress</th>
<th>N stress</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lipid Content (% g g&lt;sub&gt;DW&lt;/sub&gt;-1)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oleic acid</td>
<td>32.70</td>
<td>10.04</td>
<td>14.39</td>
</tr>
<tr>
<td>Total Lipids</td>
<td>53.43</td>
<td>17.42</td>
<td>25.80</td>
</tr>
<tr>
<td>Unsaturated Lipids</td>
<td>48.71</td>
<td>13.66</td>
<td>24.89</td>
</tr>
<tr>
<td><strong>Productivity (mg L&lt;sup&gt;-1&lt;/sup&gt; day&lt;sup&gt;-1&lt;/sup&gt;)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oleic acid</td>
<td>76.98</td>
<td>65.12</td>
<td>58.33</td>
</tr>
<tr>
<td>Total Lipid</td>
<td>132.64</td>
<td>96.47</td>
<td>98.83</td>
</tr>
<tr>
<td>Unsaturated Lipids</td>
<td>119.03</td>
<td>88.03</td>
<td>93.84</td>
</tr>
<tr>
<td><strong>Concentration (mg L&lt;sup&gt;-1&lt;/sup&gt;)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oleic acid</td>
<td>870.8</td>
<td>1023.5</td>
<td>1501.1</td>
</tr>
<tr>
<td>Total Lipids</td>
<td>1422.3</td>
<td>1469.1</td>
<td>2692.4</td>
</tr>
<tr>
<td>Unsaturated Lipids</td>
<td>1283.1</td>
<td>1329.6</td>
<td>2597.8</td>
</tr>
</tbody>
</table>

### 3.3 Carbon Sources

#### 3.3.1 VFA’s

In this set of experiments the use of Volatile Fatty Acids as a carbon source for *C. vulgaris* was studied. VFAs are metabolic products, intermediate or final, of many processes. These processes include anaerobic digestion, dark fermentation (aiming at H<sub>2</sub> production) and acidogenesis. The effluent streams of these processes, depending on the operational conditions, also contain other substances in lesser amounts, and can be further processed using microalgae. VFA’s can also be inadvertently produced, in cases where wastewaters are stored for a long period due to uncontrolled fermentation.

Thus, the goal of this part of the present study was to investigate which VFAs can be used as carbon sources by *C. vulgaris*, individually or in a combination with glucose, grown without the presence of light. Moreover, the effect of the different carbon
sources on the maximum specific growth rate was studied. All the experiments were carried out in pH 7.5, regulated using a buffer solution. However, the consumption of acetic acid resulted in increase of the pH value, and thus the pH was corrected using HCl 1M.

The VFAs tested as carbon sources were acetic, propionic, butyric and isobutyric acid. *C. vulgaris* failed to grow on propionic, butyric and isobutyric acid as sole carbon sources respectively. However it was able to grow on acetic acid. The results obtained are illustrated on the following figure (Figure 3.13):

![Figure 3.13: Biomass growth and acetic acid assimilation.](image)

The microalga did not present a lag phase. However, the maximum specific growth rate obtained during this experiment was 0.4293 days⁻¹, which was 20.63% less than the one obtained when glucose was the sole carbon source at pH 7.5. This outcome was expected as glucose possesses more energy per mol when compared to acetic acid (2.8 kJ mol⁻¹ and 0.8 kJ mol⁻¹ respectively) [Boyle et al., 2009]. The consumption of 1.476 g L⁻¹ acetic acid resulted in a biomass generation of 0.336 g L⁻¹.

The lipid content was monitored by SPV method, as because of the low concentration of biomass, large samples needed to be extracted in order to carry out the FAME analysis. The highest lipid content was observed during the 6th day of cultivation where the microalga reached stationary phase and was 14.42% and 12.52%, for SPV canola oil and oleic acid respectively. This was due to the fact that the limiting substrate was acetic acid.
There are cases where the processes mentioned above (anaerobic digestion, acidogenesis, fermentation) do not achieve the goal of eliminating the organic substances due to incorrect designing of the process. Hence, the produced effluent streams contain organic acids in addition to sugars and other organic compounds. These streams can be further treated using microalgae. In the following experiment, the combination of all the VFAs mentioned above in addition to glucose was used as carbon source for \textit{C. vulgaris}, simulating this case. The results are illustrated in the following figure (Figure 3.14):

![Figure 3.14: Biomass growth and assimilation of carbon sources by \textit{C. vulgaris}.](image)

As shown in the figure above, \textit{C. vulgaris} was again unable to assimilate propionic, butyric and isobutyric acid, while glucose and acetic acid were metabolized simultaneously. However, in another study using the same strain for the biotreatment of fermented sewage sludge, \textit{C. vulgaris} was able to assimilate all above mentioned VFAs, in addition to valeric and isovaleric acid, under the presence of light [Cho et al., 2015]. These results indicate that, even though \textit{C. vulgaris} possesses the necessary metabolic characteristics to assimilate organic acids, the increase of carbon atoms in the aliphatic chain make the acids mentioned above non-degradable for \textit{C. vulgaris}, under heterotrophic conditions. Consequently, the presence of light is essential for the assimilation of long-chained VFAs.

The maximum specific growth rate obtained in this case was 0.5338 days$^{-1}$, a value comparable to that obtained when glucose was the only carbon source. Thus, even though there were substances in the medium which could not be assimilated, they did not affect the growth rate of \textit{C. vulgaris}. The consumption of 2.94 and 1.10 g L$^{-1}$
glucose and acetate respectively, resulted in a maximum biomass generation of 1.397 g L\(^{-1}\). In this case, the limiting nutrient was the carbon source.

The lipid content was again monitored by SPV method, and the results are depicted in the following figure (Figure 3.15):

![Figure 3.15: Lipid content of C. vulgaris, monitored via SPV method.](image)

The maximum lipid content was achieved during the 14\(^{th}\) day of cultivation and was 10.28 and 8.74\%, for SPV using canola oil and oleic acid respectively. In this case the lipid content remained in low levels as the limiting nutrient was the carbon source. As previously stated, SPV method underestimates the lipid content.

### 3.3.2 Phenolic compounds

The phenolic compounds tested as potential carbon sources for \(C. vulgaris\) were vanillic, ferulic, syringic, gallic, \(p\)-hydroxybenzoic acid and catechol. The culture medium consisted of BG-11 medium, supplemented with the respective phenol. All experiments were conducted in pH 7.5, under heterotrophic conditions.

It is widely known that phenol degradation by microorganisms is generally limited by substrate (phenol) inhibition and low specific conversion rates. However, the pre-adaptation of the microorganisms in the respective phenol was found to remarkably enhance the degradation rate. Thus, before the experiments with phenol as the sole carbon source were carried out, a process of acclimation took place. The stages of this adaptation process are shown in the following table:
Table 3.6: Stages of *C. vulgaris* adaptation to phenols.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Glucose (g L(^{-1}))</th>
<th>Phenol (mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>200</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>300</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>350</td>
</tr>
</tbody>
</table>

The goal of this process was to introduce the respective phenols in a gradually increasing concentration, while reducing the amount of glucose in the culture medium. The addition of glucose in the medium allows the growth of *C. vulgaris* in a medium supplemented with a toxic for the microalga compound. Hence it promotes the adaptation to phenol, as glucose constitutes the easiest degradable organic compound by microalgae. The final concentration of phenol in the adaptation process (350 mg L\(^{-1}\)) was similar to that of the final experiment with phenol as the sole carbon source (500 mg L\(^{-1}\)) in order to obtain the best results possible.

Although the presence of 50 mg L\(^{-1}\) catechol in the culture medium allowed the growth of *C. vulgaris*, in the second stage of the acclimation process where catechol concentration was 100 mg L\(^{-1}\), the growth of the microalga was inhibited. This finding indicates that the toxicity of catechol in *C. vulgaris* starts between 50 and 100 mg L\(^{-1}\), thus confirming the high toxicity of this particular phenol to microalgae. The adaptation process of *C. vulgaris* to catechol was not continued.

All the other phenols allowed the growth of *C. vulgaris* in a medium supplemented with glucose, in all the stages of the acclimation process. However, the microalga was not able to degrade these phenols when glucose was present in the medium. Even after the depletion of glucose, the phenol concentration remained stable. The sole exception was that of gallic acid, which *C. vulgaris* was able to use as carbon source, even during the acclimation process.

After the process of pre-adaptation, the main experiments with each phenol as the sole carbon source, in a concentration of 500 mg L\(^{-1}\), were conducted. *C. vulgaris* was unable to grow with vanillic, ferulic and p-hydroxybenzoic acid as sole carbon sources in a cultivation period of 15 days. However, gallic and syringic acid were degraded by *C. vulgaris* in the respective experiments. The results obtained are presented in the following figure:
During the assimilation of gallic acid the biomass generation was 0.0341 g L$^{-1}$, resulted from the degradation of 0.3166 g L$^{-1}$ phenol. By observing Figure 3.16 one can see a big drop in gallic acid concentration during the first days of the experiment, which does not correspond to an increase in biomass concentration. At this point it should be noted that the determination of the concentration of phenolic compounds was conducted via Folin-Ciocalteu method, which is not a high resolution technique and does not offer much information about the biotransformation of compounds in the medium. It only quantifies the decrease in concentration of phenolic compounds, without detecting the production of intermediate metabolic compounds in the culture medium. Thus, this drop might be the result of the biotransformation of gallic acid to other compound(s) by the metabolic activity of C. vulgaris. As expected, the degradation of gallic acid resulted in low maximum specific growth rate, which presented the value of 0.0631 days$^{-1}$. Additionally, the microorganism presented a prolonged lag phase of 6 days.

In the case of syringic acid, a similar trend was observed. However, the performance of C. vulgaris with this particular phenol as a substrate was slightly improved. The biomass generation was 0.1066 g L$^{-1}$ as a result of the degradation of 0.263 g L$^{-1}$ phenol. The microalga again presented a lag phase of 6 days, and the specific growth rate was 0.1028 days$^{-1}$. All these results verify the inhibiting effect of phenols on microalgal growth.

### 3.3.3 Effect of carbon source on $\mu_{\text{max}}$

The maximum specific growth rate is highly affected by the provided carbon source. Compounds that are easily assimilated and are characterized by high energy content promote the growth of microorganisms and thus result in high specific growth rates.
In the present study, various compounds were tested as potential carbon sources for *C. vulgaris*. The results obtained concerning $\mu_{\text{max}}$ are summarized in the following table. All experiments were carried out in pH 7.5, with BG-11 supplemented with the respective organic substance as substrate. The results obtained are summarized in the following table:

**Table 3.7**: Maximum specific growth rate obtained during the assimilation of various compounds

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>VFAs + Glucose</th>
<th>Acetic acid</th>
<th>Syringic acid</th>
<th>Gallic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu_{\text{max}}$ (days$^{-1}$)</td>
<td>0.5409</td>
<td>0.5338</td>
<td>0.4293</td>
<td>0.1028</td>
<td>0.0631</td>
</tr>
</tbody>
</table>

Glucose is the easiest degradable organic compound among those tested. This fact is also proven by the high $\mu_{\text{max}}$ obtained when it was provided as a substrate. Acetic acid is also easily assimilated by *C. vulgaris*. However, when it is the only organic compound supplied in the medium, the specific growth rate obtained was 79.37% of the one obtained with glucose. This is due to the lower energy content of acetic acid (2.8 kJ mol$^{-1}$ of energy for glucose compared to 0.8 kJ mol$^{-1}$ for acetate) [Boyle et al., 2009]. In the experiment where the combination of organic acids with glucose was supplied, the specific growth rate was comparable to the one obtained when glucose was the sole carbon source. This result indicates that the presence of glucose in the medium has positive effects on the growth of the microalga.

On the other hand, phenols constitute a non-easily assimilated substrate. The inhibiting effect of phenols is easily noticed by the substantial drop of the maximum specific growth rate of *C. vulgaris* when gallic and syringic acid were supplied as carbon sources. More specifically, the value obtained for $\mu_{\text{max}}$ was 11.66% of the one for glucose when gallic acid was provided, while for syringic acid it was 19.00%. The aromatic ring of phenols constitutes a complex structure which demands increased amounts of energy spent by microalgae in order to be disrupted and further on assimilated. This energy demanding process results in low $\mu_{\text{max}}$ when phenolic compounds are supplied as the only carbon source.

**3.4 Olive Mill Wastewater**

**3.4.1 Qualitative Characteristics of OMW**

The physico-chemical characterization of OMW was carried out and the main features are the following:
• High organic load and solids concentration
• High concentration of phenolic compounds
• Low concentration of nitrogen
• Low pH (~5.0)

In the following table the results from the physico-chemical characterization of OMW are presented:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Raw OMW</th>
<th>Filtered OMW²</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>-</td>
<td>5.19 ± 0.21</td>
<td>5.19 ± 0.21</td>
</tr>
<tr>
<td>Alkalinity</td>
<td>mg CaCO₃ L⁻¹</td>
<td>2087.50</td>
<td>712.50</td>
</tr>
<tr>
<td>Carbohydratesᵇ</td>
<td>g L⁻¹</td>
<td>19.30 ± 0.26</td>
<td>18.30 ± 0.26</td>
</tr>
<tr>
<td>COD</td>
<td>g L⁻¹</td>
<td>134.4 ± 1.2</td>
<td>50.8 ± 0.6</td>
</tr>
<tr>
<td>Phenolsᶜ</td>
<td>g L⁻¹</td>
<td>5.26 ± 0.09</td>
<td>5.26 ± 0.09</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>mg L⁻¹</td>
<td>327.2 ± 14.6</td>
<td>215.1 ± 0.4</td>
</tr>
<tr>
<td>Total Kjeldahl Nitrogen (TKN)</td>
<td>mg L⁻¹</td>
<td>290.70 ± 3.52</td>
<td>235.10 ± 2.41</td>
</tr>
<tr>
<td>Ammonium Nitrogen</td>
<td>mg L⁻¹</td>
<td>1.60 ± 0.03</td>
<td>1.60 ± 0.03</td>
</tr>
<tr>
<td>TS</td>
<td>g L⁻¹</td>
<td>89.67 ± 1.04</td>
<td>45.23 ± 0.07</td>
</tr>
<tr>
<td>VS</td>
<td>g L⁻¹</td>
<td>70.23 ± 0.94</td>
<td>34.24 ± 0.14</td>
</tr>
<tr>
<td>TSS</td>
<td>g L⁻¹</td>
<td>30.88 ± 1.07</td>
<td>-</td>
</tr>
<tr>
<td>VSS</td>
<td>g L⁻¹</td>
<td>30.41 ± 1.0</td>
<td>-</td>
</tr>
<tr>
<td>Oils and Grease</td>
<td>g L⁻¹</td>
<td>22.33</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

² via Whatman® (Grade GF/F);ᵇ In equivalent glucose;ᶜ In equivalent syringic acid

In all experiments of microalgal growth, filtered OMW was used as a substrate.

### 3.4.2 Attempts for the biotreatment of OMW

As described earlier (§1.3.1, 3.4.1), OMW is a wastewater with complex composition. Its most distinctive features are the high concentration of phenolic compounds, and the low nitrogen content, all present in the form of organic nitrogen, which make OMW a non-easily degradable substrate for microalgae (§1.4.5.1.4). Moreover, due
to its high organic load along with the high concentration of phenols, OMW needs to be diluted aiming to be potentialy used as substrate for microalgal growth.

The first attempt for biotreatment of OMW with the microalga *C. vulgaris*, was in concentration of OMW at 10%, 25% and 50% v v\(^{-1}\), diluted with 3D water. After a cultivation period of 15 days, no growth was observed.

Based on the previous experiments of the present study, where it was observed that the addition of a sulfur source has significantly positive effects on *C. vulgaris* growth, the measurement of sulfur content in OMW was carried out. It was found that this particular wastewater has significantly low sulfur content, which is further lowered due to the dilution of OMW with 3D water. Thus, the first series of experiments was subsequently repeated, but this time the effect of addition of 350 mg L\(^{-1}\) MgSO\(_4\)\(\cdot\)7H\(_2\)O was investigated, without altering the pH. After a period of 15 days, no growth was observed.

After the failure of the second series of experiments, the speculation was that, although *C. vulgaris* can grow on pH 5 (initial pH of OMW)(§3.1), the combination of inhibiting factors (phenolic compounds, low pH, low nitrogen content) might be the reason for which *C. vulgaris* cannot grow on OMW. The experiments were then repeated again, in the optimal pH for *C. vulgaris*. The conditions were the following:

- Concentration of OMW: 10%, 25% and 50% v v\(^{-1}\).
- pH = 7.5, regulated with phosphate buffer solution.
- Addition of 350 mg L\(^{-1}\) MgSO\(_4\)\(\cdot\)7H\(_2\)O.

Again, after a period of 15 days, no growth was observed.

The next assumption was that *C. vulgaris* could not grow on OMW because it was not acclimated in this particular wastewater. In an effort to acclimate *C. vulgaris* in OMW, a batch culture of the microalga was prepared, containing 10% OMW, diluted with BG-11 culture medium (§2.2.1), supplemented with 2 g L\(^{-1}\) glucose and 350 mg L\(^{-1}\) MgSO\(_4\)\(\cdot\)7H\(_2\)O. Surprisingly, after a lag phase of 12-14 days, *C. vulgaris* started growing, resulting in a high density culture, which was subsequently used as inoculum for the next series of experiments. In this series of experiments the substrate was 10% OMW supplemented with MgSO\(_4\)\(\cdot\)7H\(_2\)O, at the initial pH of OMW and at the optimal pH for *C. vulgaris*. After 6 days of cultivation, no growth was observed.

The last assumption was that one or some of the essential nutrients for microalgal growth was missing. Since the sulfur and phosphorus sources were abundant, due to the addition of MgSO\(_4\)\(\cdot\)7H\(_2\)O and phosphate buffer, respectively, the addition of a nitrogen source was found to be requisite. Thus, KNO\(_3\) was added in the substrate as
a nitrogen source, in the same concentration that NO$_3^-$ is present in BG-11 medium. In a period of 15 days after the addition of KNO$_3$, C. vulgaris failed to grow.

In order to draw a conclusion about the reason for which OMW could not support C. vulgaris growth, the final experiment carried out was with 10% OMW supplemented with BG-11 medium at pH 7.5. During the first 9 days no growth was observed. However, during the 12$^{th}$ day of cultivation, infection of the medium was observed. Three days after, this microorganism was the dominant one in the culture. The following pictures illustrate the microorganism that grew in the medium, without being inoculated:

![Picture 3.1: Infection of OMW.](image)

This microorganism appears to be a fungus. However, it was not the first time that this kind of infection was presented when OMW was used as substrate. More specifically, this incident was observed in other attempts for the treatment of OMW and the experiments were then repeated. The fact that a microorganism that was not inoculated thrived in these conditions and dominated the medium indicates that it is more suitable for the treatment of OMW than C. vulgaris. Nevertheless, C. vulgaris cell number also increased, indicating that the minerals present in BG-11 broth had positive effects on the growth of the microalga in OMW, and that the lack of these compounds in the wastewater was the inhibiting factor for C. vulgaris in the previous experiments. The lag phase (12 days) of the microalga was the result of the acclimation process to a medium containing phenols in a concentration of approximately 0.5 g L$^{-1}$. Hence, C. vulgaris can tolerate the presence of phenols in that concentration.
3.5 Energy valorization of *C. vulgaris* biomass

Biofuel production from microalgal biomass includes harvesting and in most cases drying. However, biodiesel production requires the additional steps of transesterification and purification. These steps include the use of organic solvents which can cause harm to human health and downgrade the environment. These processes augment the cost of biofuel production, thus making it an economically unviable solution. Especially in cases where the lipid content is low, the processes that must take place in order to obtain the FAMEs cost more than the resulting biofuel itself. However, in order to obtain valid results about the process viability, techno-economical evaluations must take place.

In an effort to explore the potentials of microalgal biomass exploitation, in the present study the energy valorization of *C. vulgaris* biomass through direct combustion and anaerobic digestion were also investigated.

### 3.5.1 Direct combustion

In order to valorize energetically the microalgal biomass through direct combustion, the most indispensable process that must take place is that of harvesting. However there are some factors that lower the energy recovery of the combustion process. More specifically, the main obstacle is the high water content in the harvested biomass. During combustion, a part of the supplied energy is consumed for the evaporation of water (latent heat of evaporation), thus lowering the net energy recovery of this particular process.

Moreover, the calorific value of microalgal biomass is directly correlated with its lipid content. The variation in microalgal composition is influenced by the growth conditions. Under nutrient starvation, microalgae accumulate carbon in the form of intracellular lipids and starch. Thus, under nutrient deprivation the produced microalgae are characterized by higher calorific values.

The specific calorific value was measured in samples harvested in the stationary phase of the heterotrophic cultivation of *C. vulgaris*, in pH 7.5 under sulfur starvation. The samples were subsequently frozen and freeze dried in order to eliminate their water content. The specific calorific value of *C. vulgaris* dry biomass was found to be 24,525 ± 182 kJ kg⁻¹ or 5,861 ± 44 kcal kg⁻¹.

In the following table the specific calorific values of petroleum and coal are presented for comparison:
Table 3.8: Specific calorific values of *C. vulgaris* biomass, coal and petroleum.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Specific Calorific value (kJ kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. vulgaris</em> biomass</td>
<td>24,525 ± 182</td>
</tr>
<tr>
<td>Coal (Lignite)</td>
<td>15,000</td>
</tr>
<tr>
<td>Coal (Anthracite)</td>
<td>27,000</td>
</tr>
<tr>
<td>Petroleum</td>
<td>43,000</td>
</tr>
</tbody>
</table>

The calorific value of *C. vulgaris* dry biomass is 57% of petroleum and 94.5% of anthracite. This value also is 63.5% higher than the calorific value of lignite.

### 3.5.2 Biochemical Methane Potential (BMP)

#### 3.5.2.1 Theoretical Estimation of Parameters

The elemental composition analysis provided the C, H, O and N content of *C. vulgaris*. The S content of the microalgal biomass was below the detection limit of this method, and thus, it was determined from the sulfur consumption of the experiments presented in §3.1. Phosphorus was determined from the data presented in §3.2. Hence, *C. vulgaris* biomass composition is presented in the formula below:

\[
C_{885.252}H_{408.814}O_{505.483}N_{47.539}S_{1.214}P
\]

Thus the C:N ratio is 18.62. After establishing the composition of *C. vulgaris* biomass, the theoretical estimation of methane production was carried out:

\[
C_{885.252}H_{408.814}O_{505.483}N_{47.539} + 565.960 H_2O \rightarrow 349.530 CH_4 + 535.722 CO_2 + 47.539 NH_3
\]

The theoretical methane yield is calculated below:

\[
Y_{methane} = 395.73 \frac{mL}{g_{VS \, destroyed}}
\]

The theoretical ratio of methane to carbon dioxide is \(r = 0.65\), while the average carbon oxidation state in the substrate is \(n = 0.84\).

Finally, the theoretical ammonium production yield is evaluated below:

\[
Y_{NH_3} = 40.85 \frac{mg}{g_{VS \, destroyed}}
\]
3.5.2.2 Experimental Estimation of Parameters (BMP assays)

The following part of the present study concerns the investigation of the Biochemical Methane Potential of *C. vulgaris* biomass. The main limitations during this process are (1) the fact that microalgae contain a rigid cell wall which might remain intact during the anaerobic digestion process and (2) the high nitrogen content of microalgal biomass which results in a low C: N ratio. The latter has as a consequence the release of ammonia from the decomposition of the contained proteins and the potential toxic effect on the anaerobic microflora.

However, the growth conditions directly influence the composition of microalgae. Under nutrient starvation, they accumulate carbon in the form of intracellular lipids and starch. During this process the protein content decreases, and thus the nitrogen content is lowered. These two phenomena may therefore improve both the conversion efficiency and the stability of the process by limiting the toxic effect of ammonia.

The BMP assays were carried out in various conditions in order to establish the optimal ones for the anaerobic decomposition of microalgal biomass. More specifically, they were conducted in mesophilic (37°C) and thermophilic (55°C) temperatures. The microalgal biomass was harvested in the stationary phase of the experiment at pH 7.5 under S stress. The samples were frozen and subsequently freeze dried in order to eliminate the water content. However, this process can be considered as biomass pretreatment since the formation of ice crystals during the freezing step (prior to freeze-drying) may result to cell disruption, as well as freeze-drying itself.

The sludge which was used as inoculum in the BMP assays conducted in mesophilic conditions was derived from the anaerobic digestion reactor of the Wastewater Treatment Plant of Heraklion, Crete. Moreover, the inoculum for the BMP assays carried out in thermophilic conditions originated from an Upflow Packed Bed Reactor (UPBR), used for the biotreatment of Olive Mill Wastewater, under thermophilic conditions. In addition to the microalgal biomass, cellulose was also used for the BMP assays as a control substrate. The control assays give an idea of the inoculum response toward “standard” substrates, and thus define its activity.

The results obtained in mesophilic conditions are illustrated in the following figure:
Figure 3.17: Cumulative biogas and methane production during BMP assays of C. vulgaris biomass in mesophilic conditions (37°C).
(B: blank, C: cellulose, S: sample)

By observing the figure above one can see that the sample presented satisfying performance in mesophilic conditions. The total methane production of the microalgal biomass was significantly larger than that of cellulose (104.99 mL compared to 63.34 mL CH₄). The yields obtained in this case were 335.08 and 390.68 ml CH₄ gVS⁻¹ added for cellulose and C. vulgaris biomass respectively. However, cellulose presented its maximum potential in a period of 30 days, while for C. vulgaris biomass the maximum potential was presented after a period of 100 days. This result indicates that C. vulgaris biomass is a non-easily-degradable substrate for anaerobic microorganisms. More specifically, C. vulgaris has a remarkably rigid cell wall, mainly composed of cellulose and hemicellulose, which serves as a protective shield against invaders. Thus, anaerobic microflora struggle to decompose this robust structure, in order to unleash the high value nutrients that are accumulated inside the microalgal cells. The rate of biogas and methane production from the anaerobic decomposition of microalgal biomass was thus slower than the one obtained when cellulose was the utilized substrate. This fact indicates that the decomposition of microalgal biomass is a time consuming process for anaerobic methanogens.

The following figure depicts the anaerobic decomposition of C. vulgaris biomass under thermophilic conditions:
Figure 3.18: Cumulative biogas and methane production during BMP assays of \textit{C. vulgaris} biomass in thermophilic conditions (55°C).

(B: blank, C: cellulose, S: sample)

Under thermophilic conditions the biogas and methane production were higher with cellulose as a substrate than with \textit{C. vulgaris}. More specifically, the thermophilic methanogens produced 80 and 51 mL methane with cellulose and \textit{C. vulgaris} as a substrate, respectively. The respective yields were 389.07 and 256.57 ml gVS\(^{-1}\)added.

An additional assay was carried out, aiming at comparing the performance of a sludge acclimated in thermophilic conditions (UPBR inoculum) with the non-acclimated one (WWTP inoculum). The results obtained are illustrated in the following figure:

Figure 3.19: Cumulative biogas and methane production during BMP assays of \textit{C. vulgaris} biomass in thermophilic conditions (55°C), using non-acclimated sludge.

(B: blank, C: cellulose, S: sample)
The low biogas and methane productivity combined with the increasing tendency of biogas production verify that the temperature acclimation has a strong effect on anaerobic digestion. The cumulative methane production during 140 days in this case was 4.60 mL for cellulose and 34.14 mL for *C. vulgaris* as a substrate. Hence, the adaptation is a time consuming process which should be performed prior to the implementation of the respective experiment. The yields obtained were 21.79 and 166.38 ml CH$_4$ gVS$^{-1}$ added for cellulose and *C. vulgaris* biomass respectively.

Additionally, the effect of ultrasonication was studied as a pretreatment of *C. vulgaris* biomass. The BMP assays were again carried out in mesophillic (37°C) and thermophillic (55°C) conditions. The biomass samples used in these tests were the same as in the previous experiments. The results are illustrated in the following figures:

![Figure 3.20: Cumulative biogas and methane production during BMP assays of *C. vulgaris* biomass subjected to ultrasonication, in mesophillic conditions (37°C). (B: blank, S: sample)](image-url)

By observing the figure one can see that a significantly larger lag phase was obtained in this case, when compared to the assay performed in mesophillic conditions without biomass pretreatment. The methane production was 57.89 mL while the obtained yield was 241.55 ml CH$_4$ gVS$^{-1}$ added. Combing these results with the fact that in this case a lag phase was observed, the conclusion is that ultrasonication had a strong negative effect on anaerobic digestion. In order to establish the optimal energy input that results in higher efficiency of the anaerobic digestion of *C. vulgaris* biomass, various tests should be performed. More specifically, the biomass should be subjected to ultrasonication for various time periods and subsequently be treated.
with anaerobic digestion through BMP assays in order to determine which energy input has the strongest effect on methane production.

The effect of pretreatment was also investigated under thermophilic conditions, and the obtained results are demonstrated in the following figure:

![Figure 3.21: Cumulative biogas and methane production during BMP assays of *C. vulgaris* biomass subjected to ultrasonication, in thermophilic conditions (37°C). (B: blank, S: sample)](image)

In this case, even though the cumulative production hovered in low levels, no lag phase was observed. The overall performance of this experiment was greater than the one obtained in mesophilic conditions and comparable to the one without pretreatment, using the same sludge in thermophilic conditions. More specifically, the methane production was 41.63 mL while the obtained yield was 260.13 ml CH$_4$ gVS$^{-1}$ added. Thus, ultrasonication had almost no effects on the thermophilic anaerobic digestion of *C. vulgaris* biomass.

When comparing the results obtained for the Biochemical Methane Potential of *C. vulgaris* biomass, the best performance was observed in the assay carried out in mesophilic conditions, without pretreatment of the microalgal biomass. Thermophilic temperatures were found to have an inhibiting effect on the anaerobic digestion of *C. vulgaris* biomass. The pretreatment through ultrasonication was found to have a negative influence in the assays carried out in mesophilic conditions, while under thermophilic conditions no significant effect was observed.

The following table summarizes the results obtained, concerning the BMP assays:
Table 3.9: Cumulative methane production and methane yield of *C. vulgaris* biomass in various conditions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Substrate</th>
<th>No treatment</th>
<th>Ultrasonication</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mesophilic</td>
<td>Thermophilic</td>
</tr>
<tr>
<td>Methane mL @STP</td>
<td>Cellulose</td>
<td>63.35</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td><em>C. vulgaris</em></td>
<td>104.99</td>
<td>51</td>
</tr>
<tr>
<td>Methane Yield mL gVS(^1) added</td>
<td>Cellulose</td>
<td>335.08</td>
<td>389.07</td>
</tr>
<tr>
<td></td>
<td><em>C. vulgaris</em></td>
<td>390.68</td>
<td>256.57</td>
</tr>
<tr>
<td>Biodegradability</td>
<td><em>C. vulgaris</em></td>
<td>0.9872</td>
<td>0.6483</td>
</tr>
</tbody>
</table>

3.5.2.3 Bacterial Growth Model

The cumulative biomethane production profile from each batch experiment was fitted to a modified Gompertz bacterial growth model using OriginPro version 8. This equation has been widely used to model gas production data. The equation used was the following [Zwietering et al., 1990]:

\[
M(t) = P \exp\left\{ -\exp\left[\frac{R_m \exp(1)}{P} (\lambda - t) + 1\right]\right\}
\]

where \( M(t) \) is the cumulative methane production (mL)

\( P \) is the maximum methane production potential (mL)

\( R_m \) is the maximum methane production rate (mL d\(^{-1}\))

\( \lambda \) is the lag-phase duration (d)

\( t \) is the time (d).

At the same time, the standard error and the coefficient of determination or correlation coefficient \( R^2 \) were also obtained.

The curves obtained from the fitting are depicted on the following figure:
Comparing each set of experimental data with the relevant model simulation, the parameters of methane production potential ($P$), the maximum methane production rate ($R_m$) and lag-phase time ($\lambda$) were determined. The correlation coefficient ($R^2$) ranged between 0.948 and 0.993, with the best fitting obtained at the assay carried out in mesophilic conditions with ultrasonicated biomass. A lag phase was presented in the assay where the non-acclimated inoculum was used under thermophilic conditions and in the one where pretreated biomass was digested in mesophilic conditions. The increased values of standard deviation observed in $P$ and $\lambda$
parameterners in the case where the non-acclimated in thermophilic conditions inoculum was used contain can be attributed to the fact that during the experimental period of approximately 140 days the anaerobic microflora were not acclimated in these conditions and thus, the experimental time was not adequate for completing the sample digestion. The obtained values are summarized in the following table:

<table>
<thead>
<tr>
<th>Parameters @STP</th>
<th>No treatment</th>
<th>Ultrasonication</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mesophilic</td>
<td>Thermophilic</td>
</tr>
<tr>
<td>P (mL)</td>
<td>105.08 ± 2.78</td>
<td>45.38 ± 1.88</td>
</tr>
<tr>
<td>R_m (mL d⁻¹)</td>
<td>1.95 ± 0.17</td>
<td>1.32 ± 0.23</td>
</tr>
<tr>
<td>λ (d)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R²</td>
<td>0.981</td>
<td>0.949</td>
</tr>
</tbody>
</table>

3.5.3 Comparison of Biomass Energy Valorization Methods

In order to compare the energy valorization processes of *C. vulgaris* biomass tested in the present study, the following assumptions were made:

- Constant qualitative characteristics of the *C. vulgaris* biomass.
- Constant composition of the produced FAMEs.
- Maximum biofuel productivities in each process.
- Wastewater is used as substrate for cultivating *C. vulgaris* (zero cost of cultivation medium).

It should be noted that, since the anaerobic digestion of *C. vulgaris* in a continuous system was not studied, the data about methane productivity in this case were obtained from literature [Ras et al., 2011]. The following table summarizes the data in relation to the energy productivity, obtained from various methods of treatment of *C. vulgaris* biomass:
Table 3.11: Energy valorization of *C. vulgaris* biomass

<table>
<thead>
<tr>
<th>Biofuel</th>
<th>Specific Heating Value (kJ kg(^{-1}))</th>
<th>Biofuel Productivity(^a) (g L(_{\text{reactor}}) day(^{-1}))</th>
<th>Energy Productivity (kJ L(_{\text{reactor}}) day(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biodiesel</td>
<td>39,000</td>
<td>0.133</td>
<td>5.187</td>
</tr>
<tr>
<td><em>C. vulgaris</em> Biomass</td>
<td>24,525</td>
<td>0.829</td>
<td>20.331</td>
</tr>
<tr>
<td>Methane(^b)</td>
<td>55,530</td>
<td>0.125</td>
<td>6.918</td>
</tr>
</tbody>
</table>

\(^a\) maximum values, \(^b\) contained in produced biogass

As shown in the table above, the highest energy productivity (20.331 kJ L\(_{\text{reactor}}\) day\(^{-1}\)) was obtained from the direct combustion of *C. vulgaris* biomass. Even though the Specific Heating Values of biodiesel and methane are higher, the biofuel productivities are substantially lower, resulting in lower energy productivities.

However, in order to draw a conclusion, apart from the profit gained by the exploitation of the produced fuel, the costs of pretreatment, operation and installation must be taken into account. Biodiesel production is the most costly process, as apart from the costs of harvest and drying, the use of organic solvents and the purification process increase the expenses. Anaerobic digestion does not include the stage of drying, but the installation and operation of a reactor is necessary. However, there is the additional advantage of potential exploitation of the digested residue. The effluent of this process is nutrient rich and can be further utilized as a fertilizer, or as a substrate for microalgae production. On the other hand, direct combustion includes the processes of harvesting and drying, but also offers the advantage the produced biofuel, in the form of pellets, can be handily utilized in any solid fuel burner.

The suggested method of energy valorization of *C. vulgaris* biomass, taking into account exclusively the energetic content of biofuels tested in this study, is direct combustion. However, anaerobic digestion and biodiesel production are processes that can be further optimized.
Chapter 4.

MODELING OF C. vulgaris BIOMASS GROWTH AND LIPID SYNTHESIS

This part of the present study is dedicated to the development of a mathematical model, describing the growth and lipid synthesis of C. vulgaris. The model describes the relations between the consumption of the basic compounds of the substrate (carbon and the limiting substrate, sulfur), with the biomass growth of the microalga and the accumulation of total lipids. At the same time, the estimation of the basic kinetic parameters takes place.

In the effort to describe the behavior of C. vulgaris, grown under heterotrophic conditions in a synthetic medium containing glucose as a carbon source, the data presented in the 3rd chapter of the present study were used. More specifically, the values obtained for the growth, lipid accumulation and substrate consumption of C. vulgaris in pH 7.5 under sulfur stress were utilized. Thus, in this chapter the development of a mathematical model is discussed.

4.1 Methods

4.1.1 Matlab

MATLAB (MATrix LABoratory) is a multi-paradigm numerical computing environment and fourth-generation programming language. A proprietary programming language developed by MathWorks, MATLAB allows matrix manipulations, plotting of functions and data, implementation of algorithms, creation of user interfaces, and interfacing with programs written in other languages, including C, C++, Java, Fortran and Python. Although MATLAB is intended primarily for numerical computing; an optional toolbox uses the MuPAD symbolic engine, allowing access to symbolic computing abilities. An additional package, Simulink, adds graphical multi-domain simulation and model-based design for dynamic and embedded systems.

In 2004, MATLAB had around one million users across industry and academia [Goering, R., 2004]. MATLAB users come from various backgrounds of engineering, science, and economics. The version used for the present study for the development of the kinetic model and the simulation of experimental data was 8.2.0.29 (R2013b) which was released at 19 May 2013.
4.1.2 Numerical computation of differential equations via method ode23s

The most general form of a 1st order differential equation (ODE) can be written as follows:

\[ \frac{dy}{dt} = f(t, y(t)) \]

where \( f(t, y(t)) \) is a known and \( y(t) \) is an unknown equation. The above equation is referred to as ordinary, as the derivative is ordinary and not partial, and 1st order, as it contains only the 1st derivative of the unknown equation. Many mathematical models involve more than one unknown function, and second and higher order derivatives. These models can be handled by making \( y(t) \) a vector valued function of \( t \). Each component is either one of the unknown functions or one of its derivatives. Thus, ODEs of 2nd, 3rd ... nth order can be expressed as a system of 1st order differential equations.

It should be noted that, in cases where \( f \) is only a function of \( t \) the solution is the following:

\[ \frac{dy}{dt} = f(t) \Rightarrow y(t) = \int f(t) \, dt + C \]

where \( C \) is an arbitrary constant and thus an analytical solution is obtained (to be more specific, an infinite number of solutions is obtained).

In cases where an initial point \( t_0 \) and an initial value \( y_0 \) is provided \( (y(t_0) = y_0) \), then the solution of the ODE is unique. This type of problem is known as an Initial Value Problem (IVP):

\[ y'(t) = f(t, y(t)) \]

\[ y(t_0) = y_0 \]

Matlab software contains many library functions for the solution of IVPs, such as ode23, ode45, ode113, ode15s, ode23s, ode23t and ode23tb. The most commonly used ones are ode23 and ode45. This family of solvers is based on multi-step methods such as Runge–Kutta schemes. More specifically, the ode23s solver uses the equations of 2nd and 3rd order Runge–Kutta method, thus developing a 3rd order algorithm. On the other hand, ode45 is similar to ode23s with the exception of the solution being based on 4th and 5th order Runge-Kutta method, thus creating a more complex algorithm of 5th order. In the present study, ode23s was used as a solver.

ode23s, starting from the initial value of the independent variable and the initial conditions, proceeds step-by-step to the interval of the variable while calculating the parameters in each step. If the solution is between the limits determined by the
error criteria then the step is considered to be successful and the solver proceeds to the next step. Otherwise, the attempt is considered to be abortive and once it reduces the step size it attempts again to solve the problem (variable-step method).

4.1.2 Solving optimization problems using fmincon function

fmincon is a nonlinear programming solver used in order to find the minimum of a constrained nonlinear multivariable function. It finds the minimum of a problem specified by:

\[ \min_x f(x) \text{ such that } \begin{cases} 
  c(x) \leq 0 \\
  c_{eq}(x) = 0 \\
  A \cdot x \leq b \\
  A_{eq} \cdot x = b_{eq} \\
  l_b \leq x \leq u_b 
\end{cases} \]

where:

- \( b \) and \( b_{eq} \) are vectors
- \( A \) and \( A_{eq} \) are matrices
- \( c(x) \) and \( c_{eq}(x) \) are functions that return vectors
- \( f(x) \) is a function that returns a scalar
- \( x, l_b \) and \( u_b \) can be passed as vectors or matrices

Moreover, \( f(x), c(x) \) and \( c_{eq}(x) \) can be nonlinear functions.

fmincon uses the following algorithms:

- interior-point
- sequential quadratic programming (sqp)
- active-set
- trust-region-reflective

The selection of the algorithm for the solution of the optimization problem is conducted by calling the optimset command. In the present study, medium-scale optimization is carried out, during which the subroutine fmincon uses the “sqp” method. The Hessian matrix\(^*\) of the Lagrange function\(^*\) in the point \( x \) is calculated via a Quasi-Newton approximation\(^*\) method.

The SQP implementation consists of three main stages:
• Updating the Hessian Matrix
• Quadratic Programming Solution*
• Line Search and Merit Function

*Interpretation of terms:

• Suppose \( f : \mathbb{R}^n \to \mathbb{R} \) is a function taking as input a vector \( x \in \mathbb{R}^n \) and outputting a scalar \( f(x) \in \mathbb{R} \); if all second partial derivatives of \( f \) exist and are continuous over the domain of the function, then the Hessian matrix \( H \) of \( f \) is a square \( n \times n \) matrix, usually defined and arranged as follows:

\[
H = \begin{bmatrix}
\frac{\partial^2 f}{\partial x_1^2} & \frac{\partial^2 f}{\partial x_1 \partial x_2} & \cdots & \frac{\partial^2 f}{\partial x_1 \partial x_n} \\
\frac{\partial^2 f}{\partial x_2 \partial x_1} & \frac{\partial^2 f}{\partial x_2^2} & \cdots & \frac{\partial^2 f}{\partial x_2 \partial x_n} \\
\vdots & \vdots & \ddots & \vdots \\
\frac{\partial^2 f}{\partial x_n \partial x_1} & \frac{\partial^2 f}{\partial x_n \partial x_2} & \cdots & \frac{\partial^2 f}{\partial x_n^2}
\end{bmatrix}
\]

• The Lagrange function is defined as follows:

\[
L(x, \lambda) = f(x) + \sum_{i=1}^{m} \lambda_i \cdot g_i(x)
\]

where \( \lambda_i \) are the Lagrange multipliers and \( g_i(x) \) the problem restrictions.

• The Quadratic Programming solution procedure involves two phases. The first phase involves the calculation of a feasible point (if one exists). The second phase involves the generation of an iterative sequence of feasible points that converge to the solution. It is related to the minimization or maximization of a second order function with linear restrictions.

• The Quasi-Newton approximation is a second order method for the minimization of multivariable functions. The goal is to find the direction \( s \) which minimizes the equation:

\[
\nabla f^T \cdot s = \sum_{i=1}^{n} \frac{\partial f}{\partial x_i} \cdot s_i
\]

provided that \( s^T \cdot Q_s = 1 \), where \( Q \) is the Hessian matrix. The Quasi-Newton methods aim at the minimization of the computational demands for the determination of the Hessian matrix.
4.1.3 Estimation of Kinetic Parameters and determination of error

The method of least squares is a standard approach in regression analysis to the approximate solution of overdetermined systems, i.e., sets of equations in which there are more equations than unknowns. "Least squares" means that the overall solution minimizes the sum of the squares of the errors made in the results of every single equation. The aim of this method is the selection of a parameter which minimizes the sum of the absolute errors. The subject which undergoes minimization is defined as follows:

\[ F = \sum_{i} \left[ \sum_{j} (y^{i}_{exp,j} - y^{i}_{calc,j})^2 \right] \]

where

\( n_i \): number of dependent variables
\( y^{i}_{exp,j} \): experimental values of the dependent variable i
\( y^{i}_{calc,j} \): estimated values of the dependent variable i

However, the Relative Least Squares (RLS) criterion, which minimizes the sum of the relative residuals, is the proper choice for cases in which the relative error has an approximately constant variance, a common characteristic for many analytical assays. The superiority of the relative residuals criterion is accentuated when the magnitude of the dependent variable varies widely [Saez et al., 1992]. The regulated sum is divided by the number of measurements for each dependent variable, thus the above equation is transformed as followed:

\[ F = \sum_{i} \frac{1}{n_i} \left[ \sum_{j} \left( \frac{y^{i}_{exp,j} - y^{i}_{calc,j}}{y^{i}_{exp,j}} \right)^2 \right] \]

In the present study, the minimization of least squares for the determination of the kinetic parameters was performed using the function fmincon, the optimization tool described above.

4.2 Model development

Kinetic models describe the relations between the concentration and the rate of consumption and production of substrate and metabolic products respectively. They predict the conversion and yield of substrate to valuable metabolic products, under certain conditions. The cognitive understanding of the biological system which is going to be subjected to modeling, in addition to the correct evaluation of kinetic
expressions and interactions between the variables can be subsequently used for the accurate prediction of the behavior of all the processes that take place in a biological system. This procedure will provide a modeling tool which can subsequently be applied for the optimal design of the process.

The basic principle of a kinetic model is the development of a functional relation between the substrate compounds and the metabolic products by using the appropriate reaction rates that are incorporated in the mathematical model. These rates should be correlated with the concentration of biomass, substrate compounds and intracellular metabolic products. In the present study, a mathematical model was used in order to predict the biomass growth of *Chlorella vulgaris*, in addition to the glucose consumption, the sulfur consumption (limiting substrate) and the concurrent intracellular lipid production of the microalga.

In order to quantitatively determine the bioconversion of substrate compounds to biomass and metabolic products with accuracy, the conditions should be such, so that they promote the homogeneity of the cultivation mixture (substrate, cells and extracellular metabolites) during the experimental period. This is accomplished in liquid cultures which are subjected to continuous stirring.

In order to develop a model describing the performance of oleaginous microorganism, it is essential to establish some assumptions in relation to the basic biochemical mechanisms that take place during the biosynthesis of intracellular lipids [Papanikolaou et al., 2011]. Oleaginous microorganisms are capable of biotransforming the provided carbon source (C) into fat-free biomass ($X_f$), which consists of the active part of the microbial biomass (proteins, nucleic acids and structural compounds), and into lipids (L). It is proven that the growth of $X_f$ can be observed even after the depletion of the limiting nutrient source, possibly coming from the accumulation of other storage compounds such as polysaccharides [Papanikolaou et al., 2011]. At this point, it should be noted that the limiting substrate for *C. vulgaris* in the experiment which was used for the development of the kinetic model was sulfur (S).

During the first stages of cultivation, where the carbon in addition to the sulfur source are abundant in the medium, the synthesis of $X_f$ is observed. However, when sulfur is depleted from the cultivation medium, the biomass growth ceases, and the death of cells is subsequently observed. In addition, the biomass growth rate is decreased when the medium approaches sulfur limited conditions, while the sulfur content was never completely depleted from the medium. Hence the term $S_{\text{min}}$ should be introduced in the equation describing the growth of $X_f$, which is the following:
\[
\frac{dX_f}{dt} = \mu_{\text{max}} \cdot \left( \frac{C}{K_C + C} \right) \cdot \left( \frac{S - S_{\text{min}}}{K_S + S - S_{\text{min}}} \right) \cdot X_f - k_d \cdot X_f
\]

where \( X_f \) is the fat-free biomass (g L\(^{-1}\)), \( \mu_{\text{max}} \) (days\(^{-1}\)) is the maximum specific growth rate of fat-free biomass, \( C \) (g L\(^{-1}\)) is the carbon source, \( K_c \) and \( K_s \) are the saturation constants for C and S respectively, \( S \) (g L\(^{-1}\)) is the sulfur source, \( S_{\text{min}} \) (g L\(^{-1}\)) is the lowest S concentration presented during the experiments and \( k_d \) is the specific kinetic rate of death.

The lipid accumulation is highly dependent on the carbon source availability and is triggered under nutrient limiting conditions (in this case sulfur). However, intracellular lipids are also accumulated in a significantly slower rate during the stage of biomass growth. A typical, widely used and unstructured kinetic model for product formation is the Leudeking-Piret model, contributed to both growth and non-growth-associated phenomena for product formation and is described by the following equation [Leudeking et al., 1959]:

\[
\frac{dL}{dt} = \alpha \cdot \frac{dX_f}{dt} + \beta \cdot X_f
\]

where \( L \) are the intracellular lipids, \( X_f \) is the lipid-free biomass formation and \( \alpha, \beta \) are the kinetic constants of the Luedeking–Piret model. This two-parameter expression has proven to be extremely useful and versatile in fitting product formation data from different biological processes. This is an expected kinetic form when the product is the result of energy-yielding metabolism. According to this model, the product formation rate depends linearly on the growth rate and the cell concentration, where \( \alpha \) is a growth-associated lipid formation coefficient and \( \beta \) a non-growth-associated correlation coefficient. The Luedeking–Piret kinetic parameters, \( \alpha \) and \( \beta \), depend on and vary with the fermentation dynamics.

The carbon source is consumed for two purposes; biomass growth and lipid synthesis. The equation describing the consumption of the carbon source (glucose) is presented below:

\[
\frac{dC}{dt} = -\frac{1}{Y_{X/C}} \cdot \frac{dX_f}{dt} - q \cdot X_f \cdot \left( \frac{C}{K_C + C} \right)
\]

where \( C \) (g L\(^{-1}\)) is the carbon source, \( X_f \) (g L\(^{-1}\)) is the lipid free biomass, \( Y_{X/C} \) (g g\(^{-1}\)) is the conversion yield of lipid-free biomass formed per carbon substrate consumed, \( q \)
(days$^{-1}$) is the maximum specific rate of storage lipids production and $K_c$ (g L$^{-1}$) is the saturation constant for C.

Finally, the sulfur consumption is described by the following equation:

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}} \cdot \mu_{\text{max}} \cdot \left( \frac{C}{K_c + C} \right) \cdot \left( \frac{S - S_{\text{min}}}{K_s + S - S_{\text{min}}} \right) \cdot X_f$$

where $S$ (g L$^{-1}$) is the sulfur source, $Y_{X/S}$ (g g$^{-1}$) is the conversion yield of lipid-free biomass formed per sulfur consumed, $\mu_{\text{max}}$ (days$^{-1}$) is the maximum specific growth rate of fat-free biomass, $K_c$ and $K_s$ (g L$^{-1}$) are the saturation constants for C and S respectively, C (g L$^{-1}$) is the carbon source, S (g L$^{-1}$) is the sulfur source, $S_{\text{min}}$ (g L$^{-1}$) is the lowest S concentration presented during the experiments and $X_f$ (g L$^{-1}$) is the lipid free biomass.

The equations used for the determination of the parameters are summarized below:

**Biomass growth**

$$\frac{dX_f}{dt} = \mu_{\text{max}} \cdot \left( \frac{C}{K_c + C} \right) \cdot \left( \frac{S - S_{\text{min}}}{K_s + S - S_{\text{min}}} \right) \cdot X_f - k_a \cdot X_f \quad (1)$$

**Lipid Synthesis**

$$\frac{dL}{dt} = \alpha \cdot \frac{dX_f}{dt} + \beta \cdot X_f \quad (2)$$

**Substrate Consumption**

$$\frac{dC}{dt} = -\frac{1}{Y_{X/C}} \cdot \frac{dX_f}{dt} - q \cdot X_f \cdot \left( \frac{C}{K_c + C} \right) \quad (3)$$

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}} \cdot \mu_{\text{max}} \cdot \left( \frac{C}{K_c + C} \right) \cdot \left( \frac{S - S_{\text{min}}}{K_s + S - S_{\text{min}}} \right) \cdot X_f \quad (4)$$
4.3 Results and discussion

The mathematical model presented above was used in order to determine the kinetic parameters related to the biomass growth, lipid accumulation and substrate consumption of the microalga *C. vulgaris*, under heterotrophic conditions. More specifically, the cultivation medium consisted of BG-11 medium, supplemented with approximately 10 g L\(^{-1}\) glucose at pH 7.5, thus resulting in sulfur starvation (§3.1). The kinetic parameters were estimated by fitting the mathematical model to the experimental data, according to §4.1.3 and are presented in the following table (Table 4.1):

**Table 4.1:** Model estimated parameters based on experimental data of *C. vulgaris* cultivation in BG-11 broth supplemented with glucose at pH 7.5, under heterotrophic conditions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Estimated Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>maximum specific growth rate of fat-free biomass</td>
<td>(\mu_{\text{max}})</td>
<td>0.8998</td>
<td>days(^{-1})</td>
</tr>
<tr>
<td>saturation constant for C</td>
<td>(K_C)</td>
<td>0.8845</td>
<td>g L(^{-1})</td>
</tr>
<tr>
<td>saturation constant for S</td>
<td>(K_S)</td>
<td>0.0029</td>
<td>g L(^{-1})</td>
</tr>
<tr>
<td>conversion yield of lipid-free biomass formed per glucose mass consumed</td>
<td>(Y_{X/C})</td>
<td>0.5218</td>
<td>g g(^{-1})</td>
</tr>
<tr>
<td>conversion yield of lipid-free biomass formed per sulfur mass consumed</td>
<td>(Y_{X/S})</td>
<td>423.74</td>
<td>g g(^{-1})</td>
</tr>
<tr>
<td>growth-associated lipid formation coefficient</td>
<td>(\alpha)</td>
<td>0.4861</td>
<td>-</td>
</tr>
<tr>
<td>non-growth-associated correlation coefficient</td>
<td>(\beta)</td>
<td>0.0310</td>
<td>days(^{-1})</td>
</tr>
<tr>
<td>maximum specific rate of storage lipids production</td>
<td>(q)</td>
<td>0.1992</td>
<td>g g(^{-1}) days(^{-1})</td>
</tr>
<tr>
<td>specific kinetic rate of death</td>
<td>(k_d)</td>
<td>0.0339</td>
<td>days(^{-1})</td>
</tr>
</tbody>
</table>
The following figure (Figure 4.1) illustrates the model prediction in addition to the actual data of each experiment:
As shown in the figure above, the data fitting was satisfying in all the variables subjected to modeling of both experiments (experiments were conducted twice). The only case presenting a noticeable deviation between the model prediction and the actual data was that of sulfur consumption. More specifically, the model predicts that the depletion of sulfur takes place in a slower rate, and starvation occurs with a delay of 3 to 4 days compared to the actual data. This fact can be attributed to a possible delay in the utilization of the sequestered from the microalga sulfur, and its bioconversion to active biomass. The model, in its present form, was unable to predict such a metabolic activity. Moreover, a slight deviation from the actual data was observed in the first 4 days of lipid formation. To be more specific, the model predicts a higher rate of increase in lipid synthesis during the first 4 days of each experiment. The best fitting was observed in the case of glucose consumption. Finally, the total error is calculated as the sum of the individual errors of each parameter and was estimated at 0.8686.

The fact that the mathematical model describes the behavior of an axenic culture, containing one microalgal strain, had strong positive effects on data fitting. Thus, the use of an axenic culture helped in avoiding variations of microbial population and resulted in repeatable and stable culture performance overtime in parallel experiments. Deviations between the model prediction and the actual data can be attributed to the fact that the mathematical model was constructed from a macroscopic point of view. More specifically, in the case of lipid synthesis the logistic model of Luedeking–Piret was used, which does not take into account the metabolic activities that take place inside the cell. The formation of intermediate metabolic products was ignored and the lipid formation rate could be adequately described using a growth- and a non-growth-associated term.
The first part of the present study was focused on the investigation of the effect of pH on the growth and lipid accumulation of \textit{C. vulgaris}, under heterotrophic conditions. The pH values tested were 3, 4, 5, 6, 6.5, 7, 7.5, 8, 9.5 and 11. The substrate was BG-11 medium supplemented with glucose. The limiting nutrient in BG-11 for this particular strain was found to be sulfur. \textit{C. vulgaris} was found to grow heterotrophically at a wide variety of pH values (pH=5-8) with a final biomass concentration of 2.59 ± 0.16 g L$^{-1}$. However, in extreme pH values (pH 3, 4 and 11) it was not able to survive and at pH 9.5, although the microorganism survived, it was not able to grow and aggregation of cells was observed. The best performance of the strain in relation to growth was observed in pH 7.5 and 8, with a maximum specific growth rate of 0.5409 and 0.5626 days$^{-1}$, and maximum biomass productivity 0.2976 and 0.2825 mg L$^{-1}$ day$^{-1}$, respectively.

The fatty acid composition of \textit{C. vulgaris} was found to be unrelated to the pH, as the lipid content did not present significant variations in the pH values tested. The fatty acid profile was mainly composed of monounsaturated fatty acids (MUFAs) and the dominant one was oleic acid (C18:1). More specifically, the main fatty acids detected in \textit{C. vulgaris}, arranged in declining order, were oleic (C18:1), linoleic (C18:2), palmitic (C16:0), palmitoleic (C16:1) and stearic acid (C18:0). On the aspect of lipid accumulation, the highest lipid productivity was observed at pH 7.5 during the 18$^{th}$ day with a lipid content of 53.43%, 48.17% and 32.70% g g$_{DW}^{-1}$ (which correspond to 1422.27, 1283.07 and 870.81 mg L$^{-1}$) in total lipids, unsaturated lipids and oleic acid respectively.

Taking into account both biomass growth and lipid accumulation, the optimal pH for this strain was found to be 7.5.

The effect of nutrient starvation was also studied. More specifically, the lipid accumulation under sulfur, phosphorus and nitrogen stress was investigated. \textit{C. vulgaris} uptakes sulfur in high quantities when compared to other microalgal strains (0.2 % per mass) while the need for nitrogen is significantly lower (3.4 % per mass). Thus, high density cultures can be achieved in low nitrogen concentrations, provided that there is enough sulfur, phosphorus and carbon to support \textit{C. vulgaris} growth and that nitrogen is present in a biodegradable form for the microalga. \textit{C. vulgaris} exhibited substrate inhibition in the experiment of N starvation, where the glucose
concentration was 40 g L\(^{-1}\). Comparing its behavior in this experiment with the other ones, the substrate inhibition is presented when the initial glucose concentration exceeds 27 g L\(^{-1}\).

Different nutrient starvation did not have effect on composition of FAMEs. The lipid composition is a characteristic feature for this microalgal strain, since it is not affected by pH or by starvation from different nutrients. On the other hand, nutrient starvation had a strong effect on the intracellular lipid content. The lowest lipid accumulation was observed during phosphorus starvation (17.42, 13.66 and 10.04% g DW\(^{-1}\) in total lipids, unsaturated lipids and oleic acid respectively). The highest lipid content was obtained under sulfur stress (53.43, 48.71 and 32.70% respectively), indicating that after sulfur depletion, carbon is mainly accumulated in the form of intracellular lipids. During N stress the respective values were 25.80, 24.89 and 14.39%. Consequently, the nutrient starvation which presented the best effect on lipid accumulation of *C. vulgaris* was that of sulfur. In all cases, the main fatty acids detected in *C. vulgaris*, arranged in declining order, were oleic (C18:1), linoleic (C18:2), palmitic (C16:0), palmitoleic (C16:1) and stearic acid (C18:0). Other fatty acids were also contained in traces. Additionally, the oleic acid content in total lipids presented a pattern in all experiments. More specifically, it had an increasing course during the growth phase of the microalga, until it reached the value of 60% of total lipids in stationary phase and remained constant for the rest of the experimental period. This fact indicates that oleic acid is not accumulated in the same rate during the cell cycle.

Overall, *C. vulgaris* appears to be an eligible strain for biodiesel production due to its ability to obtain a high content of intracellular lipids. The fatty acids accumulated in this particular strain are suitable for biodiesel production.

In the next series of experiments, various substances were tested as potential carbon sources for *C. vulgaris*. More specifically, these substances were volatile fatty acids (VFAs) and phenols, which were supplemented in BG-11 broth. All experiments were performed in the optimal pH for the microalga (7.5).

The VFAs tested were acetic, propionic, butyric, and isobutyric acid. Although *C. vulgaris* was able to assimilate acetic acid, the specific microalga failed to grow with propionic, butyric and isobutyric acid as sole carbon sources. However, when the combination of all the VFAs tested in addition to glucose was supplied as carbon source, glucose and acetic acid were metabolized simultaneously. The microalga was again unable to assimilate propionic, butyric, and isobutyric acid. These results indicate that, even though *C. vulgaris* possesses the necessary metabolic characteristics to assimilate organic acids, the increase of carbon atoms in the aliphatic chain make the acids mentioned above non-degradable for *C. vulgaris*, under heterotrophic conditions.
In terms of maximum specific growth rate, the value obtained when acetic acid was the only carbon source was 20.63% less than the one obtained when glucose was the organic compound supplied (0.4293 and 0.5409 days\(^{-1}\) respectively). In the case where the combination of VFAs and glucose was supplied the \(\mu_{\text{max}}\) was not affected (0.5338 days\(^{-1}\)). Thus, the presence of glucose has positive effects on the growth rate of \(C.\ vulgaris\) as it contains more energy per mol than all the other compounds tested.

The study of phenols as potential carbon sources was subsequently carried out. The phenols tested were vanillic, ferulic, syringic, gallic, p-hydroxybenzoic acid and catechol. Before the experiments with phenol as the sole carbon source were carried out, a process of adaptation took place, which involved the addition of the respective phenol in a medium composed of BG-11 broth and glucose. This process was performed in five stages where the amount of glucose was gradually decreased (2, 2, 1.5, 1 and 1 g L\(^{-1}\)), while the amount of phenol was increased (50, 100, 200, 300 and 350 mg L\(^{-1}\) respectively).

While the presence of the plethora of phenols tested allowed the growth of \(C.\ vulgaris\) during the adaptation process, catechol inhibited the growth of the microalga from the second stage of acclimation (100 mg L\(^{-1}\) phenol). This fact indicates the highly toxic effect of catechol. The only phenol that was consumed during this process was gallic acid.

The experiments of phenol as the only carbon source were subsequently performed, with a phenol concentration of 500 mg L\(^{-1}\). The acclimated cultures were used as inoculum. \(C.\ vulgaris\) was unable to grow with vanillic, ferulic and p-hydroxybenzoic acid as sole carbon sources in a cultivation period of 15 days. However, gallic and syringic acid were degraded by \(C.\ vulgaris\) in the respective experiments. During the assimilation of gallic acid the biomass generation was 0.0341 g L\(^{-1}\), resulting from the degradation of 0.3166 g L\(^{-1}\) phenol. As expected, the assimilation of phenols resulted in low maximum specific rates, which in the case of gallic acid was 0.0631 days\(^{-1}\). In the case of syringic acid, a similar trend was observed. However, the performance of \(C.\ vulgaris\) with this phenol as a substrate was slightly better than the one observed using gallic acid. The biomass generation was 0.1066 g L\(^{-1}\) as a result of the degradation of 0.263 g L\(^{-1}\) phenol, while the specific growth rate was 0.1028 days\(^{-1}\). All these results verify the inhibiting effect of phenols on microalgal growth. In both cases, the microorganism presented a prolonged lag phase of 6 days. The value obtained for \(\mu_{\text{max}}\) was thus 11.66% of the one for glucose when gallic acid was provided, while for syringic acid it was 19.00%. The aromatic ring of phenols constitutes a complex structure which demands big amounts of energy spent by microalgae in order to be degraded. This energy demanding process results in low \(\mu_{\text{max}}\) when a phenol is supplied as the only carbon source.
The next series of experiments in the present study concerned the use of Olive Mill Wastewater (OMW) as a substrate for *C. vulgaris* growth. OMW is characterized by low pH, high organic load and high concentration of phenols. After a series of failed attempts of the biotreatment of this particular wastewater in various dilutions with 3D water using *C. vulgaris*, the effect of the addition of various nutrients was investigated. More specifically, the supplemented compounds were MgSO₄·7H₂O as sulfur source, KNO₃ as nitrogen source, and phosphate buffer for the regulation of pH to the optimal for the microalga value (7.5). In all these scenarios the microalga failed to grow during a cultivation period of 15 days.

In an effort to acclimate *C. vulgaris* in OMW, a batch culture of the microalga was prepared, containing 10% OMW, diluted with BG-11 culture medium, supplemented with 2 g L⁻¹ glucose and 350 mg L⁻¹ MgSO₄·7H₂O. After a lag phase of 12-14 days, *C. vulgaris* started growing, resulting in a high density culture, which was subsequently used as an inoculum for the next series of experiments. The final experiment carried out was with 10% OMW supplemented with BG-11 medium at pH 7.5. During the first 9 days no growth was observed. However, during the 12th day of cultivation, infection of the medium was observed. Three days after, this microorganism, which appeared to be a fungus, was the dominant one in the culture. Nevertheless, *C. vulgaris* cell number also increased, indicating that the minerals present in BG-11 broth had positive effects on the growth of the microalga in OMW. The lag phase (12 days) of the microalga was the result of the acclimation process to a medium containing phenols in a concentration of approximately 0.5 g L⁻¹.

To sum up, the growth of *C. vulgaris* in OMW supplemented with glucose and BG-11 medium showed that the microalga can tolerate the phenols present in such concentration (0.5 g L⁻¹). Additionally, the nutrients contained in BG-11 were able to support *C. vulgaris* growth in a substrate containing OMW, thus, it's the lack of nutrient bioavailability that inhibits the growth of microalgae in OMW. Even though nitrogen—an important nutrient for microalgae—is contained in low levels in OMW, the inhibiting factor for *C. vulgaris* is not the lack of nitrogen alone. Moreover, the low pH of OMW was not the reason for which the microalga could not function. Finally, the addition of D-glucose had positive effects on the growth of *C. vulgaris*. Taking all these factors into account, the conclusion drawn is that the biotreatment of OMW with *C. vulgaris* under heterotrophic conditions is not a viable option.

The last part of this particular study concerns the energy valorization of *C. vulgaris* biomass. The first conclusion drawn is related to the optimal time of the harvest. When the microalga has achieved stationary phase, it possesses the most desirable characteristics for biofuel production. At the same time, the intracellular lipids (and the most suitable for biodiesel production, oleic acid) are accumulated at the highest rate and the concentration has reached a value that is comparable to the maximum
one. More specifically, the lipid content increases approximately 5% during the period between early stationary phase and the end of each experiment. If the microalga remains further in the medium, without external nutrient supply, the lipid content is not going to be substantially increased and the assimilation of storage lipids could be triggered if there is not enough carbon in the medium. Additionally, in terms of nutrient uptake viewed as a wastewater post-treatment process, the highest rate is observed during the exponential phase of growth. Hence, in order to harness the desired characteristics of *C. vulgaris* at the greatest extent, both from biofuel production and wastewater treatment efficiency point of view, the harvest should be performed at the point of early stationary phase. In continuous systems, the microalgal specific growth rate is related to the hydraulic retention time (HRT). HRT is a parameter strongly related to the energy requirements and operational costs of a unit. Thus, HRT should be adjusted so as to achieve the dual goal of nutrient uptake and biofuel production, without increasing the operational costs.

Biodiesel production from microalgal biomass, apart from harvesting and drying, also includes the processes of extraction and transesterification. These processes augment the cost of biofuel production, thus making it an economically unviable solution. Hence, the valorization of *C. vulgaris* biomass through direct combustion and anaerobic digestion were investigated as alternative solutions. The specific calorific value and Biochemical Methane Potential were measured in samples harvested in the stationary phase of the heterotrophic cultivation of *C. vulgaris*, in pH 7.5 under sulfur starvation.

The specific calorific value of *C. vulgaris* dry biomass was found to be 24,525 ± 182 kJ kg⁻¹ or 5,861 ± 44 kcal kg⁻¹. This value is 57% of the calorific value of petroleum and 94.54% of anthracite. Additionally it is 63.5% higher than the calorific value of lignite.

In order to compare the theoretical values obtained from the anaerobic digestion of *C. vulgaris* biomass, the elemental composition of the microalga was estimated and is described by the formula C₈₈₅.₃₂₂H₄₀₈.₈₁₄O₅₀₅.₄₈₃N₄₇.₅₃₉S₁.₂₁₄P. The estimation of the methane potential of *C. vulgaris* biomass was also carried out in mesophilic and thermophilic conditions, with and without pretreatment (ultrasonication). When comparing all the results obtained, the best performance was observed in the assay carried out in mesophilic conditions, without pretreatment of the microalgal biomass, achieving a yield of 389.07 ml gVS⁻¹ added. Moreover, thermophilic temperatures were found to result in lower methane production. The pretreatment through ultrasonication was found to have a negative influence in the assays carried out in mesophilic conditions, while under thermophilic conditions no significant effect was observed.

Finally, when comparing the energy valorization methods, the highest energy productivity (20.331 kJ L⁻¹ reactor⁻¹ day⁻¹) is obtained through the direct combustion of *C.
C. vulgaris biomass. Even though the Specific Heating Values of biodiesel and methane are higher, the biofuel productivities are substantially lower, resulting in lower energy productivities. However, in order to draw a conclusion, apart from the profit gained by the exploitation of the produced fuel, the capital, operational and installation costs must be taken into account. Biodiesel production is the most costly process, as apart from the costs of harvest and drying, the use of organic solvents and the purification process increase the expenses. Anaerobic digestion does not include the stage of drying, but the installation and operation of a reactor is necessary. However, there is the additional advantage of potential exploitation of the digested residue. The incomes obtained from the commercialization of the byproducts of each energy valorization process must be taken into account. The effluent of this anaerobic digestion is nutrient rich and can be further utilized as a fertilizer, or as a substrate for microalgal production. On the other hand, direct combustion includes the processes of harvesting and drying, but also offers the advantage the produced biofuel, which can be handily utilized in any solid fuel burner. Taking into account exclusively the energetic content of biofuels tested in this study, the suggested method of energy valorization of C. vulgaris biomass is direct combustion.

A simple, macroscopic, non-structural mathematical model was developed for the description of the behavior of C. vulgaris. The use of an axenic culture helped in avoiding variations of microbial population and resulted in repeatable and stable culture performance overtime in parallel experiments. Deviations between the model prediction and the actual data can be attributed to the fact that the mathematical model was constructed from a macroscopic point of view. More specifically, in the case of lipid synthesis the logistic model of Luedeking–Piret was used, which does not take into account the metabolic activities that take place inside the cell. The formation of intermediate metabolic products was ignored and the lipid formation rate could be adequately described using a growth- and a non-growth-associated term. This model could be extended in order to accurately describe the metabolic processes that actually take place. The model resulted in the satisfying description of biomass growth, lipid accumulation and substrate consumption of the microalga C. vulgaris, at pH 7.5 under heterotrophic conditions. The kinetic parameters were estimated by fitting the model to actual data. The only case presenting a noticeable deviation between the model prediction and the actual data was that of sulfur consumption. This deviation can be attributed to a possible delay in the utilization of the sequestered from the microalga sulfur, and its bioconversion to active biomass. The model, in its present form, was unable to predict such a metabolic activity. The best fitting was observed in the case of glucose consumption and the total error was estimated at 0.8686.


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