UNIVERSIDAD DE ANTIOQUIA

PRODUCTION OF DOCOSAHEXAENOIC ACID FROM *Aurantiochytrium limacinum* SR21 USING NON-CONVENTIONAL CARBON SOURCES

Autor
Ruben Darío Múnera Soto

Universidad de Antioquia
Facultad de Ingeniería, Departamento de Química
Medellín, Colombia
2021

Production of Docosahexaenoic Acid from *Aurantiochytrium limacinum* SR21 using Non-Conventional Carbon Sources

Ruben Darío Múnera Soto

Trabajo de investigación presentado como requisito parcial para optar al título de:
Magister en Ingeniería Química

Asesores (a):
Mariana Peñuela Vásquez, PhD

Línea de Investigación:
Tecnología de las Fermentaciones
Grupo de Investigación:
Grupo de Investigación Bioprocesos

Universidad de Antioquia
Facultad de Ingeniería, Departamento de Química
Medellín, Colombia
2021.

Acceptance Note

____

____

____

____

____

President of the Jury

____

Jury

____

Jury

Medellín, 2021

Acknowledgements

First and foremost I want to state that all the work contained in this document is not the result of my own personal work, but rather the collection of many interactions with so many wonderful people who, in one way or another, helped me to pull through the so many stages, both academical and personal, that this endeavor represented for me as an engineer and as a person. I will do my best to name every person, but I am sure that I will leave amazing collaborators out despite my hardest effort to remember you all.

I want to thank Universidad de Antioquia that granted me the *Estudiante Instructor* scholarship, which allowed me to carry on with my studies while I was able to share with students and faculty members. Also, I thank COLCIENCIAS for financing this project.

I also want to thank the wonderful team of Bioprocesos research group, which can make you feel at home during the long hours that are required to pull through with this endeavor. Particularly, I thank Martin Delgado who sparked this process and opened the doors for coming back to this group after more than 10 years of absence. Individually, every single person in the group made this a more enjoyable experience: Catalina Lugo, who is a wonderful person and a great biologist; Carolina Montoya, who as a fellow chemical engineer became a friend, Jeronimo, Naydu, Mauricio, Daniela, Luisa, Vanesa, Daniel, each of whom marked every single step either by helping me or allowing me to demonstrate what little I know. Last but not least, I send my gratitude to Mariana Peñuela, who is not only the head of the group but a leader capable of guiding through the most difficult moments that can arise when walking across this path.

I want to specially express my gratitude to Dalma Marsiglia, who is one of the most admirable people I have had the honor to meet, she showed me what a professional acts like and more importantly what a person can do when tested by life alongside Camila Marín, who despite the age difference shares so many of my tastes and life philosophy. In this regards I also thank Jeferyd Yepes, who became the person to go to when everything seemed to be going awry and whose energy helped me pushed through so many of the days in which I questioned why I was working on this project. Also, my deepest thanks to Natalia Gómez for whom I feel that words fail to describe what she has done for me in so many aspects of my life.

I want to specially thank Yamid Yepes a brilliant chemical engineering student, whom I met in a classroom, for his invaluable help during the experimental phase of this work; his utter disposition and eagerness for learning allowed me to successfully finish the experimental phase. I wish him the best in his career.

However, this project was not limited to *Bioprocesos* and there were so many wonderful people that provided me with what I needed for navigating the different stages of this experience. First, my study group; Sara Dominguez, Andrés Gutierrez, Luis Carlos Ojeda, and Iván Aguas; with whom I spent most of my first year attending classes and doing our best to meet the deadlines after a challenging and hellish week. I also want to thank all the professors whose devotion and passion comes through every single class, specially Lina Gonzalez, Rigoberto Ríos, Silvia Ochoa and Aida Villa whose classes I had the honor to attend and enjoy.

Bioenergía professors Biviana and Luis Rios who opened the doors of the facilities to me, which allowed me to carry on with the experiments and a place for writing this work. Paola and Daniel provided me with advice and encouragement during the last stages of the experimental stage and the writing of this work. James Marín from PQI, who assessed us in the gas chromatography analysis for the lipids, and the *Biomateriales* research group team who made possible the freeze-dry process.

Professors Jesús Pérez from the department of civil engineering and Ronald Martinod from the mechanical engineering department at *Universidad EAFIT*, I sincerely and greatly thank them for their support acting as a connection between both universities for finding much needed solutions at critical times.

Also, many thanks to all of those who supported me albeit during a small conversation over coffee or by constantly asking me how I was doing and cheering me on. Among the so many of them, I want to mention Sandra Alfonso and Adriana Posada, with whom I spent countless hours going over the details of this work and who ended up becoming my friends.

I lack the words to express my gratitude to Doctor Luis Javier Gallego for assessing me, this work would not have come to fruition without his immense advice, assessment, patience, and diligence. I will always hold him very dear.

I want to thank my family, whom have nurtured me and walked with me during the many stages of life, ensuring my character and giving me the strength I need to set and fulfill this and many other objectives in my life. For being the one certain thing that I have, I love each one of them very much.

Finally, I want to thank the evaluators of this work, for their disposition and valuable insight. Who have taken the time to share their ideas and thoughts on the results here presented. Thank you.

Abstract

In recent years, the supply of polyunsaturated fatty acids (PUFAs) has faced two different problems. Firstly, the production from traditional sources, namely capture fish, has remained stagnant while demand continues to increase. Secondly, capture fish has been targeted as a source for heavy metals, hazardous for the human health, due to bioaccumulation of these components as a result of ocean pollution. Consequently, new sources have been studied. In this way, *Aurantiochytrium limacinum* SR21, a marine chromist, presents itself as an alternative due to its capability to accumulate up to 60% of its weight in lipids. These lipids are rich in Docosahexaenoic Acid (DHA), an essential PUFA. However, producing DHA from *A. limacinum* SR21 is expensive as it uses glucose as its carbon source. For lowering the costs in the production of DHA from *A. limacinum* SR21, this work evaluates different non-conventional carbon sources that have the potential to substitute glucose as carbon source. These materials are thought to lower the medium cost, which has shown to be one of the most representative costs in biorefineries. To do so, this work evaluated the potential of amylaceous and lignocellulosic materials. Potato and corn washes were evaluated as waste from the chips and *arepa* industries. Coffee mucilage, barley spent grain, and empty fruit bunch were used from the coffee, beer, and palm oil industries. All materials were subjected to different pretreatments. Specifically, the materials were pretreated with alkaline pretreatment (2% NaOH) and Liquid Hot Water (LHW) at 121°C and 1.5 atm. The pretreatments were performed aiming to obtain the highest yield of glucose when performing the enzymatic hydrolysis for assessing their usefulness as a replacement for glucose. Finally, the most suitable conditions were tested in a kinetic study to stablish which materials have the best potential to replace glucose as carbon source in the culture of *Aurantiochytrium limacinum* SR21 to produce DHA. From this study, it was found that all lignocellulosic materials had the potential for producing 50 g/L and above of glucose after the enzymatic hydrolysis. It was also possible to stablish that both barley spent grain and empty fruit bunch mediums were able to produce over 1 g/L of DHA in the culture. The economical estimation showed that the culture medium from empty fruit bunch can be an economical alternative to glucose when producing DHA from *A. limacinum* SR21.

Table of Content

Acknowledgements iii
Abstract v
Table of Content vi
List of Tables viii
Table of Figures ix
Chapter 1 1
Problem Statement 1
Introduction 3
Objectives 6
  General objective 6
  Specific objectives 6
Chapter 2 7
Theoretical Framework and State of the Art 7
  Fatty Acids 7
  Microbial LC-PUFAs 9
    Photosynthetic PUFA producing microorganisms 10
    Heterotrophic PUFA producing microorganisms 10
  Aurantiochytrium limacinum SR21 11
    Carbon to nitrogen ratio 11
    Sea salt concentration 12
  Polyunsaturated fatty acids synthesis 13
    Polyketide synthase pathway 13
    Fatty acid synthesis pathway 14
  Lignocellulosic materials 15
  Pretreatment of lignocellulosic materials 16
    Physical pretreatments 17
    Physical-chemical pretreatment 18
    Biological pretreatments 18
    Chemical pretreatments 19
  Hydrolysis of cellulose 21
    Enzymatic hydrolysis 21
  Economic Estimation 23
Chapter 3 25
Methodology 25
  Alternative Carbon Sources 25
    Characterization 25
  Carbone source conditioning 26
    Amylaceous materials 26
    Lignocellulosic materials 27
  Microorganism and media preparation 28

Microorganism maintenance ... 28
Media preparation ... 28
Culture of Aurantiochytrium limacinum SR21 ... 29
Analytical Methods ... 29
Glucose and nitrogen determination ... 29
Hydroxymethylfurfural determination ... 30
Dried cell weight (DCW) ... 30
Lipid extraction ... 30
Lipid profile determination ... 30
Economical estimation ... 31
Statistical analysis ... 32
Chapter 4 ... 33
Results ... 33
Alternative Carbon Sources Assessment ... 33
Characterization ... 33
Amylaceous material characterization ... 33
Lignocellulosic material characterization ... 34
Pretreatment of the non-Conventional Carbon Sources ... 35
Enzymatic hydrolysis of the pretreated materials ... 35
Culture of non-Conventional Carbon Sources with Aurantiochytrium limacinum SR21 ... 38
Amylaceous culture ... 38
Lignocellulosic cultures ... 38
A. limacinum SR21 growth under BSG hydrolysate culture ... 39
A. limacinum SR21 growth under CM hydrolysate culture ... 41
A. limacinum SR21 growth under FEB hydrolysate culture ... 42
Comparative Kinetic Study ... 43
BSG Kinetic study ... 43
CM Kinetic study ... 46
EFB Kinetic study ... 47
Lipid profile ... 48
Economical estimation ... 52
Chapter 5 ... 55
Conclusions ... 55
References ... 58
Endorsements ... 65

List of Tables

Table 1, Glucose from acid hydrolysis of amylaceous materials, potato and corn kernel washes 33
Table 2. Composition and proximate composition assays for crude BSG, CM, and EFB 34
Table 3. Glucose concentration (g/L) after performing hydrolysis on pretreated materials......... 35
Table 4. Composition and proximate composition assays for pretreated BSG, CM, and EFB 37
Table 5. Conditions selected for kinetic study and conditions after culture 43
Table 6. Kinetic parameters for the kinetic study of Aurantiochytrium limacinum SR21 using 4 different carbon sources: Glucose, hydrolyzed BSG, hydrolyzed CM, and hydrolyzed EFB 45
Table 7. Total fatty acid percentage in biomass and total fatty acid yield.................................... 49
Table 8. Fatty acid composition of Aurantiochytrium limacinum SR21 when cultured under different n-CCS hydrolysates and the basal glucose medium 49
Table 9. Total DHA percentage in TFA and DHA yield 52
Table 10. Estimation of cost of production of 1L of culture medium 53

Table of Figures

Figure 1. Chemical structure of fatty acids. a) Saturated: Palmitic acid C16:0, b) Monosaturated: Oleic acid C18:1 n-9, and c) Polyunsaturated: α-Linolenic acid C18:3 n-3 7
Figure 2. Chemical structure of the Omega 3 family. a) ALA, b) EPA, and c) DHA 8
Figure 3. Thraustochytrid isolate. A and C in bright field; B and D in epifluorescence photomicrograph showing fluorescent lipid bodies (Jain et al. 2007) 10
Figure 4. Polyketide synthase pathway for the biosynthesis of DHA (Béligon et al. 2016) 14
Figure 5. Pathway of polyunsaturated fatty acids biosynthesis (Béligon et al. 2016) 15
Figure 6. Layout of the pretreatment of lignocellulosic materials (Balat 2011) 17
Figure 7. Action of the cellulase enzymes on cellulose (Balat 2011) 23
Figure 8. Simplified scheme of single cell organism biorefinery ('t Lam et al. 2017) 24
Figure 9. Characterization process of lignocellulosic and amylaceous material 26
Figure 10. Culture stage of Aurantiochytrium limacinum SR21 in n-CCS and glucose media ... 31
Figure 11. Economical estimation of the medium using n-CCS and glucose for the culture of A. limacinum SR21 towards DHA production 32
Figure 12. Culture using A. limacinum SR21 with BSG hydrolysates using buffer solution and distilled water against basal glucose medium. Culture conditions: 23°C, 160 rpm, for 7 days 40
Figure 13. Culture using A. limacinum SR21 with CM hydrolysates using citrate solution against basal glucose medium. Culture conditions: 23°C, 160 rpm, for 7 days 41
Figure 14. Culture using A. limacinum SR21 with EFB hydrolysates using buffer solution and distilled water against basal glucose medium. Culture conditions: 23°C, 160 rpm, for 7 days 42
Figure 15. Kinetic study comparison between BSG medium and glucose medium. Culture conditions: 23°C, 160 rpm, for 216 hours 44
Figure 16. Kinetic study comparison between CM medium and glucose medium. Culture conditions: 23°C, 160 rpm, for 216 hours 46
Figure 17. Kinetic study comparison between CM medium and glucose medium. Culture conditions: 23°C, 160 rpm, for 216 hours 47
Figure 18. Sensitivity analysis for the medium cost with variations of the enzyme unit cost 53
Figure 19. Sensitivity analysis for the cost of 500 mg of DHA with variations of the enzyme unit cost 54

Chapter 1

Problem Statement

Polyunsaturated fatty acids (PUFAs); namely Docosahexaenoic acid (DHA, C22:6, n-3), Eicosapentaenoic acid (EPA, C20:5, n-3), and Alpha-Linolenic acid (ALA, C18:3, n-3); have recently been linked to a number of benefits for both infants and adults (Guo et al., 2016). In infants, PUFAs, especially DHA, have been linked to the development of the retina tissue where it makes between 35 and 60% of the rod and cone tissues, and it makes up to 40% in certain areas of the gray matter in the brain (Talbot, 2015). In adults, DHA is linked in the prevention of cardiovascular diseases as well as improving inflammatory conditions such as asthma, eczema, psoriasis and Crohn's disease (Pike & Jackson, 2010).

For obtaining these benefits, the World Health Organization (WHO) recommends a daily n-3 PUFA intake of 1 to 2% of the daily energy for the general adult population. The Food and Agriculture Organization (FAO) recommends a daily intake of EPA + DHA between 100 to 250 mg for infants between ages 1 to 10; they also recommend that pregnant and lactating women should consume no less than 200 mg/day, with 300 mg being the recommended intake. The French Agency for Food Health Security (AFSSA) indicates that a daily EPA + DHA consumption between 500 and 750 mg/day reduces the risk for metabolic syndrome diabetes-obesity, cardiovascular diseases, breast and colon cancer, neuropsychiatric disorders, and age-related macular degeneration (Goed, 2014).

Traditionally, PUFAs come from fish capture and fish oil supplements (Robles Medina et al., 1998); however, new sources are being developed to meet the increase in the demand (Béligon et al., 2016). Consequently, biotechnological processes have been developed to produce PUFAs from different lipid-producing microorganisms. Among the possible lipid producing microorganisms, Thraustochytrids stand out due to their high biomass yield (20 g/L DCW or more) and their PUFAs accumulation capacity, which is usually around 50% DCW. The lipid profile of Thraustochytrids is special as they have the capacity to produce a high percentage of DHA, which can range from 35% TFA (Total Fatty Acids) to around 50% TFA. The most common genus of the Thraustochytrid family are *Schizochytrium, Thraustochytrium, Ulkenia,* and *Aurantiochytrium* (Jye et al., 2015).

Nevertheless, these biotechnological processes are expensive as they mostly rely on glucose as carbon source. This impact is more evident when we consider that in microalgal biorefineries, the carbon source usually accounts for 50 to 60% of the medium cost and the culture stage represents between 40 to 50% in process (’t Lam et al., 2017). Consequently, cheaper carbon sources are being considered for these processes, such is the case of glycerol, which is widely used as substitute of glucose in these type of cultures (Guo et al., 2016).

Considering the above, this work aims to propose the use of non-Conventional Carbon Sources (n-CCS) that lower the production cost of DHA using the microorganism *Aurantiochytrium limacinum* SR21 while maintaining the yield levels of glucose when as carbon source.

Introduction

Given all the benefits that the PUFAs have on the human health, the lack of accessibility in most of the population is a matter of general concern. Colombia has a very low intake of DHA, 67.2 mg/day or 0.024% of daily energy (Forsyth et al., 2016), which is quite low when compared to the recommended intake values. Furthermore, the Colombian Ministry of Health and Social Welfare, in its resolution 3803 of 2016, recommends a daily intake of 500 mg/day for lactating infants and 0.6 to 1.2% of the daily energy for the remaining of the population. Therefore, the consumption of n-3 PUFAs, DHA in specific, is below the recommended values from both international organizations and the Colombian government.

Because most of Colombia's population lives inland with no easy access to the sea, and considering that DHA can be found in certain varieties of capture and farmed fish –not all species of fish are rich in DHA–; the diet of most of the population is comprised of foods that do not provide the recommended amounts of DHA for achieving its benefits.

The city of Medellín is a good example of this situation where the diet is far from meeting the daily intake requirements of foods that contain Long Chained PUFAs (LC-PUFAs) such as EPA and DHA. Medellín follows the trends of western diets were cereals (rice, beans, and *arepa* –a corn based traditional food) and meats (beef, pork, and chicken) are more prevalent in the diet (Montoya & Alcaraz, 2016) as opposed to LC-PUFA rich foods.

For adding these essentials nutrients into the diet, it is necessary to consume foods or supplements that are rich in EPA and DHA; however, these foods tend to be expensive. A study compared the cost for different sources needed to meet the intake requirements. In this study the cheapest source of 500 mg of EPA+DHA was fish/Cod oil supplements at US$0.19 in average followed by Omega 3, 6, 9 supplements at $0.44, which usually also come from fish oil. For reaching the requirement intake by incorporating fish into the diet, according to the study, it would be necessary to spend in average US$1.17 for Salmon, US$1.35 for pelagic fish, and US$10.68 for demersal fish (Watters et al., 2012). It is worth mentioning that these values, even though assessed in 2012, are a good comparison to today's market as the inflation in the USA between 2012 and 2018 was 9.37%.

Furthermore, another reason to look for alternatives to fish and fish oil to supplement PUFAs intake is the growing problem of pollution in the ocean which has resulted in the bioaccumulation of hazardous substances in the fatty tissue of wild fish (e.g., heavy metals and polychlorinated biphenol (Manikan et al., 2015)) wherefrom fish oil is mostly derived (Chanpiwat et al., 2016). To cope with this situation, some biotechnological processes have introduced microorganisms capable of producing and accumulating LC-PUFAs, commonly referred to as Single Cell Oils (SCOs) (Jye et al., 2015). In this regard, the microalga *Nannochloropsis oceanic*, *Porphhyridim cruentum*, *Phaedactyctum tricornutum*, and *Chaetoceros calcitrans* have shown the potential to produce EPA and *Isochrysis galbana* and *Crypthecodinium* spp. the potential for DHA production; however,

their need for photobiorreactors is still a hindering characteristic when using these microorganisms (Béligon et al., 2016). Thraustochytrids are another alternative for the production of DHA, and being heterotrophic microorganisms that do not depend on light for their growth, biotechnological processes are being developed to use them in the production of n-3 PUFAs. Specifically, the genera *Schizochytrium* and *Aurantiochytrium* have shown great potential for the production of DHA where feed batch processes have obtained over 100 g/L of biomass with DHA synthesis around 50% Total Fatty Acids (TFAs) (Jye et al., 2015; Li et al., 2015). However, production costs are still high, which can keep the recommended intake low for the vulnerable population (Forsyth et al., 2016).

To face this situation, different microorganisms have been studied, which have the capacity to produce and accumulate SCOs. Microalgal species, such as *Chlorella* sp or *Dunaliella bardawil* (Hu et al., 2008), as well as other microorganism that are capable of accumulating PUFAs, among them, there are some species of yeast like *Cryptococcus curvatus, Cryptococcus albidus, Lipomyces lipofera, Lipomyces starkeyi, Rhodotorula glutinis, Rhodosporidium toruloides, Trichosporom pullulan* and *Yarrowia lipolytica.* However, in recent years Thraustochytrids have arisen as the most suitable alternative in the production of PUFAs given their capacity to accumulate up to 50% of their weight in lipids, as mentioned above. Furthermore, EPA and DHA tend to be of high production in these family (Jain et al., 2007). Among the Thraustochytrids, the genera *Schizochytrium* sensu lato and *Aurantiochytrium* sensu lato receive special attention due to their production advantages and higher lipid accumulation. However, production costs continue to be still high when using these microorganisms, which makes the access difficult for most of the population ('t Lam et al., 2017)

For lowering the costs, there are commonly two strategies to be applied. One way is reducing the operational costs for making the process economically feasible; and the other way to lower costs is by increasing the productivity of the culture (Chauton et al., 2015). In this way, for lowering the operational costs of the culture, supplying the microorganism with non-Conventional Carbon Sources (n-CCSs) as an alternative to glucose, which is commonly used as carbon source and has a high cost, could be a way to lower process costs. This strategy is based on the fact that the carbon source accounts for up to 50 % of the medium cost; therefore, this strategy could impact the process costs ('t Lam et al., 2017).

In this way, several studies have tried a variety of n-CCSs for lowering the process costs, among them sweet sorghum juice (which yielded 34.28% TFA of DHA), hemp hydrolysate (38% TFA of DHA), and crude glycerol (24.86% TFA of DHA) (Gupta et al., 2012, 2015). Following this idea, and considering the agricultural vocation of Colombia, this work presents several n-CCSs for substituting glucose as carbon source in the culture of *Aurantiochytrium limacinum* SR21.

Coffee and palm oil are two of the most important agro-industrial industries in the country, and Coffee Mucilage (CM) and Empty Fruit Bunch (EFB) are two byproducts from these

industries than have great potential as carbon sources; previous studies have found that EFB hydrolysates can obtain 58 g/L of glucose and coffee waste can produces sugar rich hydrolysates (W.-K. Hong et al. 2011; Mussatto et al. 2012). Similarly, the growth of the artisanal brewing companies in the country also provides a waste that is promising for the obtention of a carbon source suitable for the microorganism. Barley Spent Grain (BSG) is a waste that can also serve in the preparation of the medium. All these materials have the potential to produce growing media for microorganism while potentially lowering the production costs for the process.

Consequently, this work presents a biotechnological alternative to produce DHA where *Aurantiochytrium limacinum* SR21 is used in the production of Single Cell Oils (SCO) rich in DHA. To do so, three different n-CCSs are evaluated against the performance of *A. limacinum* SR21 in glucose for determining the most suitable replacement as carbon source in the obtaining of a low cost DHA.

Objectives

General objective

To determine a non-conventional carbon source that allows substituting glucose as substrate for *Aurantiochytrium limacinum* SR21 cultures, while lowering production costs and keeping the levels of productivity.

Specific objectives

To determine the amount of available carbohydrates from the selected non-conventional carbon sources for the growth of *Aurantiochytrium limacinum* SR21.

To compare the growth of *Aurantiochytrium limacinum* SR21 in a glucose culture against non-conventional carbon source cultures for the production of DHA.

To estimate the culture cost based on the non-conventional carbon source cost against glucose cost in the production of the recommended intake of DHA from *Aurantiochytrium limacinum* SR21.

Chapter 2

Theoretical Framework and State of the Art

Fatty Acids

Fatty acids are a group of chemical compounds that have a great impact on the human health. Some fatty acids can be synthesized by the human body; however, some of them need to be incorporated into the diet as humans lack the capacity to synthesize them or do so in inefficient ways. Fatty acids can be divided into saturated and unsaturated fatty acids as shown in Figure 1. The later are likewise grouped as mono or poly unsaturated fatty acids.

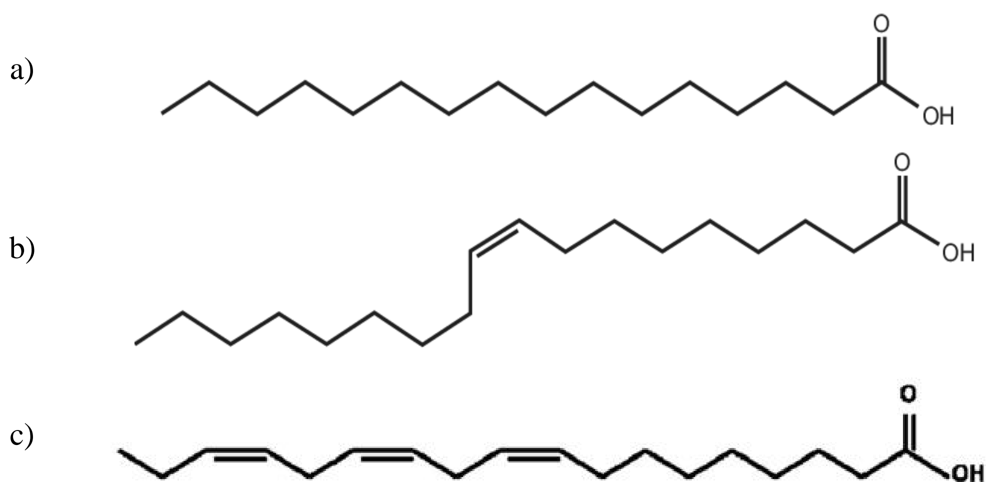

Figure 1. Chemical structure of fatty acids. a) Saturated: Palmitic acid C16:0, b) Monosaturated: Oleic acid C18:1 n-9, and c) Polyunsaturated: α-Linolenic acid C18:3 n-3

Saturated fatty acids have between 12 and 22 carbon in their chain, these compounds have no double bonds in their structure; hence, they do not have any bending in their chain. Saturated fatty acids have an even number of carbons, some examples of saturated fatty acids are Palmitic acid (C16:0) and Stearic acid (C18:0).

Monounsaturated fatty acids, which have a single carbon to carbon double bond in any position of the fatty acid chain, are between 16 and 22 carbon long. The double bond is positioned in a cis configuration, which bends the molecule and provides the molecule with a low melting point when compared to trans configuration bonds and saturated acids. These acids can be found in eggs, where Palmitoleic acid (C16:1) and Oleic (C18:1) are most prevalent.

PUFAs poses more than one double bond in their fatty acid chain. These acids are synthesized by plants and other marine microorganism, this makes them essential to all high order organisms. One defining characteristic of PUFAs is where the unsaturations are located. In this way, when the double bond in located between the third and fourth carbon from the methyl end or omega carbon (i.e., the last carbon in the aliphatic chain) the fatty acid is referred to as omega 3 (n-3). Similarly, when the first double bond is located between the sixth and seventh or the ninth and tenth carbon the fatty acid is referred to as omega 6 (n-6) or omega 9 (n-9), respectively. However, omega 9 fatty acids are not considered to be essential as they can be synthesized by the human body from saturated fatty acids.

Omega 6 fatty acids are commonly found in a great variety of vegetable oils, such as soybean oil, rich in Linoleic acid (LA). Other omega 6 rich sources are spinach, buttercrunch, red leaf lettuce, and mustard lettuce; additionally, purslane also contains ALA n-3 (A. P. Simopoulos, 2002). Other food containing both omega 6 and ALA are eggs, bovine meat, poultry meat, and milk (Forsyth et al., 2016).

However, omega 3, as shown in Figure 2, aside from ALA are mostly available in the oil of a few fish species, namely cod, salmon, mackerel, menhaden, and tuna (Rusu et al., 2017). Omega 3s are a very important group of fatty acids. They have been linked to a variety of benefits for the human health. In this way, some studies link EPA and DHA to cardiovascular disease risk reduction as well as neural disorders, arthritis, asthma, and skin disease (Jye et al., 2015).

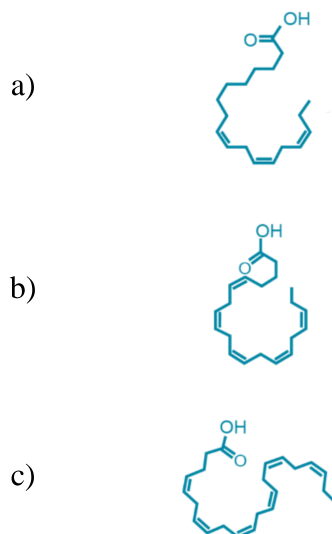

Figure 2. Chemical structure of the Omega 3 family. a) ALA, b) EPA, and c) DHA

It is important to mention that the omega 6/omega 3 ratio is an important factor for achieving the different health benefits that these molecules have on the human health. There are indications that early humans had an omega 6 to omega 3 ratio of around 1;

nevertheless, this ratio is nowadays between 15-20 to 1 due to the shift in western diets towards agriculture based fatty acid intake. It is worth mentioning that this imbalance, caused by the lower intake of n-3 PUFAs, increases the risk of suffering from cardiovascular diseases and other chronic diseases (Artemis P. Simopoulos, 2008).

Furthermore, DHA has also been linked to the development of the brain and retinal maturation partly by influencing neurotransmitter pathways (Rogers et al., 2013). DHA is also beneficial in the visual and cognitive development of low-birth-weight infants (Kris-Etherton et al., 2009).

Additionally, fatty acids are a key building block of the phospholipid bilayer surrounding the cells; moreover, they are used as energy storage in the form of triacylglycerol, which is preferred to the storage of glucose as they can produce six times more the amount of usable energy. Additionally, fatty acids intervene in the acylation of proteins aiding in the anchoring, folding, and function of many proteins. Fatty acids are also found in the regulation of the transcription genes regarding the metabolism, cellular proliferation, and apoptosis.

Synthesizing DHA through the metabolism is expensive; therefore, mammalians (humans included) have developed several mechanisms to protect the DHA molecules –which are prone to oxidation due to their high number of unsaturation– from oxidizing. For example, phospholipid fatty acid recycling removes damaged PUFAs from membranes to preserve the composition. Alternatively, to convert either Docosapentaenoic acid n-3 or n-6 (DPA n-3 or DPA n-6) into DHA, three additional steps that require translocation from the endoplasmic reticulum to the peroxisomes are needed; however, this mechanism is very inefficient in adults making preformed DHA a better source (Brenna & Carlson, 2014).

Microbial LC-PUFAs

Although fish oil is the main source of EPA and DHA, fish are not the main synthesizers of LC-PUFAs, but rather they accumulate these molecules as storage of energy. EPA and DHA are mainly synthesized by a variety of microorganisms. With this in mind, there are a number of microorganisms capable of synthesizing these components. Some bacteria can accumulate PUFAs, such is the case of *Staphylococcus aureus*, which has shown to yield up to 0.65% of its Dried Cell Weight (DCW) in DHA with cultures obtaining 0.122 g/L (Yano et al., 1994). Similarly, the fungus *Entomophthora obscura* produces up to 12% of its biomass in LC-PUFAs, with yields of 24.4 g/L (de Bievre & Latgé, 1980); similarly, *Mortierella isabellina* (21.7 to 64% DCW), and *Cunninghamella echinulate* (23.8 to 53.6% DCW) have also shown the capacity to accumulate lipids under different substrates and modes of culture (Béligon et al., 2016). Nevertheless, both bacteria and fungi do accumulate fatty acids in small amounts rendering them inadequate for up-scale processes. This is how marine microorganism such as microalgae and other single cell microorganisms stand out for their high accumulation of PUFAs, and in especial for their accumulation of both DHA and EPA, which –as has been stated above– need to be

incorporated into a healthy diet. Considering this, there are two type of microorganisms that can be used in the production of DHA, photosynthetic and heterotrophic (Brenna & Carlson, 2014).

Photosynthetic PUFA producing microorganisms

Microalgae have the capacity to synthesize and accumulate omega 6 and 3 fatty acids (Béligon et al., 2016). There is a group of photosynthetic microorganisms that are capable of producing DHA in different amounts. Namely, *Ostreoccocus tauri*, *Chroomonas salina*, *Isochrysis galbana*, *Emiliana huxleyi*, among others produce DHA to some degree (Khozin-Goldberg et al., 2011).

Heterotrophic PUFA producing microorganisms

Another source of omega 3 PUFAs is heterotrophic oleaginous microorganisms. These microorganisms are important producers of EPA and DHA, which are then carried down the marine food web until reaching the fish species where fish capture and fish oil comes from. In this regard, the Thraustochytriae family has shown great capacity for accumulating DHA as energy storage when under nutrient stress. This family is known to accumulate up to 50% of their DCW in lipids, DHA frequently constituting 25% or more of these (Jain et al., 2007).

Thraustochytrids, accumulate between 70 to 98% of their lipids in triacylglycerols to store energy. Figure 3 shows isolates of Thraustochytrids under epifluorescence micrographs where it is possible to see lipid accumulation, though in the culture used for these images, this accumulation happens when the cell is under vegetative stages. However, different Thraustochytrids produce and accumulate different amounts and types of PUFAs, in our case we will focus on one microorganism, *Aurantiochytrium limacinum* SR21, that has shown to produce high amounts of DHA.

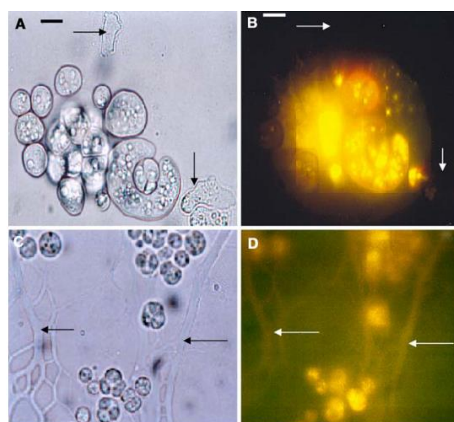

Figure 3. Thraustochytrid isolate. A and C in bright field; B and D in epifluorescence photomicrograph showing fluorescent lipid bodies (Jain et al. 2007)

Aurantiochytrium limacinum SR21

Originally, *Aurantiochytrium limacinum* SR21 was classified as *Schizochytrium limacinum* SR21. Etymologically, limacinum refers to the limacifom amoeboid cells released from vegetative cells. The microorganism is a spherical cell with 7 to 15 µm in diameter with an ectoplasmic net element. In its zoosporangium state, 12 to 24 µm in diameter, it produces 16 to 64 zoospores (ovoid, 6 to 8,5 µm long and 5 to 7 µm wide with two laterally inserted flagella). In sea water or pine pollen culture or nutrient medium, there are presence of vegetative cells forming limaciform amoeboid cells (12 to 20 µm long and 5 to 8 µm wide with pseudopodia) as well as rounded amoeboid cell forming eight zoospores 4.5 to 6 µm long and 3.5 to 5 µm wide. Vegetative cells are spherical and form clusters in liquid medium. The cells possess many spherical vacuoles 2 to 3 weeks after being inoculated in a fresh medium and after multinuclear stage, they go onto zoosporangia (Honda et al., 1998).

Further studies on *Schizochytrium* determined that this genus had some controversy in its classification; and therefore, rearrangement was needed. After performing a 18S rRNA gene sequence, three species; *S. aggregatum*, *S. minutum*, and *S. limacinum*; arouse. Therefore, it was necessary to determine how many other lineages within the *Schizochytrium* genus needed to be rearranged (Yokoyama & Honda, 2007).

In that study, *Aurantiochytrium* arouse as a new genus (*aurantius*=orange color and *chytrion*=pot. Referring to the color of the thallus). The distinctive characteristics were defined as: cells that possess astaxanthin phoenicoxanthin, canthaxanthin, and β-carotene. They possess minor arachidonic acid and mainly produces docosahexaenoic acid regarding PUFAs. This new genus forms small colonies by continuous binary cell divisions. They do not develop well ectoplasmic nets as opposed to the *Schizochytrium* genus. Regarding the zoospores, they are biflagellate heterokont and reniform to ovoid. Their 18rRNA is distinct. Resting spores were not observed. The species *Aurantiochytrium limacinum* was based on *Schizochytrium limacinum* (Yokoyama & Honda, 2007).

In recent years, more studies have been conducted for shedding more light into the behavior of *Aurantiochytrium limacinum* SR21, in this way, new sequencing studies have shown how this species uses different ways to take advantage of the carbon sources in its environment. However, not all species have the capability to produce the necessary enzymes for hydrolyzing more complex materials such as cellulose and hemicellulose. In this way, this microorganisms inhabit areas where other trophic species degrade the material for its further use by many thraustochytrids, *A. limacinum* SR21 among them. (Song et al., 2018).

Carbon to nitrogen ratio

Nitrogen is the second most important nutrient for marine microorganisms after carbon. This nutrient can be incorporated as nitrate or ammonium. Nitrogen is also a critical factor in the regulation of the lipid content (Amsler, 2008; Andersen, 2005). Typically, nitrogen

makes up to 20% of the microorganism; however, when nitrogen becomes a limiting factor in the growth of the culture, the accumulation of lipids can increase above 40%. Nevertheless, limiting the nitrogen in a medium can hinder the production of biomass; therefore, a high yield of lipids and a high yield of biomass are not simultaneously possible (Fu et al., 2017; Sakarika & Kornaros, 2017; Xin et al., 2010).

Considering that decreasing the amount of nitrogen can limit the biomass production, when the conditions for the culture are designed, it is necessary to account for conditions that favor both the growth of the microorganism while accumulating lipids. *Aurantiochytrium limacinum* SR21 has shown the capacity to grow and accumulate lipids using different nitrogen sources; however, yeast extract has shown to be one of the most assimilable nitrogen sources. Nevertheless, the microorganism also has affinity to other sources such as monosodium glutamate (Ling et al., 2015) and ammonium sulphate (Furlan et al., 2017). When yeast extract is used, *Aurantiochytrium limacinum* SR21 is capable to accumulate more than 40% of its biomass in lipids, achieving an accumulation of 50% of its lipids in DHA (Hong et al., 2011).

Moreover, as nitrogen is a critical factor that directly impacts the metabolism of the microorganism, decreasing the assimilable nitrogen in the medium can result in an increase of the accumulation of lipids, specially TAGs, this behavior can be seen in many marine microorganisms. (Singh & Sharma, 2012). The gradual shift towards a nitrogen depleted medium changes the lipid composition moving from lipids rich in free fatty acids to lipids rich in TAG; additionally, there is also an increment in pigments, which are molecules with lipid-like characteristics. (Leyva Soto, 2014).

The accumulation of secondary metabolites, such as carotenoids, is another characteristic that some microorganisms – *A. limacinum* SR21 included– exhibit when they are cultured in a medium with a low concentration of nitrogen (Yen et al., 2013). Specifically, significant increases in the accumulation of β-carotenes and a higher amount of astaxanthins are evident in these types of culture conditions (Song et al., 2018).

In heterotrophic cultures, when the carbon to nitrogen ratio varies, the biomass productivity is affected as well as the accumulation of lipids. *Aurantiochytrium limacinum* SR21 has a good biomass and lipid production when the carbon to nitrogen ration is 1:25. This ratio was stablished by a previous study in the Bioprocesos Research Group, where different rations were tested (1:5, 1:15, 1:25, and 1:35). This study was carried out with an initial concentration of 50 g/L of glucose and 50% sea water. No other nutrient was added to the medium. Under the 1:25 carbon to nitrogen ratio, the growth velocity of the microorganism was 0.017 h^{-1} with a biomass accumulation of 19.27 g/L and a productivity of 0.33 g/L/h. With this carbon to nitrogen ratio, it was possible to achieve 36.1% of the biomass in lipids (Delgado Naranjo et al., 2017).

Sea salt concentration

Being a marine microorganism, a saline environment would be important, both for the growth of the microorganism and its lipid accumulation. However, several studies show that the effects of salt concentration are neglectable concerning DCW, lipid accumulation, and DHA content when the concentration is maintained between 2 and 50 g/L artificial sea salt (Hong et al., 2011). DCW was proven to remain constant in sea salt levels of 50% and more at about 12.5 g/L; however, lipid accumulation decreased as the concentration of sea salt increased (Yokochi et al., 1998).

Therefore, to guarantee the least disruptive environmental conditions for the microorganism, it is recommended to use a culture that has 50% artificial sea salt. The concentration of salt in the oceans vary depending on several factors, but a general consensus establishes the concentration of sea salt around 37.50 g/L; therefore, 50% artificial sea water should have a concentration of 18.75 g/L.

Polyunsaturated fatty acids synthesis

As *Aurantiochytrium limacinum* SR21 arises as a candidate for producing PUFAs, it is important to understand the way in which this microorganism transforms the carbon source into PUFAs. In this way, thraustochytrids, in general, are a subject of study regarding the pathway that they use to synthesize fatty acids. There are two main pathways that these microorganisms can follow towards the production of PUFAs, these are known was the Fatty Acid Synthesis pathway (FAS) or the Polyketide Synthase pathway (PKS). As mentioned above, it is still not clear which pathway in the main one in the synthesis of PUFAs. A sequencing of the *A. limacinum* SR21 genome, showed that there are genes that can code for both PKS and FAS pathways in the microorganism, indicating that under different conditions *A. limacinum* SR21 can follow one or the other, even a combination of both pathways (Song et al., 2018).

Nonetheless, the lipogenesis starts with glucose, which is transformed into pyruvate, this process takes place in the mitochondrion. The pyruvate is then transformed into acetyl-CoA by the pyruvate decarboxylative dehydrogenase. Acetyl-CoA then reacts with oxaloacetate to produce citrate, which then enter the Krebs cycle leading to the synthesis of ATP. Acetyl-CoA is converted into Palmitic Acid by the lipogenesis pathway, which is the primer for longer chained saturated or unsaturated fatty acids. Acetyl-CoA also acts as the primer for the synthesis of malonyl-Acyl carrier protein and acetyl-ACP (Béligon et al., 2016). From here the above mentioned pathways can be followed.

Polyketide synthase pathway

The PKS system uses the acyl carrier protein as a covalent attachment for the synthesis of the chain. An acyl-ACP and a malonyl-ACP condense due to the action of the 3-ketoacyl-ACP synthase to generate 3-ketoacyl-ACP. This molecule is then reduced by a 3-hydroxyacyl-ACP reductase producing 3-hydroxyacyl-ACP. Later in the process, a dehydrase/isomerase removes a molecule of water and induces the production of enoyl-

ACP. Finally, enoyl-ACP is reduced to form a saturated acyl chain (Béligon et al., 2016). This process is illustrated in Figure 4.

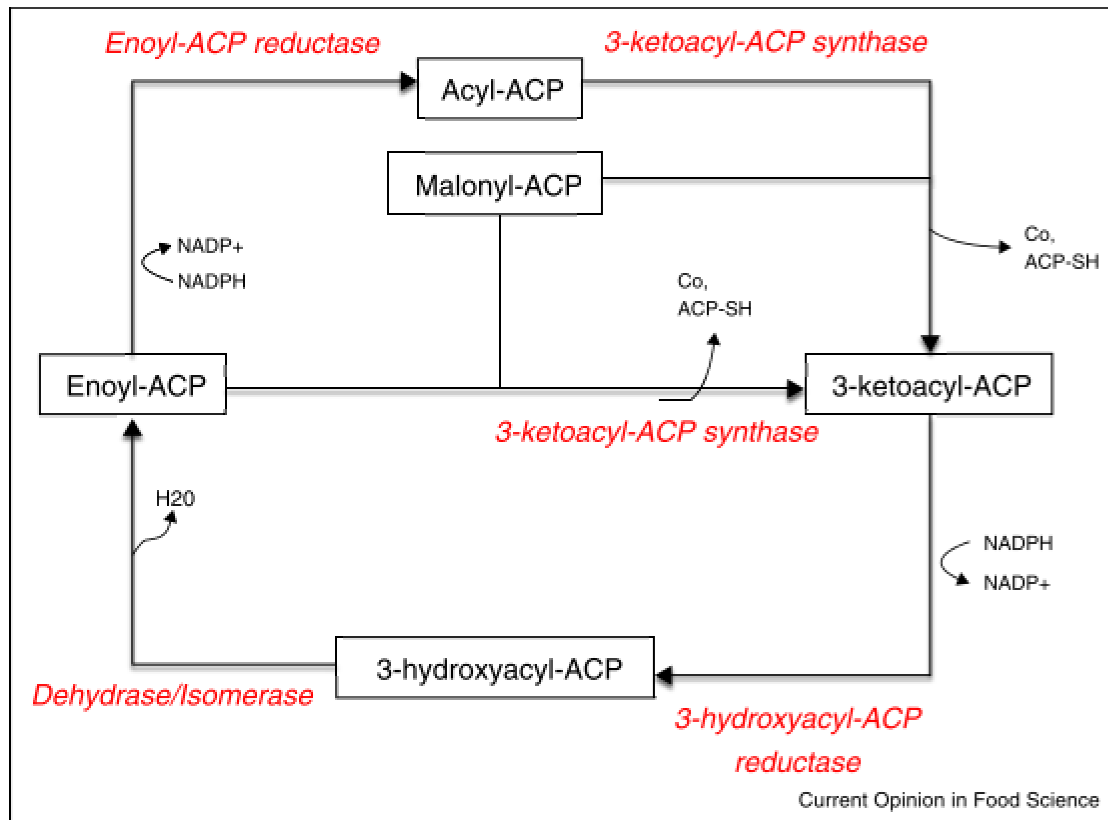

Figure 4. Polyketide synthase pathway for the biosynthesis of DHA (Béligon et al. 2016)

Fatty acid synthesis pathway

The FAS pathways starts when oleic acid is desaturated by the Δ-12 desaturase which leads to the production of linoleic acid (LA). From this point onwards, there are two pathways that can be followed, one pathway produces the omega 6 fatty acids; conversely, when LA is desaturated by Δ-15 desaturase to produce alpha-linolenic acid (ALA) the pathways follows the formation of the omega 3 fatty acids. From ALA, the Δ-6 desaturase inserts a double bond to produce the octadecatetraenoic acid (C18:4; 6, 9, 12, 15), which is an intermediate that is elongated to form the eicosatetraenoic acid (C20:4; 8, 11, 14, 17). A second desaturation mediated by the Δ-5 desaturase produces the synthesis of EPA, which is then elongated and desaturated by the Δ-4 desaturase, this process finally yields DHA (Béligon et al., 2016). This pathway is illustrated in Figure 5.

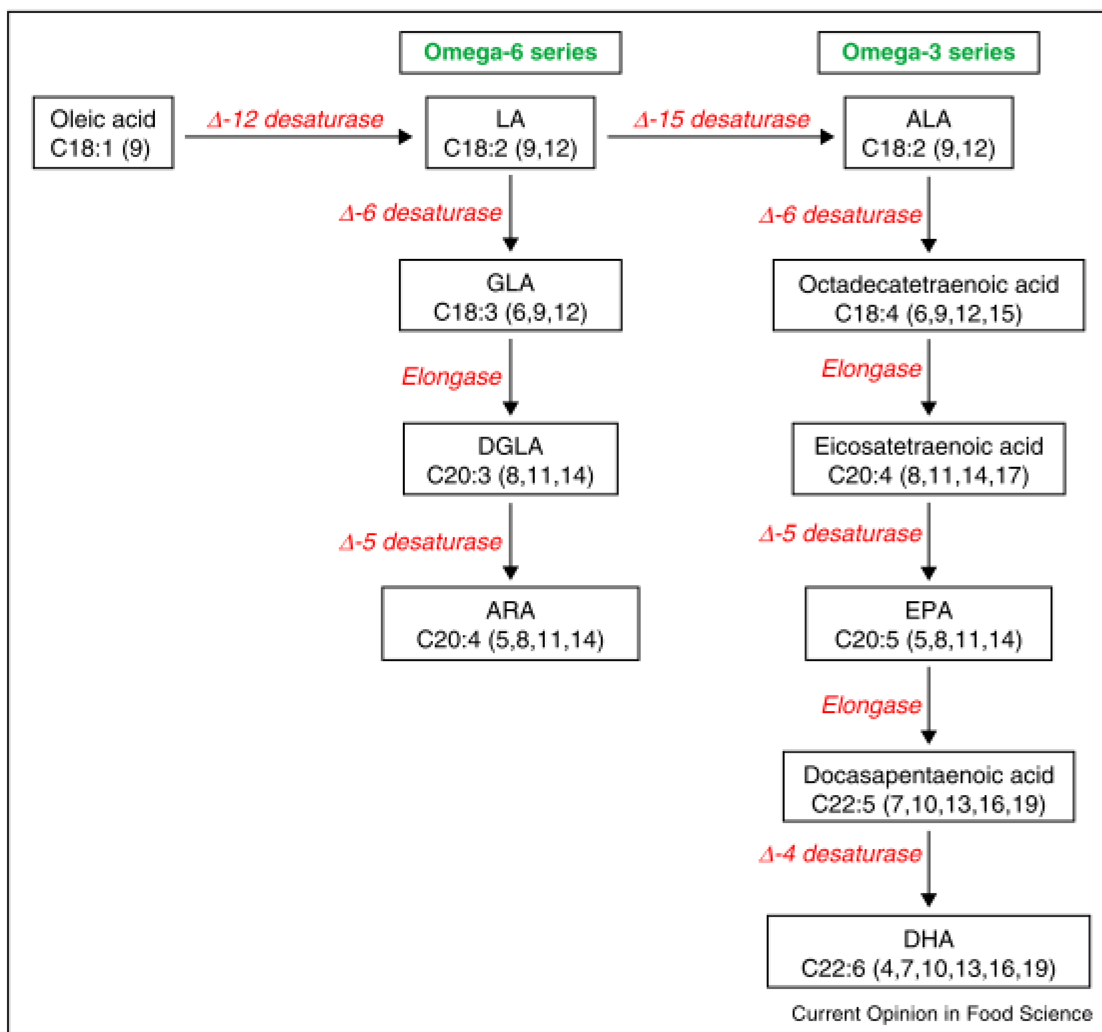

Figure 5. Pathway of polyunsaturated fatty acids biosynthesis (Béligon et al. 2016)

Lignocellulosic materials

Lignocellulosic materials are classified considering their origin. There are some categories such as: woods, grass, and agro-industrial wastes. The main advantage when using these materials is the lowering of CO_2 emissions into the atmosphere. Another advantage is the diverse source of lignocellulosic materials that are available. However, using these materials comes with the disadvantage of lack of homogeneity due to changes in regional soils and harvesting periods (Cardona et al., 2018).

The composition of the lignocellulosic material is a very important factor to produce hydrolysates rich in sugars, such as glucose. A high concentration of polysaccharides allows for a high recovery of sugars and a higher biomass concentration. However, the chemical composition of the substrates may vary significantly from one species to another

due to genetical and environmental factors. Cellulose, hemicellulose, and lignin are the main components in lignocellulosic materials with an approximated ratio of 4:3:3, which constitutes around 90% of the dry material, the remaining 10% is made of ash and extractives (Kim et al., 2016).

Cellulose is a linear biopolymer made of glucose molecules connected by glycosidic bonds (β1-4). The arrangement of these molecules in the plant's wall follows a hierarchy. The resulting tridimensional configuration is stabilized through Van der Walls interactions made of 30 linear molecules. These molecules have between 10,000 and 14,000 glucose molecules. The resulting fibers are interlaced through non-covalent interactions with the hemicellulose and lignin components. Cellulose fibers are grouped into two different regions; namely, crystalline and an amorph. The crystalline region is insoluble in most solvents, water included, and counteracts the action of microbial enzymes.

Hemicellulose is the second most abundant polysaccharide in nature. Unlike cellulose, it does not have a homogeneous structure, and it is mainly made of pentoses (xylose and arabinose), and hexoses (glucose, galactose, and mannose). Hemicellulose is polydisperse (i.e., indicating that is has a polymerization variable degree) and branched. Therefore, it is easier to hydrolyzed than cellulose (Álvarez et al., 2016).

Lignin is a complex aromatic biopolymer, and it is the third most abundant polymer in nature. It accounts for around 15% of the biomass on earth and its most common source is wood. Hard woods have been shown to have a lower content of lignin when compared to soft woods. Its chemical structure is amorph and made of aromatic alcohols, which provide the lignin macromolecule with a high polar behavior. The main function of this heteropolymer is to provide structural support, rigidity, and water resistance to the cellulose and hemicellulose components. Thanks to these characteristics, it is considered as a natural barrier. Lignin restricts the hydrolysis by protecting the surface of the cellulose or by adsorbing and inactivating the enzymes. The close intertwining between lignin and cellulose prevents the fibers from swelling; hence, the accessibility of the enzymes to the cellulose is affected.

Pretreatment of lignocellulosic materials

The structure of the lignocellulosic materials is a barrier for obtaining the sugars that can later be use as carbon source in the culture of microorganisms. Therefore, it is necessary to pretreat the material to alter the structure and make the cellulose more available to the enzymes, which transform the carbohydrate polymers into sugars. Figure 6 shows how sugars can be made readily available through pretreatment of the lignocellulosic material. The goal of the pretreatment is to break the lignin barrier and make the hemicellulos soluble while modifying the crystalline structure of the cellulose.

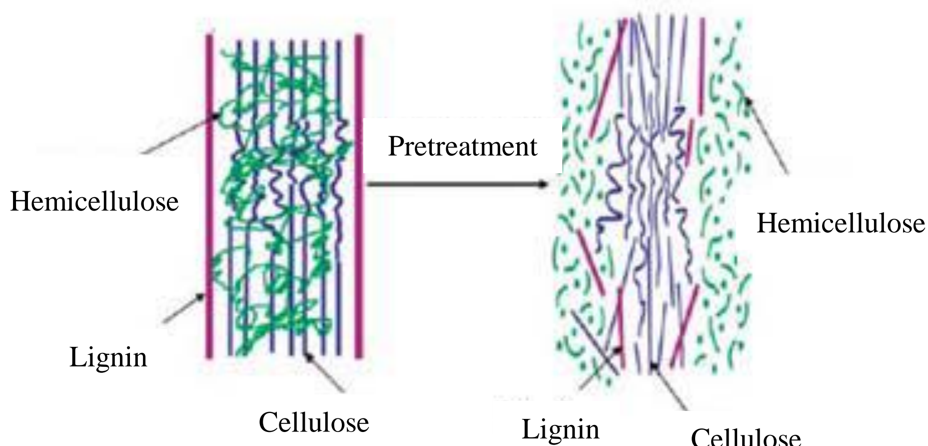

Figure 6. Layout of the pretreatment of lignocellulosic materials (Balat 2011)

The pretreatment that is used for making the polysaccharide fractions available from the lignocellulosic materials needs to have the following characteristics, as it has a high economic impact in the other stages of the process (Galbe & Zacchi, 2007): high recovery of all the carbohydrates, high digestibility of the cellulose, minimum production (or none at all) of degradation products and toxic compounds, biomass that does not require further size reduction, operation in reactors of moderate size and cost, free of solid residues, high concentration of solids and free sugars in the liquid fraction, compatibility with the culture, low consumption or demand of energy, recovery of the lignin, and low capital and operational costs. Pretreatments can be classified as follows:

Physical pretreatments

Within these pretreatments we find the following. Extrusion, were the material are subjected to heating, mixing, and shearing, resulting in physical and chemical changes during the passing through the extruder. The speed of the screw and the temperature of the barrel modify the structure of the lignocellulosic material causing changes in the fibers to increase the availability of the carbohydrates for the enzymatic attack (Balat, 2011). Pyrolysis, were cellulose rapidly decomposes in gaseous products and residual carbon when the material is treated at temperatures above 300°C. The performance of the hydrolysis process is significantly improved when in presence of oxygen. Some acid hydrolysis have been reported (1N H_2SO4 at 97°C for 2.5 hours) with a conversion of cellulose between 80 to 85% for reducing the sugars with more that 50% in glucose (Balat, 2011). Size reduction, were the cellulosic fraction is separated from the hemicellulosic and lignin shell that surrounds it. In this way, the effective superficial area available for the microorganisms is increased while decreasing the crystallinity of the cellulose. The size of the materials is around 10 to 30 mm after the splintering of the wood and 0.2 to 2 mm after the milling of the material (Sun & Cheng, 2002). The reduction of size does not add value

to the raw material, has high energy demand, and can significantly increase the production costs (Alvira et al., 2010).

Physical-chemical pretreatment

Among these pretreatments we find the following. Steam explosion, where the lignocellulosic material goes under temperatures between 190 and 230°C through the direct injection of saturated steam for 1 to 10 minutes. The mechanical effect is caused by the quick depressurizing that causes an evaporation of the water inside the material. This creates shear forces that produce the separation of the fibers, mainly, in the weakest regions (amorph cellulose). The chemical effect is caused by the hydrolysis of the acetylic fractions from the hemicellulose, which produce acetic acid and catalyzes the hydrolysis at the temperature of the process (autohydrolysis). The most important variables in this pretreatment are temperature, residence time, particle size, and humidity (Duff & Murray, 1996). Steam explosion has been recognized as a very effective method in the pretreatment of hard woods and agro-industrial wastes (Alvira et al., 2010; Balat, 2011). Steam explosion, ammonia fiber expansion (AFEX), this is a similar process to steam explosion where the material is impregnated with anhydrous liquid ammonia (1 to 2 Kg ammonia per Kg of dry material) at a temperature between 60 to 100°C and high pressure for approximately 30 minutes (Sun & Cheng, 2005). A rapid expansion of the ammonia gas swells and breaks the material as well as partially decrystalizes the cellulose (Alvira et al., 2010). The difference with steam explosion and other acid pretreatments is that in this process, the hemicellulose is not solubilized. (Hamelinck et al., 2005; Mosier et al., 2005). Carbon dioxide explosion, which is like steam explosion or AFEX. In the explosion with CO_2 as super critic fluid, carbon dioxide forms carbonic acid, which increases the rate of the hydrolysis. The CO_2 molecules are similar in size to the water and ammonia molecules and they can enter through the small pores of the lignocellulose at high pressures (Alvira et al., 2010). The advantages of the pretreatment are the relative availability at low cost, the non-toxicity, non-inflammability, easy recovery after extraction, and environmental acceptability. Microwaves, in this pretreatment, the material is submerged in diluted chemical reactants. This suspension is then exposed to microwave radiation during 5 to 20 minutes (Alvira et al., 2010). This pretreatment can be considered a physical-chemical process due to its thermic and non-thermic effects (Balat, 2011). Ultrasound, the effect of ultrasound on the lignocellulosic material has been used for the extraction of the hemicellulose, cellulose, and lignin; however, few studies have focus on the susceptibility of the lignocellulosic materials to the hydrolysis. Despite the few studies on this pretreatment, some studies indicate that the saccharification of the cellulose has improved through the ultrasound pretreatment (Alvira et al., 2010; Balat, 2011; Balat et al., 2008).

Biological pretreatments

Using cellulosic organisms that produce extracellular enzymatic systems that transform polysaccharides into sugars Hydrolases degrade cellulose and hemicellulose by the action of cellulases and hemicellulases enzymes (C. Sánchez, 2009). Ligninases depolymerize the lignin by manganese-peroxidase, lignin peroxidase, and laccase enzymatic oxidation reactions (Cuervo et al., 2009; C. Sánchez, 2009). The main lignocellulosic

microorganisms are some bacteria and fungi. Within each group of microorganisms, two main groups with cellulolytic capability have been identified. This first is an anaerobic group, composed by bacterial and fungal species that live in waste waters, rumen, and the intestinal tract of herbivore animals and some insects, such as beetles and termites. Some examples of bacteria in this group among other there is the genera *Clostridium* and *Ruminococcus*. Some identified fungi are *Anaeromyces mucronatus, Caecomyces communis, Cyllamyces aberen- cis, Neocallimastix frontalis, Orpinomyces sp.* and *Piromyces sp*.(Lynd et al., 2002; Martínez-Anaya et al., 2008).The second group includes aerobic species living on the ground, especially in forests. Some bacteria are *Cellulomonas* and *Streptomyces*, and basidiomycete fungi responsible in the rotting of wood. The white rotting of wood is carried out in 96% of the cases by fungi of the *Polyporaceae* family, this phenomenon is but the decomposition of lignin y the enzymatic systems that allow for easy access to the cellulose and hemicellulose (Lynd et al., 2002; Martínez-Anaya et al., 2008; Taniguchi et al., 2005; Zhang et al., 2007). Conversely, the degradation mechanism and the enzymatic systems in brown rotting are less known and even controverted. These fungi do not have peroxidase systems for the extensive degradation of the lignin and that the initial attack is on the holocellulose (i.e. cellulose and hemicellulose) (Martínez-Anaya et al., 2008). For the effective digestion of the cellulose, the fungal enzymes have evolved synergic mechanisms that allow them to compete with the material's resistance. This phenomenon refers to the observation that the maximum degradation activity does not happen under individual enzymes, but mixed of three or more enzymes (Lynd et al., 2002; Martínez-Anaya et al., 2008).

Chemical pretreatments

Finally, we encounter chemical pretreatments, the goal of these pretreatments is to solubilize the lignin fraction and modify the cellulose structure to facilitate the enzymatic action. Among the chemical pretreatment we find the following. Ozone, which has been used for degrading lignin and hemicellulose in several lignocellulosic materials such as wheat straw, bagasse, pine, cotton, and sawdust. (Alvira et al., 2010; Oliva Martínez et al., 2002; Pimentel, 2003; Ó. J. Sánchez & Cardona, 2008; Taniguchi et al., 2005; Thomsen et al., 2008). The degradation is basically limited to the lignin as the hemicellulose is slightly attacked and the cellulose is barely affected. This pretreatment has a series of advantages such as the effective removal of lignin, it does not generate toxic products that affect the later processes, and the reaction takes place at room temperature and atmospheric pressure. However, the use of ozone makes this process expensive (Sun & Cheng, 2002). In recent decades, the ozonolysis pretreatment has demonstrated its efficiency essentially in the degradation of the lignin polymer, but also slightly solubilizing the content of hemicellulose in the lignocellulosic material (Sun y Cheng, 2002). Organosolv, which uses mixes of organic solvents or mixes of aqueous phases, or using the addition of a acid or alkaline catalyst for breaking the internal bonds between the lignin and hemicellulose. The most commonly used organic solvents are acetone, methanol, ethanol, ethylene glycol, triethylene glycol, and tetrahydrofurfuryl alcohol (Alvira et al., 2010). Ethanol has the advantage of being easy recyclable by distillation. Nonetheless, when acid catalysts are

added (hydrochloric acid, sulfuric acid), higher yields of xylose are obtained. Additionally, organic acids such as oxalic, acetylsalicylic, and salicylic acid can be used (Mosier et al., 2005). The processes based on peracids are more specific for the removal of lignin, but are more expensive as they demand more safety measurements (Ó. J. Sánchez & Cardona, 2008).

Another chemical pretreatment is the acid hydrolysis, its main objective is to solubilize the hemicellulose in the material and make the cellulose more accessible to the enzymes. This type of pretreatment can be made with concentrated acid or diluted acid. The high maintenance and operational costs reduce the interest for applying concentrated acid pretreatment at commercial scale (Ballesteros et al., 2008; Cao et al., 2012; Hendriks & Zeeman, 2009; Karimi et al., 2006). This pretreatment can be made at high temperatures, for instance at 180°C for a given time, or at lower temperatures, 120°C for higher residence times (30 to 90 minutes). High yields from the hydrolysis have been reported when lignocellulosic materials are pretreated with diluted H_2SO_4, which is the most studied acid (Alvira et al., 2010). The diluted acid hydrolysis is probably the most common used method in chemical pretreatments. This can be used as pretreatment of lignocellulosic materials for the enzymatic hydrolysis or as the method for hydrolyzing fermentable sugars. (Ballesteros et al., 2008; Sun & Cheng, 2005). At high temperatures (140 to 190°C) and low concentrations of the acids (0.1 to 1% sulfuric acid), the diluted acid pretreatment can reach high reaction velocities and significantly improve the hydrolysis of the cellulose. The diluted acid pretreatment can be developed in short retention times (5 minutes for instance) and high temperatures (180°C) or in longer retention times (30 to 90 minutes) and low temperatures (120°C) (Taherzadeh & Karimi, 2007).

As part of the chemical pretreatments, we also find liquid hot water as well as alkaline hydrolysis, which are presented more extensively as they will be the ones used in this work.

Liquid hot water pretreatment (LHW)

In this pretreatment, the material undergoes temperatures between 160 to 240°C for a given time. The reactor must be pressurized for keeping the water in its liquid state.. (Mosier et al., 2005). For preventing the formation of inhibitors, the pH is kept between 4 and 7 during the pretreatment as the cellulosic sugars are maintained, at this pH range, in their oligomeric form and the formation of monomers is minimum. Lignin is partially depolymerized and solubilized during the LHW; however, the complete delignification is not possible using only hot water because of the re-condensation of soluble components from the lignin. In general the pretreatments are attractive for their potential cost savings (Alvira et al., 2010).

Alkaline hydrolysis

During the alkaline pretreatment, the first reactions that take place are the solvatation and saponification. The pretreatment with diluted NaOH produces a swelling of the material, which increases the internal surface area, decreases the crystallinity, separates the structural

bonds between lignin and the carbohydrates, and breaks the structure of lignin (Asgher et al., 2013). The mechanism of the alkaline hydrolysis of the material seem to be based on the saponification of the intramolecular esters that bond the xylanases from the hemicellulose and other components, such as lignin, or other components in the hemicellulose (Sun y Cheng, 2002). The effectiveness of this pretreatment depends on the lignin content in the material to be pretreated. (Chuck-Hernández et al., 2011). Sodium, potassium, calcium, and ammonium hydroxides are recommendable alkaline pretreatment. NaOH has been reported to increase the digestibility of hard woods from 14 to 55% by the reduction of lignin content in 24 to 55% at 20% (Kumar et al., 2009).

Hydrolysis of cellulose

Polymers from cellulose and hemicellulose must be converted into simple sugars for further use in cultures. Several methods or releasing these sugars have been used. Among them there is the acid hydrolysis, which can be strong or soft, and the enzymatic hydrolysis (Asgher et al., 2013; Balat, 2011; Balat et al., 2008; Oliva Martínez et al., 2002; Sun & Cheng, 2002). In the acid hydrolysis, the lignocellulosic material is exposed to acids in solution for a period and at certain temperature. This generates pentoses and hexoses from the hemicellulose and cellulose. This hydrolysis can be strong or diluted. The strong acid hydrolysis is generally carried out with concentrated sulfuric acid (H_2SO_4 70 to 90%). The reaction times are lower when compared to the diluted acid hydrolysis. If the acid and diluted acid hydrolyses are compared, it can be said that the conversion from polysaccharides into simple sugars will be more complete in the strong acid hydrolysis with yields close to 100% (Balat, 2011; Balat et al., 2008; Ballesteros et al., 2008; Duff & Murray, 1996). However, the negative environmental impacts, the high consumption of acid, and the difficulty to recover the sugars make this process not viable for industrial processes. The diluted acid hydrolysis takes place at high temperatures and pressures. Additionally, this process requires reaction times in the order of seconds to minutes. Therefore, this process can be easily turned into a continuous process. Diluted sulfuric acid is prepared at 1% concentration. The efficiency in the recovery of sugars is around 50%. The process needs two stages, the first stage takes place at low temperature, favoring the conversion of hemicellulos into pentoses. The second stage is carried out at high temperature, favoring the conversion of cellulose into glucose (Ballesteros et al., 2008; Oliva Martínez et al., 2002).

Enzymatic hydrolysis

This process is catalyzed by a group of enzymes generally referred to as cellulases, these enzymes are truly a mix of different enzymatic activities that combined together produce the degradation of cellulose. Generally, the enzymatic hydrolysis takes place at softer conditions than the acid hydrolysis, pH 4.5 to 5.0 and temperature between 40 to 50 °C. Hence, low energy consumption and low toxicity of the hydrolysates should be expected, these represent the main advantages of the enzymatic hydrolysis. Enzymatic hydrolysis of cellulose consists in the adsorption of cellulase on the surface of the cellulose and the breaking of cellulose into sugars followed by the desorption of the cellulase (Taherzadeh

& Karimi, 2007). Higher plants, some invertebrates, and, mainly, microorganisms (fungi and bacteria) are producers of these enzymes. Bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosic materials. These microorganisms can be aerobic or anaerobic, mesophilic or thermophilic. Some bacteria capable of producing cellulases are: *Clostridium*, *Cellulomonas*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, *Acetovibrio*, *Microbispora* and *Streptomyces*. *Cellulomonas fimi* and *Thermomonospora* have been widely studied for producing cellulases. However, many cellololitic bacteria, such as *Clostridium thermocellum* and *Bacteroides cellulosolvens* produce cellulases with high specific activity, but their low growth rate and anaerobic conditions cause that most of the research for the production of commercial cellulases focuses on fungi (Sun & Cheng, 2002).

Cellulases of fungal origin, mainly from the genera Trichoderma, Phanerochaete, and Fusaruim have been extensively studied and better characterized due their capacity to produce high amounts of extracellular enzymes, which makes easier the separation from the culture medium. The main advantages of the cellulases from Trichoderma are the stability of the enzymes under hydrolysis conditions as well as their resistance to chemical inhibitors; conversely, their main disadvantages are the suboptimum levels and the low activity of β-glucosidases. On the other hand, Aspergillus are very efficient producers of β-glucosidases (Taherzadeh & Karimi, 2007).

The enzymatic degradation of cellulose into glucose is generally achieved by the synergic action of at least three main types of enzymes, endoglucanases, exoglucanases, and β-glucosidases. Endoglucanases randomly act at the interior of the polymer attacking the low crystallinity regions (1,4-β-glucan bonds) in the free cellulose fiber and create free reducing ends. They can also act on cellodextrins and substitute derivates such as carboxymethylcellulose (CMC) and hydroxymethylcellulose (HMC) as well as on amorph cellulose, but they do not act on crystalline cellulose nor cellobiose. They make up around 20% of the total protein in the complex (Balat, 2011; Mosier et al., 2002; Oliva Martínez et al., 2002; Sun & Cheng, 2002). Exoglucanases slowly hydrolyze D-cellobiose, and 1,4-β-D-glucan cellobiohydrolases, which release D-cellobiose. Cellobiohydrolase act on the non-reducing ends of the chain generated by the endoglucanase, releasing cellobiose molecules. This enzyme has activity on both the crystalline and amorph cellulose. They also have activity on cellodextrins, but they do not act on their substituted derivates nor the cellobiose. Exoglucanases further degrade the sugar chain through the elimination of cellobiose units (glucose dimers) from the free reducing ends. The units of cellobiose produced are later broken into simple units of glucose by the action of β-glucosidases. These enzymes constitute between 50 t0 80% of the cellulolytic complex. The glucohydrolase is often found in small proportion and act on the non-reducing ends releasing glucose units. It has activity on amorph cellulose, cello-oligosaccharides, and CMC (Balat, 2011; Mosier et al., 2002; Oliva Martínez et al., 2002; Sun & Cheng, 2002). Finally, β-glucosidases are very important for completing the depolymerization of cellulose into glucose. The β-glucosidase hydrolyzes the cellobiose, producing monomeric units of glucose, which can be use as carbon source by microorganisms. β-glucosidase

hydrolyzes cellobiose and oligosaccharides and it is absolutely necessary for avoiding a strong inhibition that produce both endo and exoglucanases on the cellobiose if it accumulates in the reaction medium (Balat, 2011; Mosier et al., 2002; Oliva Martínez et al., 2002; Sun & Cheng, 2002) Figure 7 shows the action of cellulase enzymes.

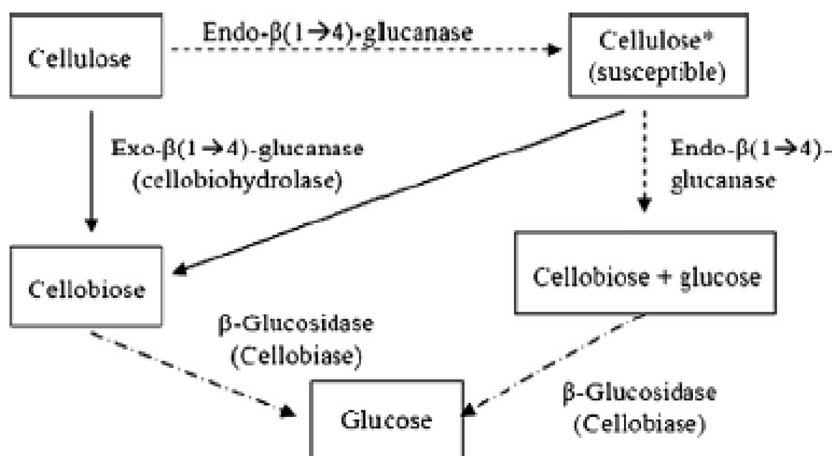

Figure 7. Action of the cellulase enzymes on cellulose (Balat 2011)

Economic Estimation

Biotechnological processes are becoming an attractive new alternative to produce traditional commodities. In this way, single cell organisms arise as an alternative to replace traditional production processes. Moreover, in the field of specialties, single cell organisms are also an alternative source. Therefore, by co-producing bulk and special products it is possible to make these processes economically viable. Moreover, when analyzing the structure of a biorefinery, it is possible to distinguish three main components: upstream processes, cultivation process, and downstream processes. The cultivation process plays a significant role in the economic viability of a biorefinery. The cultivation stage usually accounts for 40 to 50% of the production costs; furthermore, the carbon source represents around half the cost of the medium. Figure 8 shows a simplified overview of a biorefinery, where the first stage (culture and harvest) accounts for 50% of the process cost, from this structure, the low-bulk production of culture derived products; such as proteins, food/feed applications, or biofuels is costly and commonly economically unfeasible (’t Lam et al., 2017).

Currently, this makes the biotechnological production of single cell products costly; and therefore, products from this sector tend to be expensive which hinders their accessibility by large sectors in the population. Despite this situation, it is possible to find in the market a number of products that offer PUFA supplements from biotechnological sources, but they are significantly more expensive than traditional sources of PUFAs, which are mainly

represented in fish capture. A study found that 500 mg of PUFA from fish oil supplement costs US$0.19 while it would be necessary to spend four times more (US$0.44) for plant oil supplements; moreover, microalga oil supplemented products cost between US$3.15 to US$10.40 per 500 mg (Watters et al., 2012).

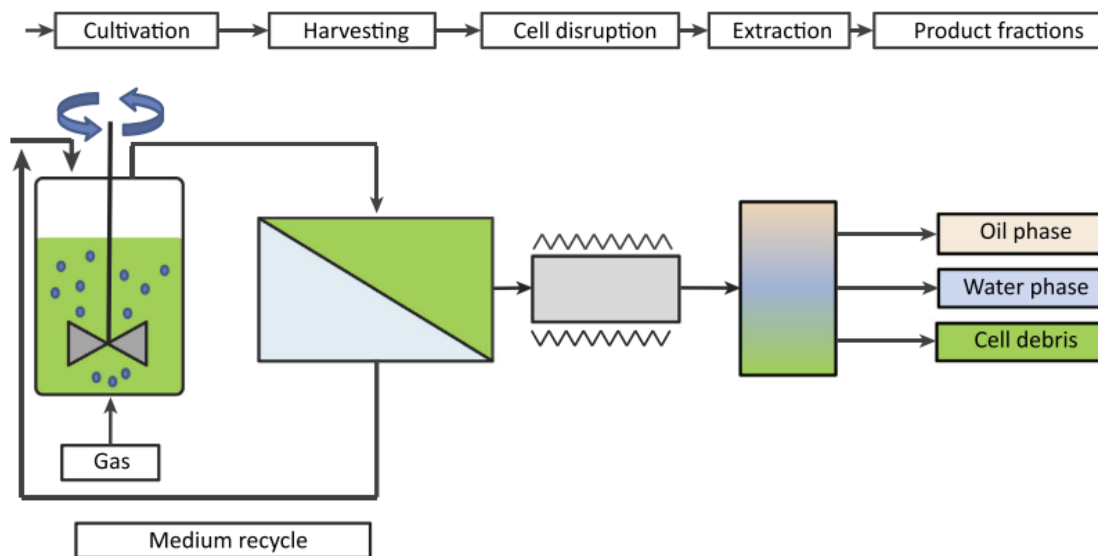

Figure 8. Simplified scheme of single cell organism biorefinery ('t Lam et al. 2017)

With this in mind, an economical estimation for comparing the performance of traditional carbon sources against the performance of non-Conventional Carbon Sources (n-CCSs) is important for establishing the real commercial value of the final cultures. To establish the production cost per unit of product (Cardona et al., 2018), we take the cost of producing 1000 mg of DHA for establishing the production costs, considering the recommended intake of DHA, from each of the media, basal glucose medium and n-CCS media, namely Barley Spent Grain (BSG), Coffee Mucilage (CM)), and Empty Fruit Bunch (EFB).

Chapter 3

Methodology

Alternative Carbon Sources

Previous studies performed at *Bioprocesos* Research Group determined that *Aurantiochytrium limacinum* SR21 grows well in a basal medium that has a glucose concentration of 50g/L; however, using glucose as carbon source has a high impact on the culture costs of glucose based media. Consequently, for reducing the costs of the culture medium, several non-Conventional Carbon Sources (n-CCSs) were considered for achieving this glucose concentration. When selecting the n-CCSs, we took into consideration the following parameters: availability of the product, saccharification possibility, and transportation readiness. With this in mind and based on the group's expertise, five materials were selected for this work. Of these materials, two types of n-CCSs were accounted for: amylaceous (*i.e.*, potato wash and corn kernel wash) and lignocellulosic material (*i.e.*, Barley Spent Grain (BSG), Coffee Mucilage (CM), and Empty Fruit Bunch (EFB)).

The potato wash was obtained from a local potato chips company. The corn wash was obtained from *Universidad de Antioquia*'s Sensory Analysis research group. BSG was obtained from a local beer brewery and CM was donated by a local coffee farm. Finally, the EFB material was donated by the Bioenergía project from RutaN, Medellín, Colombia.

Characterization

After receiving the materials, a characterization was performed for establishing the potential glucose yield by the materials.

The material's composition was characterized following two methods, according to the material's state (*i.e.*, solid or liquid). For assessing the composition of the solid materials, the NREL Determination of Structural Carbohydrates and Lignin in Biomass test was used. For determining the amount of extractives in the samples, 5 g of materials were used following the particle size protocol, the hydrolysis of the sample was carried out with 300 mg of each lignocellulosic material; finally, 1 g o material was used for determining the ash composition (Sluiter et al., 2008).

Both liquid materials (potato and corn kernel washes) were characterized by strong acid hydrolysis as a strong acid hydrolysis has the potential for yielding 100% of the sugars from the polysaccharide materials (Ballesteros et al., 2008). For assessing all the glucose from the available starch, 10mL of each material were loaded into 50 mL flasks. Then, 2 mL of 96% H_2SO_4 were added and the mixture was loaded for 60 minutes digestion under autoclave conditions (121°C and 1.5 atm) (Sun & Cheng, 2005). After the acid hydrolysis

ended, the sample was neutralized using NaOH until pH 7.0. Glucose was determined after the neutralization was completed.

Subsequent to the material characterization and considering the glucose concentration of 50 g/L threshold for further media preparation, at this stage it is necessary to determine which of the materials will continue with the second characterization stage.

For the materials with the potential glucose concentration threshold (50 g/L), a proximate composition analysis is performed. The proximal composition analyses were performed by the *Nutrición y Tecnología de Alimentos* Research Group associated to the Food Engineering department, *Unversidad de Antioquia*. The physicochemical characterization of the n-CCS materials was carried out in accordance with the provisions of the AOAC (Association of Official Analytical Chemists). The moisture content was determined using the methodology established in standard 930.15(AOAC, 2000b), by drying the sample at 105 ° C for 8 hours. The protein was determined by the Kjeldahl method (standard 954.010) (AOAC, 2000a). The ashes were analyzed according to standard 942.05 (AOAC, 1995). Fat analysis was conducted according to standard 920.39 (AOAC, 2003). The carbohydrate determination was performed by subtracting one hundred percent of the previously mentioned components from the sample (Spanopoulos-Hernandez et al., 2010).

The characterization process is shown in Figure 9.

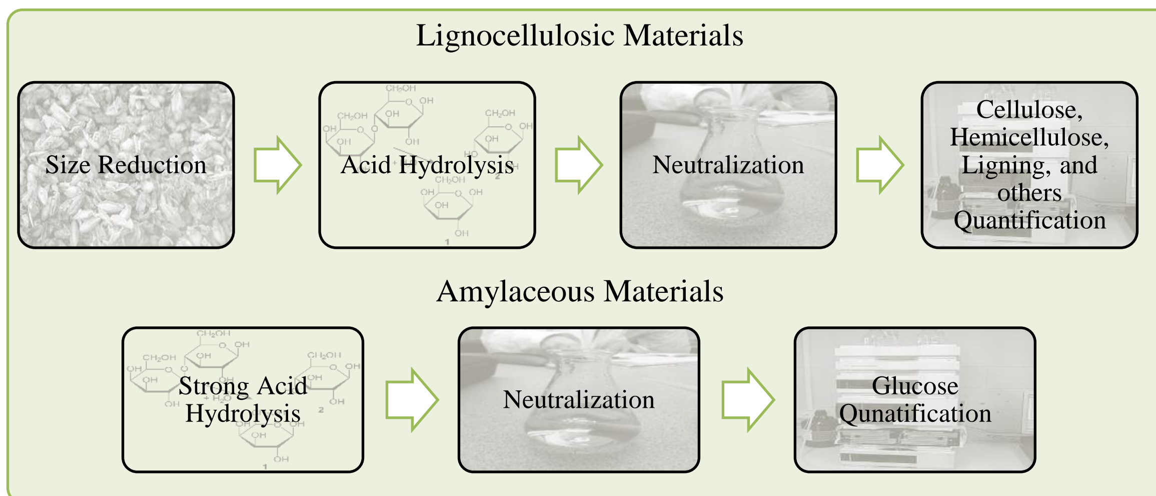

Figure 9. Characterization process of lignocellulosic and amylaceous material

Carbone source conditioning

Amylaceous materials

Given that the characterization of the amylaceous material will provide a precise estimation of the glucose concentration in these n-CCSs no further conditioning will be carried out for the materials that comply with the glucose concentration threshold.

Lignocellulosic materials
Given that glucose is not immediately available for the microorganism in this type of materials, it is necessary to further condition them. For making the cellulose more available from the lignocellulosic n-CCSs, two different pretreatments were selected. The pretreatments were selected based on their capacity to remove the most lignin from the material without damaging the cellulose fibers. These conditions were met by alkaline and LHW pretreatments.

Alkaline pretreatment

This pretreatment was chosen given its efficacy in removing lignin from the lignocellulosic matrix, this is important as lignin can hamper the enzyme's capacity to hydrolyze the cellulose; therefore, lowering the glucose yield after the enzymatic hydrolysis. For the development of this study, other chemical pretreatments were discarded based on the damage that they could cause to the lignocellulosic matrix; the potential risk for producing inhibiting or toxic compounds for the microorganism; or their high cost, which would make them unfeasible to achieve one of the goals of this work.

For making the sugars more available for the hydrolysis step, the alkaline pretreatment was performed for every lignocellulosic n-CCS. The pretreatment was conducted in a 2% NaOH solution, using a liquid to solid ratio of 20:1. The material was then taken to an autoclave pot at 121°C for 1 hour. The remaining liquor was discarded, and the pretreated material was washed twice using tap water and the pH was adjusted for further enzymatic hydrolysis conditions. The material was then dried at 60°C. The material was hydrolyzed after the pretreatment was done and the content of glucose was determined.

Liquid Hot Water (LHW)

LHW was also performed on the lignocellulosic materials as this pretreatment only uses energy and water, at high temperature and pressure conditions, with no further use of chemicals. For this pretreatment, 125 g of each material were loaded into a 1000 mL flask, then 500 mL of distilled water were added. The conditions for the pretreatment were 121°C, 1.5 atm, and 20 min (Cardona et al., 2018). Likewise, the material was hydrolyzed after the pretreatment was done and the content of glucose was determined.

Enzymatic hydrolysis

For the enzymatic hydrolysis, the enzyme CEB3L (Sinobios, Shanghai, China) was used. The activity was determined to be 54 FPU/mL_{enzyme}. Every pretreated material was loaded at 30 $FPU/g_{material}$ and 10 g of material were loaded per 100 mL of solution. According to the manufacturer, this enzyme is recommended to work at a pH of 5.0 for highest performance. Since this would represent an extra cost in the process, two different condition of the enzymatic hydrolysis were tested. In the first condition, we assessed the need to use a buffer solution (sodium citrate) to maintain the pH under control at the given

value of pH 5.0. For the second condition, only distilled water was used, based on the information from the manufacturer, which disclaims that the enzyme performs well in a pH range of 3.5 to 7.0. However, the second condition was only tested when the first condition yielded a concentration of glucose above 50 g/L. This strategy was thought of aiming to lowering the costs of the process. Before adding the enzyme to both processes, the material was adjusted to the enzymatic hydrolysis condition. The pH of the material was adjusted to 7.0 when distilled water was used and, similarly, the pH was adjusted to a value of 5.0 when sodium citrated was used for the enzymatic hydrolysis. The enzymatic hydrolysis conditions were 50°C, 180rpm, and 60 hours. The hydrolysate was then sedimented and centrifuged at 10.000rpm for 5 minutes to recover the sugar-rich supernatant. At the end of the hydrolysis, glucose contend was determined.

After the enzymatic hydrolysis, the characterization tests were newly performed for the materials which conditions yielded a concentration of glucose equal of higher than 50 g/L.

Microorganism and media preparation

Microorganism maintenance

Aurantiochytrium limacinum SR21, ATCC ® MYA 1381, was used for this research work. For preservation, the microorganism was stored at -80 °C in a 790+N medium with 40% glycerol. The storage medium was made of 10 g/L glucose, 2 g/L yeast extract, 1 g/L peptone, and 100% natural sea water. *A. limacinum SR21* was grown at 23°C, and 160rpm. 2 mL vials were used for storage.

Media preparation

In this study, for the preparation of the media only the suitable n-CCSs are considered for assessing microorganism's growth as well as a basal medium using glucose for compering the growth of *A. limacinum* SR21 under standard conditions against all suitable n-CCSs. The n-CCS media are prepared from the best performing materials (*i.e.*, those with 50 g/L of glucose after the pretreatment and enzymatic hydrolysis processes). All n-CCS media are prepared aiming for an initial glucose concentration of 50 g/L, to do so, distilled water is added to adjust the concentration when needed (*i.e.*, for hydrolysates with a concentration higher than 50 g/L). The Carbon to Nitrogen ratio was 25, thus, 22.9 g/L of Yeast Extract were added to all media. Artificial Sea Water (ASW) was used at 50% w/w by adding 18.75 g/L of sea salt into the medium for providing the marine environment. Both Yeast Extract and ASW are added separately; hence, two solutions are prepared containing half the distilled water used for adjusting the concentration. All hydrolysates are autoclaved at 121°C for 20 minutes prior to the hydrolysis step for avoiding denaturalization of the medium after adjusting the glucose concentration; likewise, both Yeast Extract and ASW solutions are autoclaved under the same conditions before being added to the n-CCS hydrolysates. The resulting culture media are handled under sterile conditions hereafter, no further autoclaving is performed for avoiding possible degradation of the glucose from the hydrolyses.

The basal glucose medium is prepared at a concentration of 50 g/L of glucose, 22.9 g/L of Yeast Extract, and 18.75 g/L of ASW, respectively. The medium is autoclaved at 121°C for 20 minutes. Glucose concentration is measured for all media before adding the inoculum.

Culture of **Aurantiochytrium limacinum** *SR21*

For preparing the inoculum, 20 mL of glucose-based medium are prepared in a 100 mL Erlenmeyer flask. The composition of the inoculum medium is 10 g/L glucose, 2 g/L yeast extract, and 1 g/L peptone using 50% natural sea water. The medium is autoclaved at 121°C for 20 minutes. The inoculum is then incubated for 48 hours at 160 rpm and 23°C in an orbital shaker (Thermo Scientific, MaxQ6000).

For assessing the growth of *A. limacinum* SR21 with the potential n-CCS media, two different cultures were carried out. First, a batch culture was performed for assessing the capacity of the microorganism to grow in a medium prepared from the n-CCSs. This culture aims to dilucidated the possible inference of any component left over from the pretreatment that could inhibit the microorganism, in which case the n-CCS would be discarded. The batch cultures were performed in 100 mL flasks with 20 mL of medium inoculated with 3% inoculum. The flasks were place in an orbital shaker (Thermo Scientific, MaxQ6000) at 23°C and 160 rpm for 168 hours. For measuring the growth of biomass, Dry Cell Weight (DCW) was determined at the end of the culture. Glucose was measured at the beginning and end of the culture and nitrogen was not measured as the culture was left long enough to guarantee full nitrogen depletion.

After determining which n-CCS media were apt for the microorganism’s growth, a comparative kinetic study was performed for all suitable media For the culture, every medium was inoculated with 3% v/v inoculum. Afterwards, 250 mL flasks were loaded with 50 mL of medium. Likewise, the flasks were place in an orbital shaker (Thermo Scientific, MaxQ6000) at 23°C and 160 rpm. The culture was carried out for 216 hours to guarantee nutrient depletion (glucose and nitrogen) from the media. Sampling was done every 6 hours on day one, every 12 hours on days 2, and 3, and every 24 thereafter until the end of the kinetic study. Glucose, nitrogen, and DCW were measured at every point.

For the kinetic cultures, all media are prepared and inoculated simultaneously, and cultures take place at the same time for preventing any possible incidence from the inoculum.

Analytical Methods

Glucose and nitrogen determination

For verifying the glucose concentration, a Glucose-oxidase kit is used (BioSystems, Barcelona), where 10 µL of the sample are added to 300 µL of the Glucose-oxidase solution. The mix is left to digest for 10 min at 37°C then the sample is read at 500 nm using a Synergy spectrophotometer.

Nitrogen is measured through a modified YAN method (Muik et al., 2002) where a sample of 400 µL is titrated with a normalized NaOH solution (0.01N). The pH of the sample is previously adjusted to a value of 8.0 using a NaOH solution (0.1N). Then, 2mL of formaldehyde previously adjusted to pH 8.0 are added to the sample for releasing the nitrogen in the sample for its titration.

Hydroxymethylfurfural determination

The hydroxymethylfurfural formed during the strong acid hydrolysis, is determined by HPLC using a Bio-Rad Aminex HPX-87H column with a sample volume of 10 mL, mobile phase 0.005M sulfuric acid, flow rate 0.6 mL/minute, column temperature 65°C, detector temperature close to column temperature and refractive index detector. Liquid samples were neutralized and filtered through 0.25 mm filter before the HPLC analysis (Cardona et al., 2018).

Dried cell weight (DCW)

For assessing the biomass production in the culture, Dried Cell Weight (DCW) was used (Jye et al., 2015). To harvest the biomass, 2 mL are taken from the media, the samples are then centrifuged at 10000 rpm for 10 minutes. The resulting pellet is resuspended using isotonic solution and centrifuged again under the same conditions. The samples are stored at -80°C for 24 hours and then freeze dried for 24 hours. The freeze-dried sample is then weighed.

Lipid extraction

For the lipid extraction, the biomass was previously freeze dried as a oppose to any other drying method for preventing any degradation of the lipids, given that DHA is prone to degrade at 50°C and up. Likewise, for preventing oxidation of the molecule, a nitrogen atmosphere is used throughout the extraction process. For recovering the lipids produced from the culture, a lipid extraction was performed. The extraction followed a modified Bligh and Dyer (Burja et al., 2007) method tuned for the necessities of the culture. For recovering the lipids from the cell, freeze dried cells are subjected to 2 phases.

In the first phase, a tertiary system is stablished using Potassium Phosphate buffer solution (50mM and pH 7.4) / Methanol (99%) / Chloroform (99%) in a 1 / 2.5 / 1.25 ratio. The system is then agitated at 250rpm during 60minutes.

In the second phase, both Potassium Phosphate buffer solution and Chloroform are added so that the final ration of the system is 0.9 / 1 / 1. In this phase the system is taken to a high speed homo mixer at 20,000rpm for releasing the lipids into the solution.

The solution is sat for a minimum of 3hours and the organic phase is then recovered.

For separating the organic phase (chloroform and lipids) the mixture is taken to a rotoevaporator system Rotavapor R-215, Buchi, Switzerland). The system is heated to 55°C, and the Chloroform is distilled at 389mbar of vacuum.

Lipid profile determination

For the lipid composition, the extracted lipid phase from the biomass is taken to a Gas Chromatographer coupled with a Flame Ionization Detection (GC-FID). The lipid in the sample is protected by using a modified nitrogen atmosphere. The GC-FID was equipped with a capillary column and the injector is maintained at 250°C with a sample volume of 1 µL injected at a 50;1 split ratio. Helium is used as the carrier gas with a flow rate of 1.5 mL/min. The oven is programmed between 140°C and 240°C at a rate of 4°C/min. the lipid peaks were identified using Chemstation chromatography software (Agilent Technologies, CA, USA) compared against external standards (Sigma-Aldrich, MO, USA).

The culture process is summarized in Figure 10.

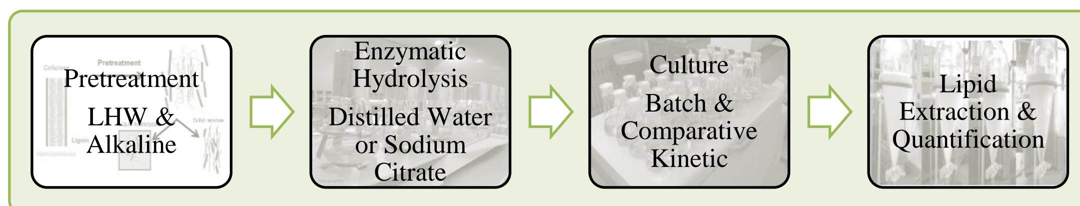

Figure 10. Culture stage of Aurantiochytrium limacinum *SR21 in n-CCS and glucose media*

Economical estimation

This estimation only pretends to compare the production cost of DHA from *Aurantiochytrium limacinum* SR21 accounting for the cost of the carbon source. The economical estimation is based on the cost of the glucose source. To perform the estimation, a mass balance is made for both the pretreatment and hydrolysis process for the n-CCSs that are suitable for producing DHA from the previous stages. The amount of sodium hydroxide and enzyme are also calculated from this mass balance. The cost of the glucose for each material accounted these two variables. Sodium hydroxide cost was obtained from a quotation from a local supplier. The enzyme cost is initially assumed using an enzyme unit price of 4.24 $/Kg protein (Liu et al., 2016). Due to the uncertainty of the enzyme cost, a sensitivity analysis is performed to assess the impact of this unit price on the cost of the n-CCS glucose. The medium cost is calculated as the sum of glucose, yeast extract, sea salt, and water costs. Glucose cost for the basal medium was obtained from USDA as 45.14 cent/lbs or 3000 COL$/Kg. All other reactant prices for the medium were also obtained from a local supplier quotation. After performing the sensitivity analysis on the glucose cost for the n-CCS, the cost for 500 mg of DHA is stablished along a new sensitivity analysis to stablish how the cost from the n-CCS compares to the cost from the basal medium (Klein-Marcuschamer et al., 2012)

Figure 11 presents the process followed for the medium economical estimation.

Statistical analysis

For assessing the number of required assays to be performed to achieve the objectives of the project, the following experimental design is proposed. To determine the non-conventional carbon source that meets the reference conditions of production (CDW, DHA) a Completely randomized One Factor Design is proposed. After obtaining these results, an Analysis of Variance (ANOVA) will be carried out to determine which of the sources is better for producing DHA rich biomass, by determining if there is any significant statistical difference between the means for each level (non-conventional carbon source). Afterwards, a Multiple Range Test (MRT) will be carried out for determining if there is any statistical difference between the levels.

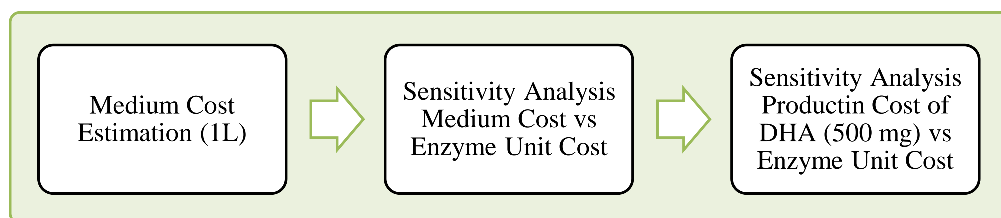

Figure 11. Economical estimation of the medium using n-CCS and glucose for the culture of A. limacinum SR21 towards DHA production

The information will be analyzed through a Hypothesis Test (HT), and a Correlation Analysis will be done in order to stablish if there is a correlation between the CDW and DHA.

Chapter 4

Results

Alternative Carbon Sources Assessment

Characterization

Originally thinking in the reduction of costs of the culture medium and based on the experience of *Bioprocesos* research group, we considered replacing the carbon source for lignocellulosic and/or amylaceous substrates. In this way, within the work network of the group, there are several collaborators for obtaining said substrates. Consequently, from the experience of the group, we opted for working with potato wash and corn kernel wash, as amylaceous sources, and Barley Spent Grain (BSG), Coffee Mucilage CM), and Empty Fruit Bunch (EFB), as lignocellulosic sources; which were initially considered as viable carbon sources for the microorganism. Previous works had shed light on the possible capability for obtaining glucose from BSG, CM, and EFB by using pretreatment and hydrolysis processes. Conversely, the microorganism, according to literature reports, had the capacity of synthesizing an enzymatic arsenal capable of consuming amylaceous materials.

Potato wash from the potato chips industry showed an interesting potential as a starch rich water disposed from the washing of potatoes at the beginning of the process. Similarly, corn kernels need to be washed before entering the *arepa* (a traditional food in the Andean region) production process. BSG is a waste product from the beer brewing industry; CM represents a significant issue for coffee growers, who need to find ways for disposing this waste; and finally EFB is originated in the production of palm oil.

Amylaceous material characterization

After performing a strong acid hydrolysis in both potato and corn kernel washes, for determining the glucose concentration, we found that the content of glucose was significantly lower. Table 1. shows the results from the strong acid hydrolysis of both materials. Considering the glucose degradation to hydroxymethylfurfural, given the conditions of the hydrolysis, a chromatography analysis was carried out for quantifying hydroxymethylfurfural and correcting the sugars value. However, there was not a peak indicating the presence of hydroxymethylfurfural, which indicated that the concentration of glucose was, indeed, very low.

Table 1, Glucose from acid hydrolysis of amylaceous materials, potato and corn kernel washes

Material	Concentration of glucose
Potato wash	< 1 g/L

Corn kernel wash	< 1 g/L

Based on previous results obtained from *A. limacinum* SR21 using glucose as carbon source, we considered working with a concentration of 50 g/L. The sugar concentrations obtained from the amylaceous materials (*i.e.*, potato and corn kernel washes) are very low; hence, further concentration operations would be required, which would increase the power costs, contrary to the aim of these work, making the process economically infeasible. Therefore, these materials were not considered as viable n-CCSs to culture *A. limacinum* SR21, and were discarded; hence, no further analyses were performed on them.

Lignocellulosic material characterization

For assessing the potential of the lignocellulosic materials as carbon source, the materials were initially characterized following the NREL composition assay as shown in Table 2. This characterization was performed on the crude material. From this assay, it is possible to see that both Barley Spent Grain (BSG) and Coffee Mucilage (CM) have a similar percentage of cellulose and hemicellulose, which could be a good source of glucose, their percentage of lignin is also similar. In this regards, Empty Fruit Bunch (EFB) has the highest percentage of both cellulose (37.37%) and lignin (24.37%), which is very promising, but at the same time the high percentage of lignin indicates that the cellulose and hemicellulose would be more difficult to access without any pretreatment on this material. The selection of the pretreatment is discussed in the following section.

Table 2. Composition and proximate composition assays for crude BSG, CM, and EFB

Composition Assay				
Material	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**	**Others* (%)**
BSG	11.92 ± 1.59	17.32 ± 0.51	11.43 ± 2.18	57.06 ± 6.51
CM	10,38 ± 1.87	15.02 ± 0.38	14.28 ± 0.90	66.99 ± 7.64
EFB	37.37 ± 1.72	15.12 ± 2.29	24.37 ± 2.46	24.02 ± 2.74

Proximate Composition Assay					
Material	**Humidity (%)**	**Protein (%)**	**Ash (%)**	**Fats (%)**	**Carbohydrates (%)**
BSG	6.50 ± 0.02	17.56 ±0.16	2.83 ± 0.06	2.83 ± 0.06	70.29 ± 0.15
CM	8.50 ± 0.25	7.86 ± 0.03	10.03 ± 0.02	3.76 ± 0.09	69.85 ± 0.14
EFB	9.04 ± 0.08	6.13 ± 0.03	4.12 ± 0.16	1.84 ± 0.01	78.86 ± 0.08

* This value comprises percentage of humidity, ash, and extractives (w/w).

Following the composition assay on the n-CCSs, a proximate composition assay was performed for assessing the presence of other potential sources of nutrients for the microorganism. This information is also presented in Table 2.

From the assay, it is important to highlight that BSG has a high content of protein (17.56%), the highest of the evaluated materials, which could be an additional source of nitrogen in

the culture of *A. limacinum* SR21.. This assay also shows a homogeneous percentage of carbohydrates, ranging from 69.85% for CM to 78.86 for EFB, the latter agrees with the results of the composition assay wherein EFB presented the a higher percentage of cellulose and hemicellulos when compared to the other materials.

Both characterization assays show how all three materials have the potential be use as substitute of glucose when culturing *Aurantiochytrium limacinum SR21* to produce DHA as they have the potential to yield a glucose rich medium, and it is possible to have several media with an initial load of glucose that would allow for the generation of SCO.

In this context, none of the lignocellulosic material is discarded due to the characterization contrary to the case of the amylaceous materials. all three of the materials move forward to the pretreatment comparison (alkaline against HLW pretreatments) for exposing the cellulosic matrix as discussed in the section below. However, these characterizations do not account for other components that can be produced downstream and that could or could not be harmful to the microorganism.

Pretreatment of the non-Conventional Carbon Sources

Once the materials were characterized, and it was clear that the materials had the potential for yielding the amount of glucose needed for the medium, two different pretreatments were applied on the materials (*i.e.*, alkaline and LHW) The pretreatments were performed prior to the enzymatic hydrolysis –given that the strain of *Aurantiochytrium limacinum* SR21 does not produce enough enzymes to retrieve the glucose from the material by itself– as the main purpose of the pretreatment was to make the cellulose readily available for the enzymatic hydrolysis to recover as much glucose as possible.

Enzymatic hydrolysis of the pretreated materials

Once the pretreatments were performed and the materials dried, the pretreated materials were subjected to an enzymatic hydrolysis for releasing the sugars from the cellulose and the hemicellulose for further use by *Aurantiochytrium limacinum* SR21 towards the production of PUFAs, specifically DHA.

For the enzymatic hydrolysis results, it is important to consider that the enzyme was loaded in excess to all the materials (*i.e.*, an enzymatic load of 30 FPU/g of material was used in all the enzymatic hydrolyses) as an optimization of the enzymatic hydrolysis was not a part of this work; by doing so, we guaranteed the maximum glucose yield possible.

Table 3 presents the results for the enzymatic hydrolyses for all three n-CCS materials under two different pretreatment conditions, LHW and alkaline.

Table 3. Glucose concentration (g/L) after performing hydrolysis on pretreated materials

Material	Pretreatment		
	LHW	Alkaline	
		Citrate solution	Distilled water
BSG	28.7 ± 3.1	57.3 ± 3.9	56.3 ± 1.7
CM	22.9 ± 1.3	70,2 ± 4.4	12.6 ± 0.7
EFB	16.3 ± 2.2	52.2 ± 3.2	56.5 ± 6.3

After the enzymatic hydrolyses were performed, the LHW pretreatment underperformed for all the materials, as no material was able to achieve the 50 g/L threshold. The highest amount of glucose obtained for a material under LHW pretreatment was BSG. This material only managed to retrieve 28.7 g/L of glucose after the enzymatic hydrolysis. EFB had the poorest performance with only 16.3 g/L of glucose after the enzymatic hydrolysis.

It is possible that the conditions used for the LHW pretreatment were not strong enough to release all the fibers from the lignocellulosic matrix, which could explain that EFB yielded the lowest glucose concentration while being the material with the highest percentage of lignin before the LHW pretreatment (24.37%). A previous study of LHW pretreatment on EFB showed that between 75 to 82% of glucose could be recovered when performed at 200°C for 20 min (Cardona et al., 2018). Although the conditions in this word could not be optimal for making the cellulose and hemicellulose fibers fully available, there is an indication that LHW could work for the process. However, stronger conditions would have a direct impact on the process costs and this work focuses on the less costly alternatives; hence, this pretreatment was discarded for further analyses.

Conversely, the alkaline pretreatment had a better performance in the recovery of glucose after the enzymatic hydrolysis. Only CM with distilled water as hydrolysis medium yielded less than 50 g/L after the enzymatic hydrolysis. The concentration of glucose after the enzymatic hydrolysis was well above 50 g/L with all the other evaluated scenarios.

Both BSG and EFB presented no significant difference between the enzymatic hydrolyses using the sodium citrate solution and the enzymatic hydrolysis using distilled water. From this result and accounting that using distilled water instead of sodium citrate buffer solution has a direct impact on the cost for the hydrolysis process, distilled water is selected as the hydrolysis medium for the saccharification of both BSG and EFB.

On the other hand, CM hydrolysis showed to be sensitive to changes in the pH; therefore, sodium citrate solution was selected for performing the hydrolysis of CM. This material also had the highest concentration of glucose at 70.2 g/L. This high yield of post hydrolysis glucose could absorb the effects that the use of citrate could have on the medium cost. This will be assessed later in this work.

Once the alkaline pretreatment was selected as the most suitable pretreatment, the characterization assays were carried out again for elucidating how the materials would change after the pretreatment.

The composition assay after the alkaline pretreatment, presented in Table 4, shows that the pretreatment was capable of disposing the lignin in the materials as intended. The reduction of lignin in the materials was 67.6, 26.2, and 45.1% for BSG, CM, and EFB respectively. From these results, we can say that the pretreatment allowed for exposing the cellulose and hemicellulose fibers for the enzymatic hydrolysis.

Moreover, after assessing the composition for all three materials, it was possible to notice a significant increase of the cellulose content after the pretreatment. This result is encouraging as all of them showed potential to produce more than 50 g/L of glucose. After the pretreatment, BSG increased more than 50% of the cellulose content, CM increased almost by threefold, and EFB increased in 12% the amount of cellulose.

Table 4. Composition and proximate composition assays for pretreated BSG, CM, and EFB

Composition Assay				
Material	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**	**Others* (%)**
BSG	46.22 ± 1.52	33.08 ± 1.43	3.70 ± 0.81	20.18 ± 2.30
CM	58.31 ± 1.11	9.34 ± 0.29	10.54 ± 1.16	21.42 ± 2.44
EFB	39.97 ± 2.61	34.61 ± 0.78	13.39 ± 0.50	14.37 ± 1.64

Proximate Composition Assay					
Material	**Humidity (%)**	**Protein (%)**	**Ash (%)**	**Fats (%)**	**Carbohydrates (%)**
BSG	3.39 ± 0.07	4.18 ± 0.02	6.93 ± 0.09	2.45 ± 0.03	83.04 ± 0.01
CM	6.09 ± 0.12	1.01 ± 0.01	3.40 ± 0.15	0.99 ± 0.01	88.51 ± 0.11
EFB	8.34 ± 0.21	1.54 ± 0.03	1.39 ± 0.06	0.67 ± 0.02	88.05 ± 0.17

* This value comprises percentage of humidity, ash, and extractives (w/w).

Similarly to the composition assay, the proximate composition assay was also performed again on the alkaline pretreated materials These results are also presented in Table 4. It is important to highlight that BSG has a highest content of protein both for the crude (17.56%) state and after being pretreated (4.18%). Also, it is important to notice how the fat content did not change significantly after the pretreatment, going from 2.83% to 2.45%. As previously mentioned, these results could be an indication that the remaining protein in the pretreated BSG could be an additional source of nitrogen in the culture of *A. limacinum* SR21; however, since the aim of this project is to evaluate the effect of n-CCSs, further studies should look into this.

Conversely, both CM and EFB were almost completely stripped from their nutritional value, in the form of protein and fats, after the alkaline pretreatment was performed. CM lost little more that 87% of its proteins and EFB lost close to 75%. In this regards, it is

important to highlight that, even though the amino acids could have been present after the pretreatment, the washes that are performed after pretreating the materials would have rinsed any free amino acid from the material. Regarding fat content, CM had the most at 3.76%; however, it lost 74% of its content. Similarly, EFB lost 64% of its fat percentage.

Regarding the loss of proteins and fats due to the pretreatments, which were intended for facilitating the saccharification of the materials, some studies have focused on the use of n-CCSs as nitrogen source, to do so, variation on the pretreatments are included aiming to protect the nitrogen sources disregarding the potential as carbon source. Hence, the final use of the material as carbon or nitrogen source plays an important role when defining the pretreatment characteristics. Consequently, a study using the same LHW conditions used in this study for pretreating BSG as nitrogen source found that, under this conditions, it was possible to use the hydrolysate as nitrogen source by achieving 262.8 mg/L of ammoniacal nitrogen; however, glycerol had to be used as carbon source for achieving a C:N ratio of 20 (Ryu et al., 2013).

Culture of non-Conventional Carbon Sources with Aurantiochytrium limacinum SR21

Amylaceous culture

For evaluating the capacity of *Aurantiochytrium limacinum* SR21 for growing in amylaceous media, a preliminary culture was done using both potato and corn kernel washes directly as medium solution, both supplemented with artificial sea salt and yeast extract. No *A. limacinum* SR21 growth was evidenced in these cultures.

It is important to highlight that both potato wash and corn kernel wash were originally selected considering the capacity of Thraustochytrids to metabolize amylaceous materials (Honda et al., 1998). However, as further studies demonstrated, not all Thraustochytrids have the same capabilities to produce the necessary enzymatic arsenal for hydrolyzing complex materials. This is the case of *A. limacinum* SR21, which lacks the enzymatic arsenal for hydrolyzing starch-based materials as opposed to *Schizochytrium limacinum* SR21 (Yokoyama & Honda, 2007). In the early stages of this work, *S. limacinum* SR21 was considered to be the strain used in the study. However, it was later stablished that it was not the case, and that *A. limacinum* SR21 was the actual strain being used. Consequently, it was possible to understand why the microorganism did not grow in the medium when using amylaceous materials as originally expected.

Similarly, a recent study shows that *A limacinum* SR21 has a reduced capacity for cellulose and hemicellulose degradation (Song et al., 2018). Thus, lignocellulosic materials could not also be directly used by the microorganism, and a previous step are needed for releasing the sugars into the culture media as shown above.

Lignocellulosic cultures

After determining the amount of glucose that was possible to recover from the different pretreated n-CCSs, several batched were prepared (*i.e.*, from basal glucose medium and n-CCS hydrolysates) for testing the capability of *A. limacinum* SR21 to grow in these environments. As from the pretreatment results, most of the lignocellulosic materials reached the threshold for moving into this culture stage when going under alkaline pretreatment, the batch culture was carried out for the materials and conditions that obtained a 50 g/L glucose concentration or higher –as presented in Table 3–; namely, BSG and EFB under distilled water; and BSG, CM, and EFB under citrate solution. LHW pretreatment does not meet the minimum of glucose concentration for any of the materials; thus, no medium for batch culture was prepared from this pretreatment nor further analysis.

For preparing the batches, all the lignocellulosic materials were hydrolyzed, and different media were prepared from the hydrolysates for comparing the growth of the microorganism in the basal medium against all other n-CCS media. To this end, the batch culture media are named after the hydrolysis solutions. The media prepared from alkaline pretreatment and sodium citrate solution hydrolyses are referred to as *Citrate solution*, likewise, those from alkaline pretreatment and distilled water hydrolysis are referred to as *Distilled water*.

***A. limacinum* SR21 growth under BSG hydrolysate culture**

After assessing the growth of *A. limacinum* SR21 when cultured in a medium prepared using BSG hydrolysates, which were obtained from two different solutions (one media was obtained using a sodium citrate buffer hydrolysate and the other using a distilled water hydrolysate), both obtained from alkaline pretreatment; we compared the final biomass production (DCW) for elucidating, in terms of biomass, how different or similar the microorganism grew. The biomass was left to grow for 7 days to guarantee both nitrogen depletion from the medium and the end of the exponential growth phase for the microorganism. The comparative results are shown in Figure 12. The comparative parameters were: initial glucose concentration, final glucose concentration, and biomass accumulation. All the parameters were accounted for the hydrolysate media against the basal medium prepared from pure glucose.

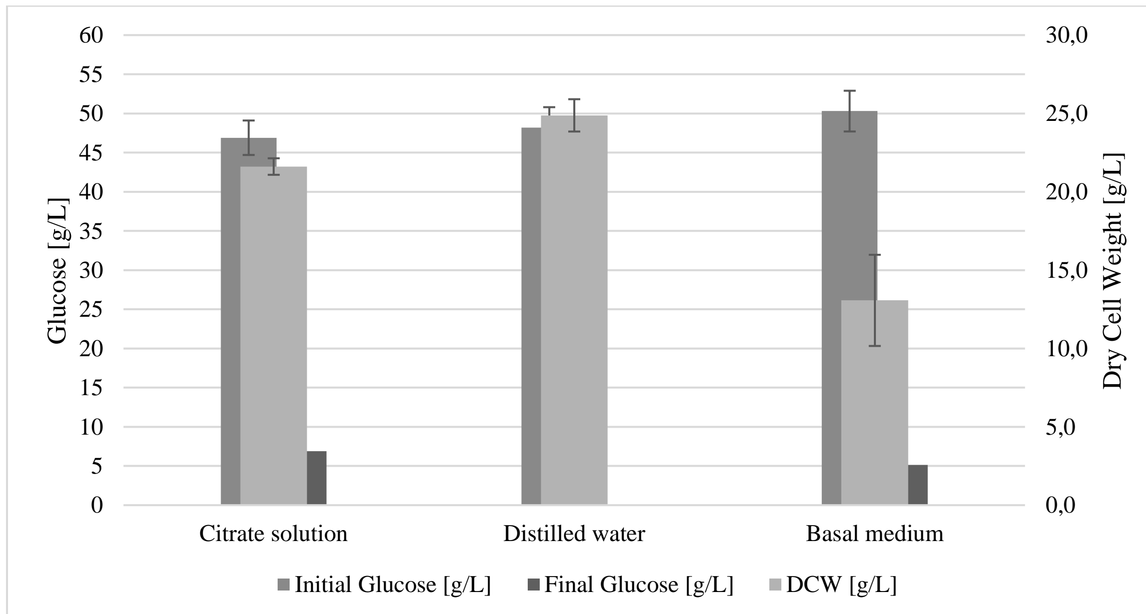

Figure 12. Culture using A. limacinum *SR21 with BSG hydrolysates using buffer solution and distilled water against basal glucose medium. Culture conditions: 23°C, 160 rpm, for 7 days*

From Figure 12 we can see that the biomass growth of *A. limacinum* SR21 with the BSG hydrolysate was significant higher when compared to the growth of the basal medium. The glucose residual was also noticeably lower when distilled water was used. A residual of glucose is expected given that the microorganism should use this residual for further transformation into lipids during the stationary stage; nevertheless, this discussion is beyond the scope of this work. As mentioned above, the assumed presence of extra components, which can act as nutrient source, from the BSG hydrolysates such as proteins and fats, can contribute to the continuous growth of *A. limacinum* SR21 beyond the expected nitrogen depletion point, which would be expected to cause a shift in the microorganism's metabolism from growth to lipid production. Nevertheless, it is important to point out that provided the microorganism actually consumes nitrogen from the hydrolysate, this would shift the C:N ratio with a direct impact on the depletion of the nutrient and the growth of the microorganism. It is also important to point out that, initially, all n-CCSs were considered solely as carbon source, their potential as nutrient source was not considered; furthermore, to the best of our knowledge, the carbon to nitrogen ratio has been widely studied and defined as an important factor in the high yield of lipids from Thraustochytrids in general (Huang et al., 2012; Yokochi et al., 1998). However, this literature mainly focuses on the study of the carbon to nitrogen ratio when using glucose as carbon source in the medium. Comparative studies on the carbon to nitrogen ratio for n-CCSs are scarce; nevertheless, a study on the use of BSG as nitrogen source that evaluated the C:N ratio found similar results to this study, meaning, when the nitrogen concentration on the medium increased, the biomass concentration also increased (Ryu et al., 2013). However, we did not find a comparative study for n-CCS against glucose medium, but

independent studies show that even when n-CCSs are said to have the same concentration for both materials (*i.e.*, 20 g/L for glucose and n-CCS), a similar behavior occurs. This behavior carries onto the other n-CCSs

With these comparative results it is possible to say that *A. limacinum* SR21 grows adequately when cultured in the BSG hydrolysate medium. Therefore, BSG hydrolysate medium could be a substitute for glucose when growing *A. limacinum* SR21 towards the production of DHA.

A. limacinum **SR21 growth under CM hydrolysate culture**

The same comparison was performed for the CM medium (sodium citrate medium was not considered as it did not meet the glucose concentration threshold). The results from this comparison are shown in Figure 13. In this figure, it is possible to see that, again, the microorganism obtained a higher biomass concentration, this could be explained by the presence of extra nutrients in the medium as presented by the bromatological study where, though in a lesser amount compared to BGS hydrolysates, there is a residue of proteins and fats.

Furthermore, the glucose residual was under 1 g/L of glucose for the CM hydrolysate at 0.05 g/L after the 7 days of culture. Hence, most of the carbon source was used for the microorganism for growing and incorporated in its metabolism.

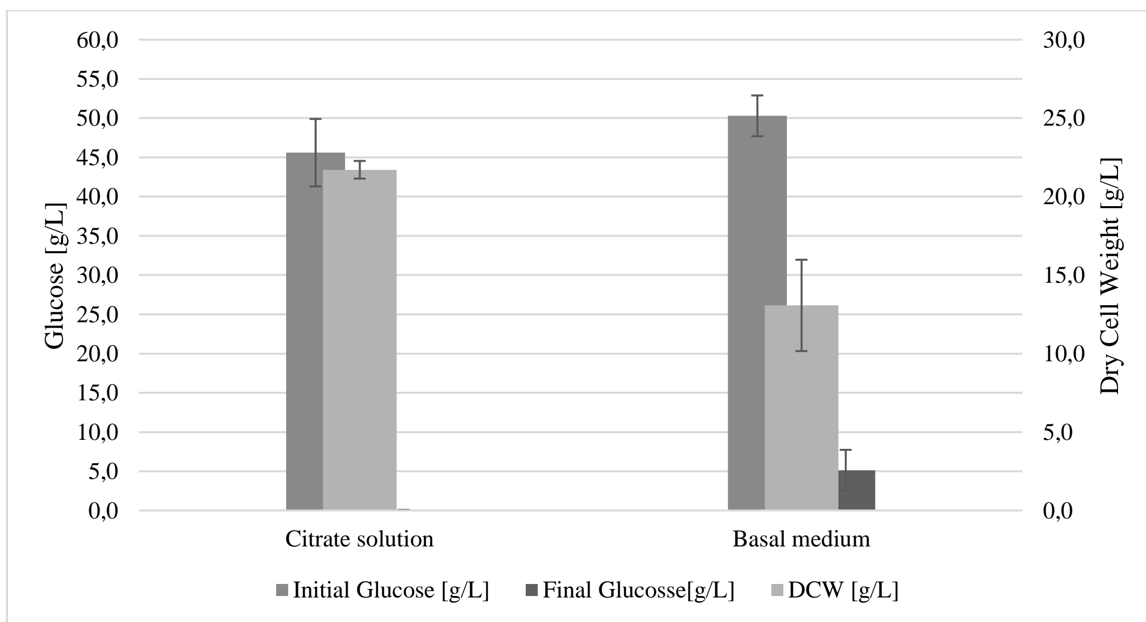

Figure 13. Culture using A. limacinum *SR21 with CM hydrolysates using citrate solution against basal glucose medium. Culture conditions: 23°C, 160 rpm, for 7 days*

Similarly, to BSG, CM hydrolysate showed to be a suitable growing environment replacement for pure glucose as a carbon source in the culture of *A. limacinum* SR21.

A. limacinum **SR21 growth under FEB hydrolysate culture**

Finally, the same culture comparison was conducted to determine how *A. limacinum* SR21 behaves when glucose from EFB hydrolysates is used in the culture. In this regards, Figure 14 presents the results for the cultures for both Citrate solution EFB medium and Distilled water EFB medium against the basal medium using glucose.

The culture of EFB showed a similar behavior to the basal medium when distilled water hydrolysates was used. In this case, 12.27 g/L of biomass were produced from the EFB hydrolysate against 10.53 g/L from the basal medium. On the contrary, in this comparison, the growth of *A. limacinum* SR21 when the citrate solution was lower than the growth from the basal medium, this was the only case where this behavior could be seen. All other cultures exceed the biomass production from the basal medium.

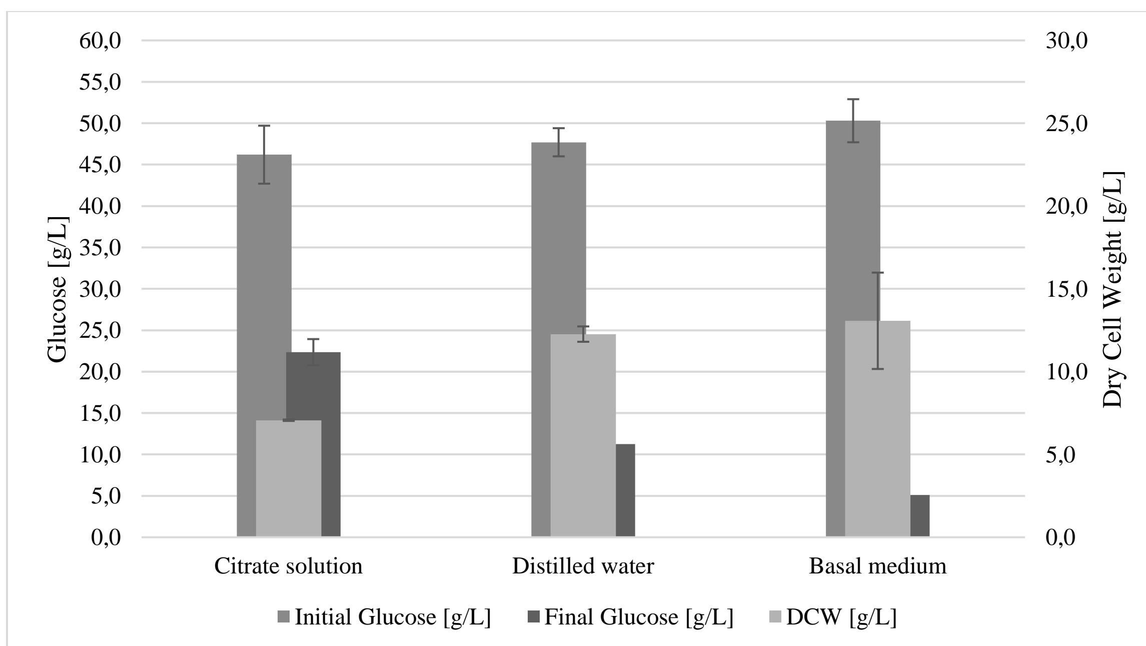

Figure 14. Culture using A. limacinum *SR21 with EFB hydrolysates using buffer solution and distilled water against basal glucose medium. Culture conditions: 23°C, 160 rpm, for 7 days*

Also, when using EFB hydrolysates, all cultures had a residual of glucose after performing the cultures unlike the other two materials, which were able to deplete all the glucose available in the medium. In this regard, the Citrate solution had the highest glucose residual and consequently the lowest biomass concentration; hence, the Distilled Water EFB hydrolysate is the one that better acts as a replacement for glucose.

With these results, all three hydrolysates from these non-Conventional Carbon Sources, Barley Spent Grain, Coffee Mucilage, and Empty Fruit Bunch, could be used as replacement for glucose when growing *Aurantiochytrium limacinum* SR21 towards the production of Docosahexaenoic acid. From this comparison, we establish the conditions to perform the comparative kinetic study. Table 5 summarizes the selected conditions as well as the parameters that were obtained for the materials.

Table 5. Conditions selected for kinetic study and conditions after culture

Material	***Pretreatment***	***Hydrolysis***	***Residual Glucose [g/L]***	***DCW [g/L]***
BSG	*Alkaline*	*Distilled water*	*0.1 ± 0.0*	*24.9 ± 1.0*
CM	*Alkaline*	*Citrate solution*	*0.0 ± 0.0*	*21.7 ± 0.6*
EFB	*Alkaline*	*Distilled water*	*11.2 ± 1.7*	*12.3 ± 0.5*

Comparative Kinetic Study

After the type of hydrolysis for each of the hydrolysates were stablished, a kinetic study was performed, which allowed us to compare the growth of the microorganism under the selected conditions towards the production of lipids and finally the assessment of DHA.

Considering that all media were prepared simultaneously as well as the cultures, any interference from the inoculum is prevented. Hereafter, a comparison between each material and the basal medium is presented, and at the end, a global comparison will take place in terms of the lipid accumulation and DHA percentage to contrast against the results from the medium using glucose.

In this way, we expect to select the better material for replacing glucose as carbon source in the culture of *Aurantiochytrium limacinum* SR21 for producing SCO rich in DHA.

BSG Kinetic study

For the Barley Spent Grain (BSG) kinetic study, BSG was hydrolyzed using distilled water for lowering the process costs; in agreement with the previous results that showed how using distilled water had little impact on the saccharification of the material as previously discussed.

The results from this kinetic study comparison are shown in Figure 15. In this case, it is important to remark that the initial concentration of glucose after preparing the medium was of 60.5 g/L. This happed as the medium was prepared from a dilution process from the glucose concentration obtained from the enzymatic hydrolysis, and further adjustment of the medium was not possible. The glucose concentration of the BSG hydrolysate was 67.6 ± 6.8 g/L. Conversely, the glucose medium was prepared *in-situ*, with a fixed concentration of glucose of 50 g/L and a carbon to nitrogen ratio of 1:25.

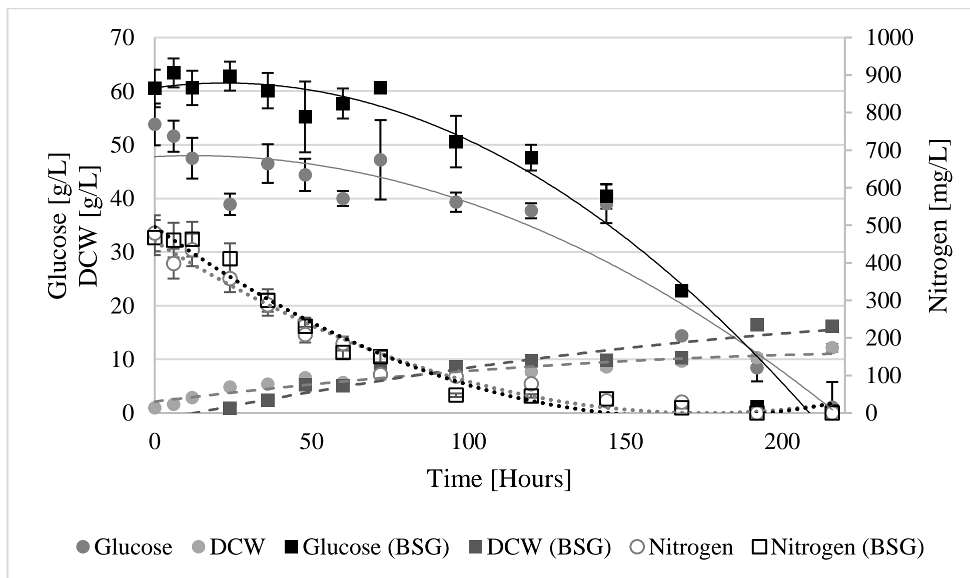

Figure 15. Kinetic study comparison between BSG medium and glucose medium. Culture conditions: 23°C, 160 rpm, for 216 hours

In this case, the conversion of glucose into biomass followed a similar trend in both media. The biomass yield on glucose for the basal medium was 0.213, which was 31.0% lower than the biomass yield on glucose for the BSG medium (with a value of 0.279). These results indicate that more glucose was transformed into biomass in the BSG hydrolysate medium than in the basal medium.

The higher rate of biomass production can also be seen in the depletion of nitrogen from the medium where nitrogen was depleted from the BSG medium after 140 hour. Similarly, nitrogen was completely consumed from the glucose medium after 165 hour. Moreover, the transformation of glucose into biomass happened at a slower rate in glucose medium than in the BSG medium. Given that nitrogen was depleted from the BSG before the glucose medium, one might be inclined to proposed that lipid accumulation would be higher in the hydrolysate medium; however, when comparing the total percentage of lipid accumulation (42.69% of DCW in the basal medium and 17.94% of DCW in the BSG medium) it is clear the carbon is transformed into lipids for the glucose medium whereas

the n-CCS medium continues to transform carbon into biomass, even after nitrogen has been depleted.

This phenomenon can be explained by the presence of other sources of nutrient in the BSG medium different from the yeast assimilable nitrogen, which is the one measured in this study, as we have suggested above by the proximate composition analysis of the materials. This represents a difficult scenario as it is quite difficult to precisely predict the real carbon to nitrogen ratio when using a n-CCS under the methodology proposed for the study. Moreover, the Kjeldahl method used in the proximate composition analysis measures total nitrogen regardless of its procedence. Therefore, it is not possible to determine the nitrogen available for the microorganism within the n-CCSs. Nevertheless, given the nitrogen consumption curves, it is possible to infer that BSG has a higher amount of available nitrogen than the measured for the YAN method used during this stage as it is specific for nitrogen associated to yeast extract, which was supplemented to the medium.

Additionally, when considering the biomass productivity in both cultures, glucose and BSG, is more than twofold. The biomass productivity of the basal medium was 0.051 g/L/h against 0.108 g/L/h for BSG medium. Therefore, the kinetic parameters (the kinetic parameters are listed for all the cultures in Table 6) confirm that *A. limacinum* SR21 continues to grow after nitrogen has been depleted in the BSG medium. This affects the accumulation of lipids. However, the higher amount of biomass with a lower accumulation could compensate for the total amount of lipids produced, expressed as Total Fatty Acids (TFA), presented in the Table 7 enclosed in the following section (*i.e.*, Lipid profile). Nevertheless, thought the total amount of lipids can be similar in both cultures (5.19 g/L for basal medium and 2.90 g/L for BSG medium), the extraction costs would be higher for the BSG medium due to the higher amount of biomass to be processed.

Table 6. Kinetic parameters for the kinetic study of *Aurantiochytrium limacinum* SR21 using 4 different carbon sources: Glucose, hydrolyzed BSG, hydrolyzed CM, and hydrolyzed EFB

Kinetic parameter	**Medium**			
	Glucose	**Hydrolyzed BSG**	**Hydrolyzed CM**	**Hydrolyzed EFB**
$Y_{X/S}$	0.213	0.279	0.287	0.228
$Y_{P/S}$	0.091	0.050	0.029	0.060
μ_{max} [h^{-1}]	0.004	0.007	0.007	0.011
Productivity [g/L/h]	0.051	0.108	0.115	0.122

Finally, as the scope of the culture is to obtain DHA, for its nutraceutical properties, rather than biomass; a lipid profile characterization was performed. These results are presented in the following section.

CM Kinetic study

Coffee Mucilage (CM) was hydrolyzed using sodium citrate for performing the enzymatic hydrolysis unlike BSG and EFB hydrolysates. After the medium was prepared, the initial concentration of glucose was 56.9 g/L. Figure 16 shows the behavior for the growth of *Aurantiochytrium limacinum* SR21 when using CM against the basal glucose medium. After growing both cultures, the biomass yield on glucose for CM was 0.287, which is even higher (34.7% higher) than the one obtained by the BSG medium. This indicates that when using CM as material more carbon was transformed into biomass.

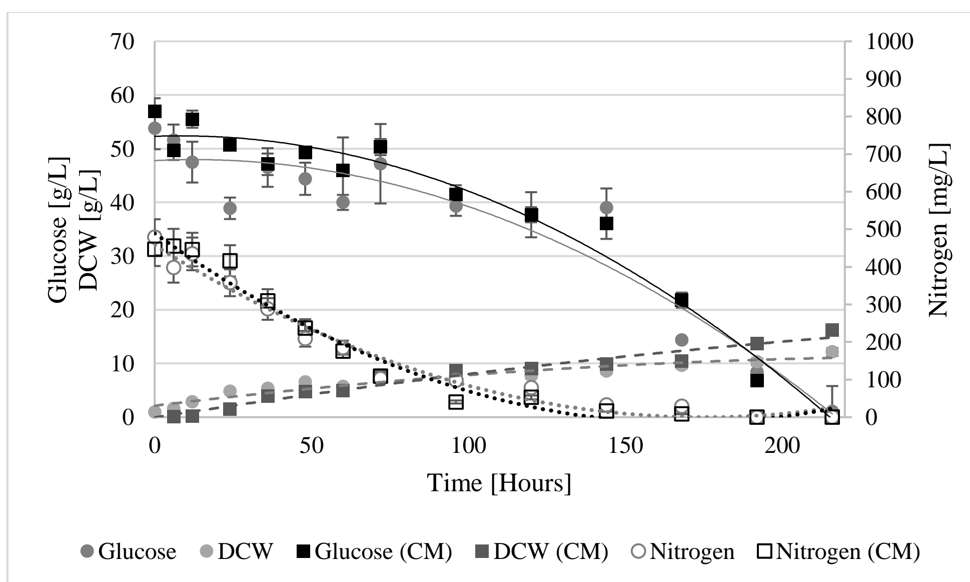

Figure 16. Kinetic study comparison between CM medium and glucose medium. Culture conditions: 23°C, 160 rpm, for 216 hours

In this regard, the CM medium has a similar behavior compared to the BSG medium, both media transform more glucose into biomass than the basal glucose medium. However, the proximate composition analysis did not show a significant amount of possible extra nutrient sources for the pretreated CM that could be used by *A. limacinum* SR21 as nutrient sources. A better characterization of the material would shed light into this behavior with the microorganism.

Regarding nitrogen depletion, this medium depleted nitrogen from the culture sooner than the basal medium. Nitrogen depletion happened after 140 hours, similarly to BSG medium. Accumulation of lipids in the CM medium was 9.94% of DCW, the lowest for all the media

that were prepared. However, it had the highest biomass accumulation at 16.2 g/L of DCW. This result agrees with what was found with the BSG medium where we presumed that glucose is being transformed into biomass instead of lipids despite the depletion of measurable nitrogen from the media. Again, this finding could be attributed to the approach of the different methods used for measuring nitrogen in the culture (*i.e.*, YAN) and the estimation of the nitrogen in the proximate composition analysis (*i.e.*, Kjeldahl).

As far as biomass productivity, CM had a value of 0.115 g/L/h, which was higher than the productivity of BSG. However, the higher biomass productivity contrasted against the lower lipid yield does not compensate the total lipid accumulation. In this regard, CM manages to obtain a substrate to product yield of 0.029, which corresponds to a production of 1.610 g/L of lipids. This concentration of lipids was also the lowest for all the tested media.

EFB Kinetic study

When preparing the medium based on Empty Fruit Bunch (EFB), the enzymatic hydrolysis yielded 62.2 ± 3.6 g/L of glucose, after diluting the hydrolysate for preparing the medium, the concentration was 58.6 g/L. This medium was also complemented to a carbon to nitrogen ration of 1:25 for assuring nitrogen depletion. The kinetic study is presented in Figure 17.

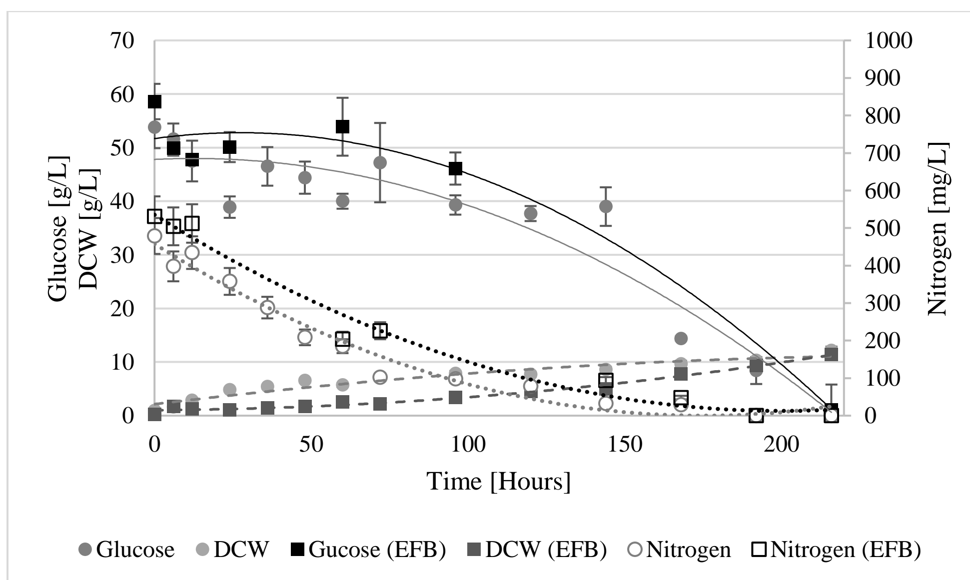

Figure 17. Kinetic study comparison between CM medium and glucose medium. Culture conditions: 23°C, 160 rpm, for 216 hours

For the EFB hydrolysate, the biomass yield on glucose was significantly lower, 0.228, but closer to the glucose medium $Y_{X/S}$ (0.213), this could be explained by the presumption that the EFB medium lacks the complex composition, as presented by the proximate composition analysis, that can incorporate extra nutrients into the medium. In this regard, the EFB medium had a closer behavior to the glucose medium when comparing the biomass production (11.38 g/L for the EFB medium against 12.15 g/L for the basal glucose medium).

Concerning the consumption of nitrogen from the medium, it stands out that the consumption rate of nitrogen was slower for the EFB medium than the other prepared media. Figures 12 and 13 show how the consumption of nitrogen for the glucose, BSG and CM happens at a faster rate that the consumption of the nitrogen in the EFB medium (Figure 17). However, nitrogen depletion happened after 200 hour of culture, significantly later than the 165 hours when nitrogen was depleted from the basal glucose medium, and even more than the 140 hours needed by the BSG and CM media for depleting the nitrogen from the media. This could explain the lesser production of biomass. The concentration of biomass from the EFB medium was 29.75, which is lesser than the CM medium, 29.54% lesser than the BSG medium, and 6.34% lesser than the basal glucose medium. When looking at the amount of additional nitrogen in CM and EFB after the pretreatment, we can see that the estimated protein is similar in both materials; however, given the culture behaviors, we can also infer that the nitrogen available for *A. limacinum* SR21 could be higher for CM than EFB.

Nevertheless, the apparent lack of extra nutrients in the medium from the proximate composition analysis favors the conversion of glucose into lipids. EFB presented the second higher lipid accumulation at 26.31% DCW just behind the basal medium (42.69% DCW). Therefore, EFB had the second TFA accumulation by yielding a total of 2.994 g/L of TFA.

It is also important to highlight that the EFB medium had the highest maximum growth speed at 0.011 h^{-1} with a biomass productivity of 0.122 g/L/h compared to 0.051 g/L/h for glucose. This result is a little contradictory given the low accumulation of biomass in the medium.

Lipid profile

After performing the kinetic studies with the best-found conditions for the studied n-CCS materials and extracting the lipids from the produced biomass, a lipid profile was performed for better understanding of the culture. For avoiding the oxidation and further degradation of the lipids, a nitrogen atmosphere was used throughout the extraction process. The recovered and freeze-dried biomass from all four media (basal glucose medium and n-CCS BSG, CM, and EFB media) prevented any degradation of the lipids in the biomass due to temperature. The characterization was made with the intention of

assessing the influence of the n-CCS media on the DHA accumulation in the microorganism.

In this way, the Total Fatty Acid (TFA) percentage, which were present in the biomass produced from the media, were determined from the solvent extraction (using chloroform and methanol) these results are presented in Table 7.

Table 7. Total fatty acid percentage in biomass and total fatty acid yield

Hydrolysate	TFA percentage in biomass	TFA yield [g/L]
Glucose	42,69%	5.187
BSG	17.94%	2.897
CM	9.94%	1.610
EFB	26,31%	2.994

It is important to highlight that the accumulation of Total Fatty Acids (TFA) in each of the n-CCS culture media is lower than the accumulation for the glucose medium. These results could be explained considering that the media from the n-CCSs can have a lower carbon to nitrogen ratio to the one initially estimated by incorporating additional nitrogen sources available to the microorganism. Lowering the carbon to nitrogen ratio favors the growth of the microorganism; nevertheless, a higher biomass production reduces the amount of carbon available for the synthesis of lipids by postponing the depletion of nitrogen from the medium (Delgado Naranjo et al., 2017). Consequently, there is a lower accumulation of lipids as evidenced in Table 7. EFB accumulates 38% less than the glucose medium; BSG and CM accumulate around 58% and 77% less, respectively. This TFA reduced accumulation agrees with the apparent availability of nitrogen in the different n-CCS materials associated to the biomass growth, where CM yielded the highest biomass production, followed by BSG and EFB.

Once the lipids were extracted from the biomass, the recovered lipid fraction was characterized using Gas Chromatography (GC), the results from the characterization as listed in Table 8 for all the media.

Table 8. Fatty acid composition of Aurantiochytrium limacinum SR21 when cultured under different n-CCS hydrolysates and the basal glucose medium

Triglyceride	Non-Conventional Carbon Source			Glucose
	BSG	CM	EFB	
tri dodecanoic	0.27	0,37	0.43	0,52
tri tetradecanoic	2.68	2,64	0.76	1,13
tri pentadecanoic	12.86	11,38	15.06	12,93
tri hexadecanoic	30.57	24,46	9.78	17,22

tri heptadecanoic	2.98	3,38	4.68	3,70
tri octadecanoic	0.99	1,78	0.74	0,60
tri oleic	0.68	0,00	3.51	0,38
tri linoleic	0.00	0,00	0.46	0,00
tri eicosanoic	0.13	0,00	0.18	0,25
C20:5 (EPA)	1.78	11,99	2.15	0,94
C22:5	8.60	7,92	10.48	10,12
C22:6 (DHA)	**38.30**	**36,46**	**51.77**	**52,46**

As shown in Table 8, it is possible to see that DHA was the most accumulated fatty acid in all the media. The glucose medium accumulated 52.46% TFA in DHA for a total DHA yield of 2.721 g/L.

Similarly, the BSG medium accumulated 38.30% TFA in DHA with 1.110 g/L of DHA in the culture. The basal medium produces a larger amount of DHA than this n-CCS medium. For the CM medium, Table 8 shows that this medium accumulated 36.46% TFA in DHA. The total DHA yield for this culture was 0.587 g/L. With this perspective, CM presented the lowest values for lipid and DHA accumulation, which is the main scope of the *Aurantiochytrium limacinum* SR21 culture. Lastly, when analyzing the accumulation of DHA for the EFB medium, Table 8 shows that the DHA accumulation was 51.77% TFA. This was the second higher accumulation of DHA, only 1.32% lesser than the DHA accumulated with the basal glucose medium when studying the lipid profile. The DHA accumulation of the EFB medium represented a total yield of DHA of 1.550 g/L against 2.721 g/L of DHA produced in the glucose medium. Therefore, this medium was the closer to the production of total DHA when compare to the production of the BSG medium (1.110 g/L) and the production of the CM medium (0.587 g/L).

From the characterization results, it is possible to infer that the medium, in which the hydrolysis takes place, influences the lipid profile, the CM medium, which was the only one that used sodium citrate as hydrolyzing medium, has a higher production of Eicosapentaenoic Acid (EPA) with 11.99% TFA as opposed to the 1.78% TFA from the BSG medium and 2.15% TFA from the EFB medium. The accumulation of EPA by the basal glucose medium is significantly lower at 0.94% TFA. These results agree with the literature, where *Aurantiochytrium limacinum* SR21 is not considered a source of EPA. Some studies show that the concentration of EPA in *A. limacinum* SR21 is $<1\%$ TFA when grown in glucose, and a little more than 1% TFA when grown using n-CCSs (Gao et al., 2013; Gupta et al., 2012).

The lipid characterization can shed light into the metabolic pathway that *Aurantiochytrium limacinum* SR21 uses to accumulate PUFAs. Even though, it is still not fully understood what is the metabolic pathway used by microorganism, the FAS and PKS pathways are thought to be the ones used, or at least a combination of both (Song et al., 2018). The high accumulation of palmitic acid (30.57% TFA for BSG, 24.46% TFA for CM, and 17.22% TFA for the basal medium) in three of the four medium tested, which is the second

most accumulated acid in all the media excepting EFB, could indicate that the FAS pathways (which starts with the elongation of palmitic acid into stearic acid) is being used by *Aurantiochytrium limacinum* SR21 for the synthesis of DHA. The second most common compound for the EFB medium was the pentadecanoic acid with 15.06% TFA.
Overall, it is important to point out that the most accumulated PUFA in all the media was DHA. This result was expected as Thraustochytrids, and *Aurantiochytrium limacinum* SR21 in particular, are known as great producers of PUFAs with high accumulation of DHA. In this way, the basal glucose medium accumulated the most amount of DHA with 52.46% TFA followed by the EFB medium, this medium accumulated 51.77% TFA in DHA.

EFB has extensively been studied as a carbon source for different purposes and with different microorganisms. In this way, a study using *Aurantiochytrium* sp KRS101 found that, when grown from EFB from the palm oil industry, the microorganism was able to accumulate 0.90 g/L of lipids with 58.1% TFA of DHA (Hong et al., 2012). These results are not far from the results from this work regarding DHA accumulation where *A. limacinum* SR21 accumulated 2.994 g/L with 51.77% TFA; furthermore, lipid accumulation was significantly higher in this study. This can be a result of the medium composition, which is not presented in the study, and the culture time. Their culture time was lesser than the time used in this study (96 hour against 216 in this study), which should influence the time the microorganism has to accumulate lipids.

The other two media, BSG and CM accumulated similar amounts of DHA. BSG accumulated 38.30% TFA in DHA; this result is similar to previous studies done using BSG hydrolysates but using another Thraustochytrid, in this study, *Aurantiochytrium* sp KRS101 is used. In this study, which uses BSG as a substitute for nitrogen instead of carbon, they obtained 18.95 g/L of biomass with 34.04% TFA of DHA. These results could indicate that the use of use of BSG hydrolysates could lower the accumulation of DHA in the microorganism. Moreover, this study evaluated the nutritional value of the hydrolyzed BSG and they concluded that no further supplementation was needed, given that the hydrolysis of BSG (under different conditions: alkaline hydrolysis at 120°C and 1 atm for 20 minutes) yielded the necessary nutrients to supplement the medium (Ryu et al., 2013). This result supports our initial consideration that the presence of nutrients from the hydrolysates could promote the continuous growth of the microorganism beyond the expected period and limit the accumulation of lipids.

Similarly to the BSG medium, the CM medium, which had the second most rich medium after pretreatment according the proximate composition analysis, also accumulated less DHA in the lipid profile. The total accumulation of DHA for the CM medium was 36.46% TFA, but the TFA accumulation was the lowest as described above. Therefore, even if the composition of the lipids is similar to the other used media, the lower production of total lipids, which is the main goal of the culture, is very discouraging when using this type of medium. Moreover, to our understanding, there are no reported studies that use any coffee byproduct for the production of DHA using *Aurantiochytrium limacinum* SR21, nor

any other Thraustochytrid. In this way, more exploration on the use of this material could provide further understanding of its potential as a replacement of glucose as carbon source.

It is important to point out that the lipid profile does not vary much from previous studies reported in the literature, as can be seen in Table 8. In these studies, the percentage of DHA in TFA takes values of 32.5, 36.2, 48,5, and 50.3 (Li et al., 2015; Manikan et al., 2015; Yokochi et al., 1998; Zhu et al., 2007) when glucose in being used as carbon source. Therefore, varying the carbon source, be it BSG, CM, or EFB, though impacts the composition of fatty acids in the cultures, it is not far from what is expected. As discussed above, our results agree with previous results from other authors, though these results come from another species of *Aurantiochytrium*, despite this fact, it is important to verify the lipids profiles within the genus, given that our profiles followed the profiles when using similar hydrolysates. Moreover, the results from this study mimic quite well the results from several studies when using glucose as a carbon source. And, since glucose is the default carbon source when studying the production of DHA using *Aurantiochytrium limacinum* SR21, these results can be seen as satisfactory. Table 9 summarizes the yield on DHA from each of the hydrolysates.

Table 9. Total DHA percentage in TFA and DHA yield

Hydrolysate	DHA [%TFA]	DHA yield [g/L]
Glucose	52,46%	2.271
BSG	38.30%	1.110
CM	36.46%	0.587
EFB	51,77%	1.550

From Table 9, it is clear that the production of DHA from the CM hydrolysate is significantly lower than the DHA production from the BSG and EFB hydrolysates. This, added to the fact that the CM hydrolysate requires sodium citrate for its saccharification process, makes us discard CM as a viable material for the production of DHA from *Aurantiochytrium limacinum* SR21. Therefore, only BSG and EFB will be considered for the economical estimation.

Economical estimation

To further understand the relevance of these results, and the potential of these sources as potential replacements of glucose as carbon sources when culturing *Aurantiochytrium limacinum* SR21 for producing DHA, we performed an economical estimation. However, this estimation does not pretend to serve as a base line for establishing the economic feasibility of the different processes. This estimation only pretends to compare the production cost of DHA from *Aurantiochytrium limacinum* SR21 accounting for the cost of the carbon source. This estimation does not account for upstream nor downstream costs and assumes constant the cost of the nitrogen source and sea salt for all the media (Glucose,

BSG, and EFB). Table 10 presents the costs for each of the media, this cost was calculated using an enzyme unitary price of 4.24 proposed by Liu, Zhang, and Bao (2016).

Table 10. Estimation of cost of production of 1L of culture medium

Material	**Fixed components [COL$/L]**			**Carbon source [COL$/L]**	**Medium cost [Col$/L]**
	Yeast Extract	**Sea Salt**	**Water**		
Glucose	466.47	37.50	6.35	178.50	688.82
BSG	466.47	37.50	6.35	202.67	712.98
EFB	466.47	37.50	6.35	81.95	592.26

From Table 10, it is possible to infer that the cheapest medium would be EFB, followed by the basal glucose medium, and BSG would have be the most expensive. However, the cost of glucose can change based on the unit price for enzyme that is applied, this factor can vary significantly based on the source of the enzyme; which affects the enzymatic hydrolysis cost in the price. To evaluate how sensitive the cost of the medium is to this factor, several factors were taken into consideration (1.25, 4.24, 23, 80, 100, and 121). This sensitivity analysis is presented in Figure 18. The Figure shows that the medium cost for BSG medium is above the basal glucose medium cost for all the evaluated factors. For enzyme unit cost lower than 23 $/Kg protein, the EFB medium has a lower value than the basal medium.

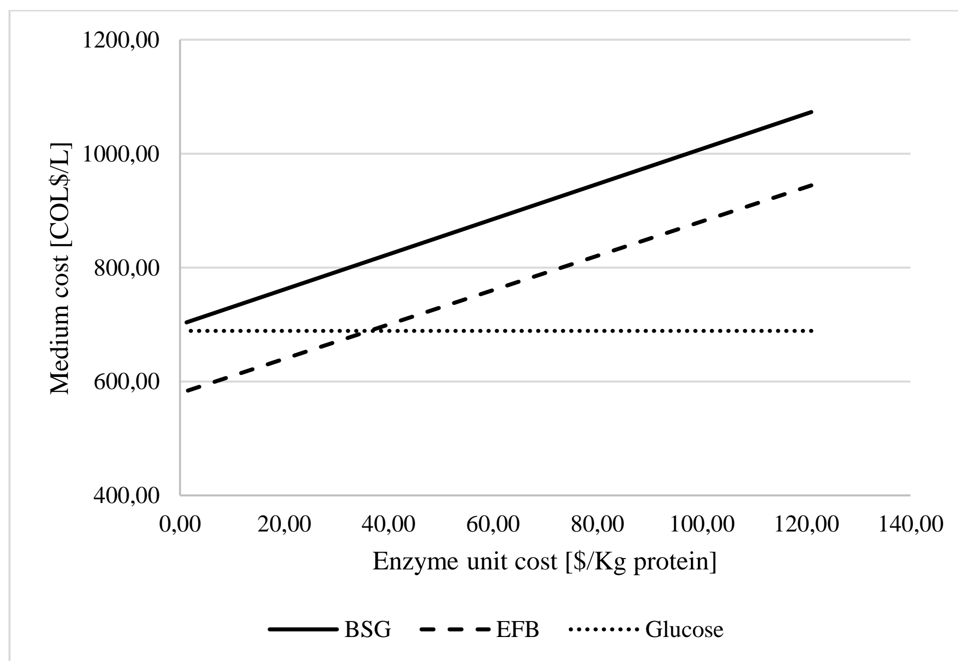

Figure 18. Sensitivity analysis for the medium cost with variations of the enzyme unit cost

Now, given that the BSG medium does not present any value in which it can be competitive against the basal glucose medium, we assume that EFB is a good candidate to substitute glucose as a carbon source in the production of DHA from *A. limacinum* SR21. However, it is still necessary to verify if there is an incidence of the final cost of DHA from both media. To do so, we considered the cost of producing 500 mg of DHA from the basal glucose medium and the EFB medium for the enzyme unit cost values of 1.25, 4.24, and 23. These values were selected because the cost of the EFB medium is lower than the cost of the basal medium at these values. It is important to remark that the cost of 500 mg of DHA when the enzyme unit cost value is 4.24, was COL$ 528.69 for the EFB culture and 151.65 COL$ for the basal medium. Figure 19 shows how the price of 500 mg of DHA vary for the values of the enzyme unit cost under 23 for EFB medium, which showed to be competitive against the production cost of the basal medium.

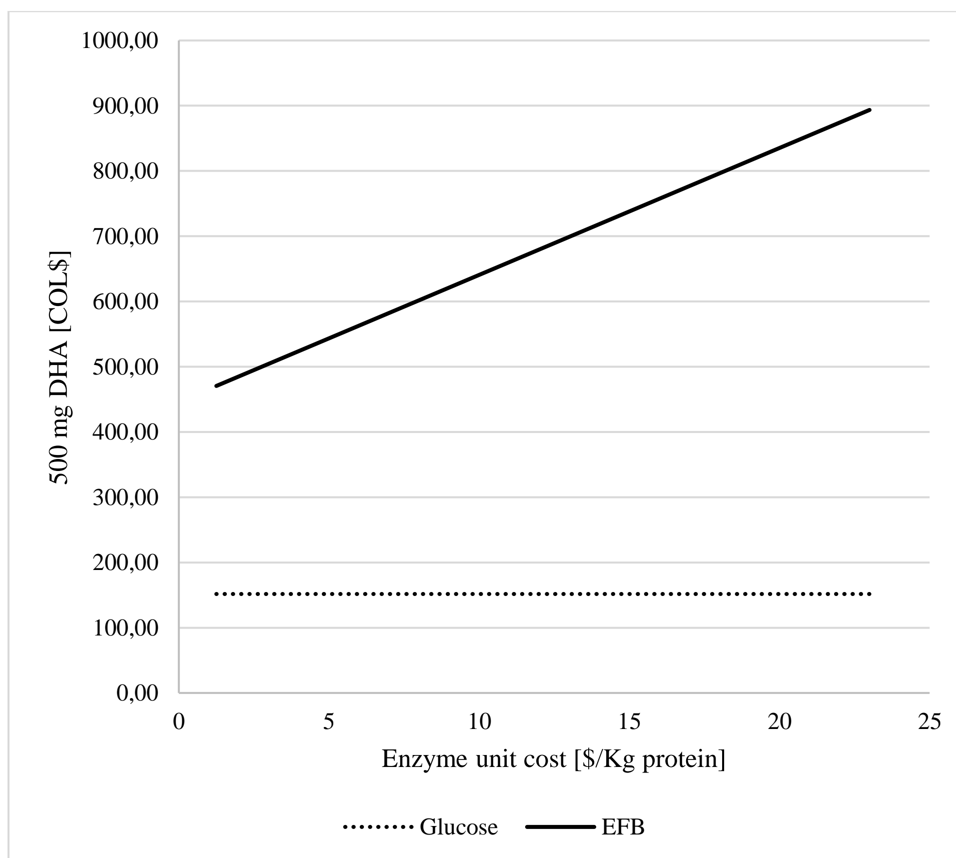

Figure 19. Sensitivity analysis for the cost of 500 mg of DHA with variations of the enzyme unit cost

The sensitivity analysis presented in Figure 19 shows that for the production cost of 500 mg of DHA from EFB medium using *Aurantiochytrium limacinum* SR21 under the condition for this study, it is not possible to obtain a production cost that is lower than 500 mg of DHA produced from glucose as a carbon source or the conditions of the study.

Chapter 5

Conclusions

This study allowed us confirming that *Aurantiochytrium limacinum* SR 21 is a good strain for obtaining rich DHA lipids. In most of the cultures, the strain was able to accumulate close to 50% of its weight in lipids when using glucose as a carbon source, which agrees with the reports in the literature. However, the lipid accumulation of the microorganism when using non-conventional carbon sources was lesser than the accumulation under glucose. This phenomenon, although not widely discussed in the literature, can be attributed to the fact that n-CCSs are complex media than have the potential to bring into the medium other nutrients that cause the microorganism to grow beyond the theoretical point established by the carbon to nitrogen ratio. In this study, CM and BSG accumulated significantly more biomass than glucose and EFB. In this way, the glucose and EFB media accumulated a similar amount of biomass (12.15 g/L and 11.38 g/L respectively). This is reflected in their lipid accumulation, 42.690% DCW for glucose and 26.310% DCW for EFB; moreover, the DHA percentage, which is quite similar for both sources, was 52.460% TFA for glucose and 51.770% TFA for EFB.

After assessing the different non-Conventional Carbon Sources, it was possible to see that amylaceous waste washes were not suitable for the culture of *Aurantiochytrium limacinum* SR21 as these byproducts cannot yield high amounts of glucose after being hydrolyzed. Both waste washes yielded under 1 g/L of glucose. As the targeted initial concentration of glucose was 50 g/L, these results did not meet the expected parameters. Nevertheless, this does not mean that amylaceous waste should be dismissed as a possible carbon source.

On the other hand, lignocellulosic materials; barley spent grain, coffee mucilage, and empty fruit bunch; have shown to be a good source of glucose, both for their high cellulose content and their glucose yield after performing the enzymatic hydrolysis. It is important to highlight that the selection of the alkaline pretreatment allowed for most of the lignin in the materials to be removed. BSG lost 67.63% of the lignin after the alkaline pretreatment, CM and EFB lost 26.19 and 45.06%, respectively. Hence, alkaline pretreatment is a good option for stripping the materials of the lignin, which can produce undesired components during the enzymatic hydrolysis while obstructing the cellulosic fibers and lowering the hydrolysis yields.

For performing the enzymatic hydrolysis, the selected enzyme had the ability to saccharify the materials under two different conditions. The first condition, using distilled water, which was intended for lowering the process costs. Here, BSG and EFB were able to produce more than 50 g/L of glucose; therefore, it was not necessary to use a buffer solution when hydrolyzing these materials.

Conversely, when CM was hydrolyzed using distilled water for lowering the process costs, it was not possible to obtain a concentration of glucose of more than 50 g/L, which was stablished as a threshold for determining the success of the saccharification process. Therefore, the second condition was selected for CM, where a sodium citrate was used for guaranteeing a pH 5.0 condition for the hydrolysis. Under these conditions, the hydrolysate from CM had a glucose concentration above the 50 g/L threshold.

Consequently, both BSG and EFB can be hydrolyzed using distilled water as hydrolyzing medium whereas CM requires the use of a buffer solution, sodium citrate in this case, for performing the hydrolysis.

After the hydrolysis conditions where stablished, it was possible to determine if the hydrolysis condition had an effect on the growth of the *Aurantiochytrium limacinum* SR21 culture. To do so, all the media that met the 50 g/L glucose concentration were used in the preparation of culture media. At the end of the cultures, it was possible to determine that the distilled water hydrolysates were not significantly different from the sodium citrate hydrolysates for BSG and EFB; therefore, these media are suitable for growing the microorganism. The CM hydrolysate, grown only with sodium citrate, also showed a good performance with the microorganism.

Hence, we concluded that all three lignocellulosic hydrolysates were suited for growing *Aurantiochytrium limacinum* SR21. BSG and EFB media came from a hydrolysate in distilled water. CM requires the use of a sodium citrate buffer solution to hydrolyze the material and reach the desired concentration of glucose for the preparation of the medium, which remains in the medium and can also alter the composition of the medium when compared to the base glucose medium.

Following the established hydrolysis conditions and the culture behavior, it was possible to compare how the media performed for the production of DHA. In this case, all the tested media presented the following different performances.

When using CM hydrolysates as carbon source in the culture, the total accumulation of lipids was affected, being this medium the one that accumulated the least amount of DHA because of the amount of fatty acids accumulated by the microorganism. Only 9.94% of the Dried Cell Weight was accumulated in lipids, this result is very distant from the expected close to 50% accumulation that is common in Thraustochytrids.

On the other hand, when BSG and EFB hydrolysates were used as carbon source, the growth of the microorganism was closer to the growth of *A. limacinum* SR21 when using glucose as carbon source. The accumulation of lipids when BSG was used was 17.94%, which is more representative than the medium under CM. Also, having a higher biomass production (as a consequence of having a richer medium) compensates the lower accumulation. In this way the yield of lipids when using BSG was 2.90 g/L against 2.99 g/L

from the EFB culture, which had a lower biomass accumulation (11.38 g/L) with a higher lipid accumulation 26.310 g/L.

When the yield of DHA is assessed, it is clear that the glucose medium was able to accumulate the most with a total DHA yield of 2.72 g/L followed by the EFB medium with 1.55 g/L. BSG and CM had the lesser accumulations with 1.11 g/L and 0.59 g/L of DHA, respectively. From these results, we concluded that CM was not a suitable material from producing DHA using *A. limacinum* SR21.

In this way, an economical estimation, based on the cost of the carbon source and culture media comparison, was performed for the n-CCS BSG and EFB. From this estimation it was possible to dilucidated that the enzyme unit cost plays a very important role in the determination of the carbon source cost. In this way, it was possible to see that it was not possible to obtain a enzyme unit value for the BSG medium that performed better than the glucose medium. In this way, an enzyme unit cost of 23 and lower produces a EFB medium cost that is lower than the cost of the basal glucose medium.

After evaluating the production cost of 500 mg of DHA using values of the enzyme unit cost of 23 and lower for the EFB medium, it was possible to verify that the production cost showed that the cost varied from 470 to 893 COL$. When stablishing the cost with the basal medium, the cost was 152 COL$. From these results, we can conclude that, under the conditions tested in this study, it is not possible to produce DHA at a lower cost from the studied n-CCS against a medium using glucose.

Nevertheless, it is important to notice that the conditions used in this study were not optimized, but rather they were excess conditions that were selected aiming to guarantee that highest performance in every of the stages of the study.

It would be interesting to test the growth of *Aurantiochytrium limacinum* SR21 under optimized conditions that guarantee the lowest possible costs and preserve the production conditions of the cultures. Additionally, it would also be interesting to study the carbon to nitrogen ration when using non-conventional carbon sources and its influence on the growth of *Aurantiochytrium limacinum* SR21 and the accumulation of lipids.

References

't Lam, G. P., Vermuë, M. H., Eppink, M. H. M., Wijffels, R. H., & van den Berg, C. (2017). Multi-Product Microalgae Biorefineries: From Concept Towards Reality. *Trends in Biotechnology*, *36*(2), 216–227. https://doi.org/10.1016/j.tibtech.2017.10.011

Álvarez, C., Reyes-Sosa, F. M., & Díez, B. (2016). Enzymatic hydrolysis of biomass from wood. *Microbial Biotechnology*, *9*(2), 149–156. https://doi.org/10.1111/1751-7915.12346

Alvira, P., Tomás-Pejó, E., Ballesteros, M., & Negro, M. J. (2010). Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresource Technology*, *101*(13), 4851–4861. https://doi.org/10.1016/j.biortech.2009.11.093

Amsler, C. D. (2008). Algal Sensory Chemical Ecology. In *Algal Chemical Ecology* (pp. 297–309). Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-540-74181-7_14

Andersen, R. A. (2005). *Algal Culturing Techniques*. Elsevier Academic Press.

Asgher, M., Ahmad, Z., & Iqbal, H. M. N. (2013). Alkali and enzymatic delignification of sugarcane bagasse to expose cellulose polymers for saccharification and bio-ethanol production. *Industrial Crops and Products*, *44*, 488–495. https://doi.org/10.1016/j.indcrop.2012.10.005

Balat, M. (2011). Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. *Energy Conversion and Management*, *52*(2), 858–875. https://doi.org/10.1016/j.enconman.2010.08.013

Balat, M., Balat, H., & Oz, C. (2008). Progress in bioethanol processing. *Progress in Energy and Combustion Science*, *34*(5), 551–573. https://doi.org/10.1016/j.pecs.2007.11.001

Ballesteros, I., Ballesteros, M., Manzanares, P., Negro, M. J., Oliva, J. M., & Sáez, F. (2008). Dilute sulfuric acid pretreatment of cardoon for ethanol production. *Biochemical Engineering Journal*, *42*(1), 84–91. https://doi.org/10.1016/j.bej.2008.06.001

Béligon, V., Christophe, G., Fontanille, P., & Larroche, C. (2016). Microbial lipids as potential source to food supplements. *Current Opinion in Food Science*, *7*, 35–42. https://doi.org/10.1016/j.cofs.2015.10.002

Brenna, J. T., & Carlson, S. E. (2014). Docosahexaenoic acid and human brain development: Evidence that a dietary supply is needed for optimal development. *Journal of Human Evolution*, *77*, 99–106. https://doi.org/10.1016/j.jhevol.2014.02.017

Burja, A. M., Armenta, R. E., Radianingtyas, H., & Barron, C. J. (2007). Evaluation of Fatty Acid Extraction Methods for. *Journal of Agricultural and Food Chemistry*, *55*(10), 4795–4801.

Cao, W., Sun, C., Liu, R., Yin, R., & Wu, X. (2012). Comparison of the effects of five pretreatment methods on enhancing the enzymatic digestibility and ethanol production from sweet sorghum bagasse. *Bioresource Technology*, *111*, 215–221.

https://doi.org/10.1016/j.biortech.2012.02.034

Cardona, E., Llano, B., Peñuela, M., Peña, J., & Rios, L. A. (2018). Liquid-hot-water pretreatment of palm-oil residues for ethanol production: An economic approach to the selection of the processing conditions. *Energy*, *160*, 441–451. https://doi.org/10.1016/j.energy.2018.07.045

Chanpiwat, P., Sthiannopkao, S., Widmer, K., Himeno, S., Miyataka, H., Vu, N. U., Tran, V. V., & Pham, T. T. N. (2016). Assessment of metal and bacterial contamination in cultivated fish and impact on human health for residents living in the Mekong Delta. *Chemosphere*, *163*, 342–350. https://doi.org/10.1016/j.chemosphere.2016.08.003

Chauton, M. S., Reitan, K. I., Norsker, N. H., Tveterås, R., & Kleivdal, H. T. (2015). A techno-economic analysis of industrial production of marine microalgae as a source of EPA and DHA-rich raw material for aquafeed: Research challenges and possibilities. *Aquaculture*, *436*, 95–103. https://doi.org/10.1016/j.aquaculture.2014.10.038

Chuck-Hernández, C., Pérez-Carrillo, E., Heredia-Olea, E., & Serna-Saldívar, S. O. (2011). Sorghum as a multifunctional crop for bioethanol production in Mexico: Tecnologies, advances and improvement opportunities. *Revista Mexicana de Ingeniería Química*, *10*(3), 529–549.

Cuervo, L., Folch, J. L., & Quiroz, R. E. (2009). Lignocelulosa Como Fuente de Azúcares Para la Producción de Etanol. *BioTecnología*, *13*(3), 11–25.

de Bievre, C., & Latgé, J.-P. (1980). Lipid Composition of *Entomophthora obscura* Hall & Dunn. *Microbiology Society*, *121*(1), 151–158. https://doi.org/10.1099/00221287-121-1-151

Delgado Naranjo, J. M., Múnera-Soto, R. D., Lugo de Ossa, C. A., & Peñuela Vasquez, M. (2017). Effect of carbon to nitrogen ratio in the growth of *Schizochytrium limacinum* towards DHA production. *Journal of Biotechnology & Biomaterials*, *07*(04), 22. https://doi.org/10.4172/2155-952X-C1-079

Duff, S. J. B., & Murray, W. D. (1996). Bioconversion of forest products industry waste cellulosics to fuel ethanol: A review. *Bioresource Technology*, *55*(1), 1–33. https://doi.org/10.1016/0960-8524(95)00122-0

Forsyth, S., Gautier, S., & Salem, N. (2016). Global Estimates of Dietary Intake of Docosahexaenoic Acid and Arachidonic Acid in Developing and Developed Countries. *Annals of Nutrition & Metabolism*, *68*, 258–267. https://doi.org/10.1159/000446855

Fu, L., Cui, X., Li, Y., Xu, L., Zhang, C., Xiong, R., Zhou, D., & Crittenden, J. C. (2017). Excessive phosphorus enhances *Chlorella regularis* lipid production under nitrogen starvation stress during glucose heterotrophic cultivation. *Chemical Engineering Journal*, *330*(August), 566–572. https://doi.org/10.1016/j.cej.2017.07.182

Furlan, V. J. M., Maus, V., Batista, I., & Bandarra, N. M. (2017). Production of docosahexaenoic acid by *Aurantiochytrium* sp. ATCC PRA-276. *Brazilian Journal of Microbiology*, *48*(2), 359–365. https://doi.org/10.1016/j.bjm.2017.01.001

Gao, M., Song, X., Feng, Y., Li, W., & Cui, Q. (2013). Isolation and characterization of *Aurantiochytrium* species: high docosahexaenoic acid (DHA) production by the newly isolated microalga, *Aurantiochytrium* sp. SD116. *Journal of Oleo Science J.*

Oleo Sci, *62*(3), 143–151. https://www.jstage.jst.go.jp/article/jos/62/3/62_143/_pdf/-char/en

Goed. (2014). *Global recommendations for EPA and DHA intake* (Issue April, pp. 1–20). http://www.goedomega3.com/

Guo, D.-S., Ji, X.-J., Ren, L.-J., Li, G.-L., Yin, F.-W., & Huang, H. (2016). Development of a real-time bioprocess monitoring method for docosahexaenoic acid production by *Schizochytrium* sp. *Bioresource Technology*, *216*, 422–427. https://doi.org/10.1016/j.biortech.2016.05.044

Gupta, A., Abraham, R. E., Barrow, C. J., & Puri, M. (2015). Omega-3 fatty acid production from enzyme saccharified hemp hydrolysate using a novel marine thraustochytrid strain. *Bioresource Technology*, *184*, 373–378. https://doi.org/10.1016/j.biortech.2014.11.031

Gupta, A., Barrow, C. J., & Puri, M. (2012). Omega-3 biotechnology: Thraustochytrids as a novel source of omega-3 oils. *Biotechnology Advances*, *30*(6), 1733–1745. https://doi.org/10.1016/j.biotechadv.2012.02.014

Hamelinck, C. N., van Hooijdonk, G., & Faaij, A. P. (2005). Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. *Biomass and Bioenergy*, *28*(4), 384–410. https://doi.org/10.1016/j.biombioe.2004.09.002

Hendriks, A. T. W. M., & Zeeman, G. (2009). Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technology*, *100*(1), 10–18. https://doi.org/10.1016/j.biortech.2008.05.027

Honda, D., Yokochi, T., Nakahara, T., Erata, M., & Higashihara, T. (1998). *Schizochytrium limacinum* sp. nov., a new thraustochytrid from a mangrove area in the west Pacific Ocean. *Mycological Research*, *102*(4), 439–448. https://doi.org/10.1017/S0953756297005170

Hong, W.-K., Kim, H. C., Rairakhwada, D., Kim, S., Hur, B.-K., Kondo, A., & Seo, J.-W. (2012). Growth of the oleaginous microalga *Aurantiochytrium* sp. KRS101 on cellulosic biomass and the production of lipids containing high levels of docosahexaenoic acid. *Bioprocess and Biosystems Engineering*, *35*(1–2), 129–133. https://doi.org/10.1007/s00449-011-0605-0

Hong, W.-K., Rairakhwada, D., Seo, P.-S., Park, S.-Y., Hur, B.-K., Ho Kim, C., & Seo, J.-W. (2011). Production of Lipids Containing High Levels of Docosahexaenoic Acid by a Newly Isolated Microalga, *Aurantiochytrium* sp. KRS101. *Applied Bichemistry and Biotechnology*, *164*, 1468–1480. https://doi.org/10.1007/s12010-011-9227-x

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., & Darzins, A. (2008). Microalgal triacylglycerols as feedstocks for biofuel production: Perspectives and advances. *Plant Journal*, *54*(4), 621–639. https://doi.org/10.1111/j.1365-313X.2008.03492.x

Huang, T. Y., Lu, W. C., & Chu, I. M. (2012). A fermentation strategy for producing docosahexaenoic acid in *Aurantiochytrium limacinum* SR21 and increasing C22:6 proportions in total fatty acid. *Bioresource Technology*, *123*, 8–14. https://doi.org/10.1016/j.biortech.2012.07.068

Jain, R., Raghukumar, S., Sambaiah, K., Kumon, Y., & Nakahara, T. (2007). Docosahexaenoic acid accumulation in thraustochytrids: Search for the rationale.

Marine Biology, *151*(5), 1657–1664. https://doi.org/10.1007/s00227-007-0608-1

Jye, K., Chang, L., Paul, H., Nichols, P. D., Koutoulis, A., & Blackburn, S. I. (2015). Australian thraustochytrids: Potential production of dietary long-chain omega-3 oils using crude glycerol. *Journal of Functional Foods*, *19*, 810–820. https://doi.org/10.1016/j.jff.2015.01.039

Karimi, K., Emtiazi, G., & Taherzadeh, M. J. (2006). Ethanol production from dilute-acid pretreated rice straw by simultaneous saccharification and fermentation with *Mucor indicus*, *Rhizopus oryzae*, and *Saccharomyces cerevisiae*. *Enzyme and Microbial Technology*, *40*(1), 138–144. https://doi.org/10.1016/j.enzmictec.2005.10.046

Khozin-Goldberg, I., Iskandarov, U., & Cohen, Z. (2011). LC-PUFA from photosynthetic microalgae: occurrence, biosynthesis, and prospects in biotechnology. *Applied Microbiology and Biotechnology*, *91*(4), 905–915. https://doi.org/10.1007/s00253-011-3441-x

Kim, J. S., Lee, Y. Y., & Kim, T. H. (2016). A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. In *Bioresource Technology*. https://doi.org/10.1016/j.biortech.2015.08.085

Klein-Marcuschamer, D., Oleskowicz-Popiel, P., Simmons, B. A., & Blanch, H. W. (2012). The challenge of enzyme cost in the production of lignocellulosic biofuels. *Biotechnology and Bioengineering*, *109*(4), 1083–1087. https://doi.org/10.1002/bit.24370

Kris-Etherton, P. M., Grieger, J. A., & Etherton, T. D. (2009). Dietary reference intakes for DHA and EPA. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *81*, 99–104. https://doi.org/10.1016/j.plefa.2009.05.011

Leyva Soto, L. A. (2014). *Estudio de la acumulación de lípidos y ácidos grasos en la microalga* Chlorella vulgaris *inmovilizada junto a* Azospirillum brasilense *en esferas de alginato, en relación a las condiciones de crecimiento* [Centro de investigaciones Biológicas del Noroeste]. http://www.remeri.org.mx/portal/REMERI.jsp?id=oai:tesis.cib.mx:2014/leyva_l

Li, J., Liu, R., Chang, G., Li, X., Chang, M., Liu, Y., Jin, Q., & Wang, X. (2015). A strategy for the highly efficient production of docosahexaenoic acid by *Aurantiochytrium limacinum* SR21 using glucose and glycerol as the mixed carbon sources. *Bioresource Technology*, *177*, 51–57. https://doi.org/10.1016/j.biortech.2014.11.046

Ling, X., Guo, J., Liu, X., Zhang, X., Wang, N., Lu, Y., & Ng, I.-S. (2015). Impact of carbon and nitrogen feeding strategy on high production of biomass and docosahexaenoic acid (DHA) by *Schizochytrium* sp. LU310. *Bioresource Technology*, *184*, 139–147. https://doi.org/10.1016/j.biortech.2014.09.130

Liu, G., Zhang, J., & Bao, J. (2016). Cost evaluation of cellulase enzyme for industrial-scale cellulosic ethanol production based on rigorous Aspen Plus modeling. *Bioprocess and Biosystems Engineering*, *39*(1), 133–140. https://doi.org/10.1007/s00449-015-1497-1

Lynd, L. R., Weimer, P. J., van Zyl, W. H., & Pretorius, I. S. (2002). Microbial Cellulose Utilization: Fundamentals and Biotechnology. *Microbiology and Molecular Biology Reviews*, *66*(4), 739–739. https://doi.org/10.1128/MMBR.66.4.739.2002

Manikan, V., Nazir, M. Y. M., Kalil, M. S., Isa, M. H. M., Kader, A. J. A., Yusoff, W. M.

W., & Hamid, A. A. (2015). A new strain of docosahexaenoic acid producing microalga from Malaysian coastal waters. *Algal Research*, *9*, 40–47. https://doi.org/10.1016/j.algal.2015.02.023

Martínez-Anaya, C., Belcázar-López, E., Dantán-González, E., & Folch-Mallol, J. L. (2008). Celulasas Fúngicas: Aspectos Biológicos y Aplicaciones en la Industria Energética. *Revista Lationoarmericana de Microbiología*, *50*(3–4), 119–131. https://www.medigraphic.com/pdfs/lamicro/mi-2008/mi08-3_4i.pdf

Montoya, L. M. A., & Alcaraz, P. A. V. (2016). Preferencias alimentarias en los hogares de la ciudad de Medellín, Colombia. *Saude e Sociedade*, *25*(3), 750–759. https://doi.org/10.1590/S0104-12902016149242

Mosier, N., Ladisch, C., & Ladisch, M. (2002). Characterization of acid catalytic domains for cellulose hydrolysis and glucose degradation. *Biotechnology and Bioengineering*, *79*(6), 610–618. https://doi.org/10.1002/bit.10316

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y., Holtzapple, M., & Ladisch, M. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology*, *96*(6), 673–686. https://doi.org/10.1016/j.biortech.2004.06.025

Muik, B., Edelmann, A., Lendl, B., & Ayora-Cañada, M. (2002). Determination of yeast assimilable nitrogen content in wine fermentations by sequential injection analysis with spectrophotometric detetection. *Analytical and Bioanalytical Chemistry*, *374*(1), 167–172. https://doi.org/10.1007/s00216-002-1418-4

Mussatto, S. I., Machado, E. M. S., Carneiro, L. M., & Teixeira, J. A. (2012). Sugars metabolism and ethanol production by different yeast strains from coffee industry wastes hydrolysates. *Applied Energy*, *92*, 763–768. https://doi.org/10.1016/j.apenergy.2011.08.020

Oliva Martínez, J. M., Negro Álvarez, M. J., Manzanares Secades, P., Ballesteros Perdices, I., & Ballesteros Perdices, M. (2002). Simultaneous saccharification and fermentation process for converting the cellulosic fraction of olive oil extraction residue into ethanol. *Grasas y Aceites*, *53*(3), 282–288. https://doi.org/10.3989/gya.2002.v53.i3.318

Pike, I. H., & Jackson, A. (2010). Fish oil: production and use now and in the future. *Lipid Technology*, *22*(3), 59–61. https://doi.org/10.1002/lite.201000003

Pimentel, D. (2003). Ethanol Fuels: Energy Balance, Economics, and Environmental Impacts are Negative. *Natural Resources Research*, *12*(2), 127–134. https://doi.org/https://doi.org/10.1023/A:1024214812527

Robles Medina, A., Molina Grima, E., Giménez Giménez, A., & Ibánez Gonzalez, M. J. (1998). Downstream Processing of Algal Polyunsaturated Fatty Acids. *Biotechnology Advances*, *16*(3), 517–580.

Rogers, L. K., Valentine, C. J., & Keim, S. A. (2013). DHA supplementation: Current implications in pregnancy and childhood. *Pharmacological Research*, *70*, 13–19. https://doi.org/10.1016/j.phrs.2012.12.003

Rusu, B., Bindea, M., Dulf, F. V., & Vodnar, D. C. (2017). DHA production by *Schizochytrium limacinum* SR-21 using crude glycerol as carbon source. *Lucrari Stiintifice Seria Agronomie*, *60*(1), 21–26.

https://www.researchgate.net/publication/325949322

Ryu, B.-G., Kim, K., Kim, J., Han, J.-I., & Yang, J.-W. (2013). Use of organic waste from the brewery industry for high-density cultivation of the docosahexaenoic acid-rich microalga, *Aurantiochytrium* sp. KRS101. *Bioresource Technology*, *129*, 351–359. https://doi.org/10.1016/j.biortech.2012.11.049

Sakarika, M., & Kornaros, M. (2017). Kinetics of growth and lipids accumulation in *Chlorella vulgaris* during batch heterotrophic cultivation: Effect of different nutrient limitation strategies. *Bioresource Technology*, *243*, 356–365. https://doi.org/10.1016/j.biortech.2017.06.110

Sánchez, C. (2009). Lignocellulosic residues: Biodegradation and bioconversion by fungi. *Biotechnology Advances*, *27*(2), 185–194. https://doi.org/10.1016/j.biotechadv.2008.11.001

Sánchez, Ó. J., & Cardona, C. A. (2008). Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresource Technology*, *99*(13), 5270–5295. https://doi.org/10.1016/j.biortech.2007.11.013

Simopoulos, A. P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomedicine & Pharmacotherapy*, *56*(8), 365–379. https://doi.org/10.1016/S0753-3322(02)00253-6

Simopoulos, Artemis P. (2008). The Importance of the Omega-6/Omega-3 Fatty Acid Ratio in Cardiovascular Disease and Other Chronic Diseases. *Experimental Biology and Medicine*, *233*(6), 674–688. https://doi.org/10.3181/0711-MR-311

Singh, R. N., & Sharma, S. (2012). Development of suitable photobioreactor for algae production - A review. *Renewable and Sustainable Energy Reviews*, *16*(4), 2347–2353. https://doi.org/10.1016/j.rser.2012.01.026

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. (2008). *Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP) (Revised July 2011)*. http://www.nrel.gov/biomass/analytical_procedures.html

Song, Z., Stajich, J. E., Xie, Y., Liu, X., He, Y., Chen, J., Hicks, G. R., & Wang, G. (2018). Comparative analysis reveals unexpected genome features of newly isolated Thraustochytrids strains: on ecological function and PUFAs biosynthesis. *BMC Genomics*, *19*(1), 541. https://doi.org/10.1186/s12864-018-4904-6

Spanopoulos-Hernandez, M., Ponce-Palafox, J. T., Barba-Quintero, G., Ruelas-Inzunza, J. R., Tiznado-Contreras, M. R., Hernández-González, C., & Shirai, K. (2010). Production of biological silage from fish waste, the smoked yellowfin tuna (*Thunnus albacares*) and fillet of tilapia (*Oreochromis* sp), for feeding aquaculture species. *Revista Mexicana de Ingeniera Quimica*, *9*(2), 167–178.

Sun, Y., & Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology*, *83*(1), 1–11. https://doi.org/10.1016/S0960-8524(01)00212-7

Sun, Y., & Cheng, J. (2005). Dilute acid pretreatment of rye straw and bermudagrass for ethanol production. *Bioresource Technology*, *96*(14), 1599–1606. https://doi.org/10.1016/j.biortech.2004.12.022

Taherzadeh, M. J., & Karimi, K. (2007). Enzyme-based hydrolysis processes for ethanol

from lignocellulosic materials: a review. *Bioresources*, *2*, 707–738.

Talbot, G. (2015). *Specialty Oils and Fats in Food and Nutrition: Properties, Processing and Applications*. Woodhead Publishing Limited.

Taniguchi, M., Suzuki, H., Watanabe, D., Sakai, K., Hoshino, K., & Tanaka, T. (2005). Evaluation of pretreatment with *Pleurotus ostreatus* for enzymatic hydrolysis of rice straw. *Journal of Bioscience and Bioengineering*, *100*(6), 637–643. https://doi.org/10.1263/jbb.100.637

Thomsen, M. H., Thygesen, A., & Thomsen, A. B. (2008). Hydrothermal treatment of wheat straw at pilot plant scale using a three-step reactor system aiming at high hemicellulose recovery, high cellulose digestibility and low lignin hydrolysis. *Bioresource Technology*, *99*(10), 4221–4228. https://doi.org/10.1016/j.biortech.2007.08.054

Watters, C. A., Edmonds, C. M., Rosner, L. S., Sloss, K. P., & Leung, P. (2012). A Cost Analysis of EPA and DHA in Fish, Supplements, and Foods. *Journal of Nutrition & Food Sciences*, *02*(08). https://doi.org/10.4172/2155-9600.1000159

Xin, L., Hong-ying, H., Ke, G., & Ying-xue, S. (2010). Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga Scenedesmus sp. *Bioresource Technology*, *101*(14), 5494–5500. https://doi.org/10.1016/j.biortech.2010.02.016

Yano, Y., Nakayama, a, Saito, H., & Ishihara, K. (1994). Production of docosahexaenoic acid by marine bacteria isolated from deep sea fish. *Lipids*, *29*(7), 527–528. https://doi.org/10.1007/BF02578252

Yen, H.-W., Hu, I.-C., Chen, C.-Y., Ho, S.-H., Lee, D.-J., & Chang, J.-S. (2013). Microalgae-based biorefinery--from biofuels to natural products. *Bioresource Technology*, *135*, 166–174. https://doi.org/10.1016/j.biortech.2012.10.099

Yokochi, T., Honda, D., Higashihara, T., & Nakahara, T. (1998). Optimization of docosahexaenoic acid production by *Schizochytrium limacinum* SR21. *Applied Microbiology and Biotechnology*, *49*(1), 72–76. https://doi.org/10.1007/s002530051139

Yokoyama, R., & Honda, D. (2007). Taxonomic rearrangement of the genus *Schizochytrium* sensu lato based on morphology, chemotaxonomic characteristics, and 18S rRNA gene phylogeny (*Thraustochytriaceae*, *Labyrinthulomycetes*): emendation for *Schizochytrium* and erection of *Aurantiochytrium* and . *Mycoscience*, *48*(4), 199–211. https://doi.org/10.1007/S10267-006-0362-0

Zhang, X., Xu, C., & Wang, H. (2007). Pretreatment of bamboo residues with *Coriolus versicolor* for enzymatic hydrolysis. *Journal of Bioscience and Bioengineering*, *104*(2), 149–151. https://doi.org/10.1263/jbb.104.149

Zhu, L., Zhang, X., Ji, L., Song, X., & Kuang, C. (2007). Changes of lipid content and fatty acid composition of *Schizochytrium limacinum* in response to different temperatures and salinities. *Process Biochemistry*, *42*(2), 210–214. https://doi.org/10.1016/j.procbio.2006.08.002

Endorsements

Student:

____
Ruben Darío Múnera Soto

Advisor

____
Mariana Peñuela Vásquez

Endorsement

____
Mariana Peñuela Vásquez
Approved Bioprocesos Research Group
Date: June 15, 2019