MASTER THESIS

Reprocessing Waste into *Chlorella vulgaris* Biomass – A Strategy of Developing Next-Generation Food and Feed

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xx.yy.2022

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Acknowledgements

First of all, I want to thank Dr. Oliver Spadiut for granting me the opportunity to perform my master thesis in his welcoming working group and to be part of this interesting project. I appreciated his expertise and enthusiasm for the research.

Moreover, I owe my gratitude to my supervisor Ricarda, who guided me through the whole process of this thesis with information, practical hints and trust in working independently. I am thankful for her time especially in terms of practical instructions and data analysis.

I also want to thank my lab colleagues for their helpfulness and pleasant atmosphere.

Last but not the least, I want to express my thanks to my family for their support, belief and patience. The completion of my studies would not have been possible without them.

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Abstract

Humanity is confronted with overpopulation joined with increasing greenhouse gas emissions and advancing depletion of the world's resources. Thus, alternative food and feed sources are strongly demanded. Microalgae arouse attention due to their capability of converting light into highly nutritious biomass and fixating the greenhouse gas CO_2. However, the cultivation of microalgae in a sustainable and economically-viable manner is still in the developing phase.

In this thesis, a new approach of using wheat straw hydrolysates (WSH) as a substrate for *Chlorella vulgaris* was studied. While *C. vulgaris* is a well-known food and feed microalgae, wheat straw is an abundantly occurring lignocellulosic waste product. Reprocessing WSH into *C. vulgaris* biomass has the potential to reduce cultivation cost and enhance sustainability.

Growth parameters and biochemical composition of *C. vulgaris* in different concentrated WSH under mixotrophic and heterotrophic conditions were studied. Results were compared to the cultivation of *C. vulgaris* in a defined medium and to a photoautotrophic control culture. While mixotrophic conditions in WSH showed 1.5-fold higher biomass concentration compared to photoautotrophic cultivation, heterotrophic cultivation resulted in 4-times lower biomass concentration. The cultivation of *C. vulgaris* in WSH had no adverse effect on the protein and chlorophyll content, being crucial parameters for the food and feed market. Hence, the usage of WSH as a substrate for *C. vulgaris* still has the potential to reduce processing costs, but research on optimising growth performance is required.

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Zusammenfassung

Die Menschheit hat mit einem exponentiellen Bevölkerungszuwachs, steigenden Treibhausgasemissionen und dem fortschreitenden Verlust natürlicher Ressourcen zu kämpfen. Daher ergibt sich ein Streben nach alternativen, umweltschonenden Lebens- un Futtermittel. Mikroalgen scheinen eine vielversprechende Lösung zu sein, da sie Lichtenergie in nährstoffreiche Biomasse umwandeln und gleichzeitig CO_2 fixieren. Allerdings bedürfen Mikroalgenkultivierungen noch Optimierungen, um sie in Zukunft ökonomischer zu gestalten.

In dieser Arbeit wurde die Kultivierung der Mikroalge *Chlorella vulgaris* in Weizenstroh-Hydrolysat (WSH) unter heterotrophen und mixotrophen Bedingungen untersucht. *C. vulgaris* ist aufgrund der nährstoffreichen Biomasse in der Lebens- und Futtermittelindustrie bereits bekannt. Weizenstroh wurde deshalb gewählt, weil es ein häufiges und günstiges Abfallprodukt ist. Die Nutzung von WSH als Substrat für *C. vulgaris* hat das Potential die Wirtschaftlichkeit und Nachhaltigkeit des Kultivierungsprozesses zu erhöhen.

Sowohl Wachstumsparameter als auch die Biomassezusammensetzung von *C. vulgaris* in verschieden konzentrierten WSH wurden unter heterotrophen und mixotrophen Bedingungen untersucht. Die Ergebnisse wurden mit der Kultivierung in einem definierten Medium und mit einer photoautotrophen Kultivierung verglichen. Während die Kultivierung im WSH unter mixotrophen Bedingungen 1.5-fach höhere Biomassekonzentrationen als die photoautotrophe Kultivierung zeigte, waren diese 4-mal niedriger unter heterotrophen Bedingungen. Der Protein- und Chlorophyllgehalt wurde durch das WSH in Anbetracht der Lebens- und Futtermittelindustrie nicht negativ beeinflusst. Die Nutzung von WSH als Substrat für *C. vulgaris* hat das Potential die Kosten der Kultivierung zu reduzieren, allerdings muss noch an optimalen Wachstumsbedingungen geforscht werden.

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1 Introduction

1.1 Definition of microalgae

Evolutionary biologists estimate that algae evolved about 3.5 billion years ago representing the ancestor of higher plants[1]. Nowadays, algae comprise a huge polyphyletic group of eukaryotic and prokaryotic organisms making them the most genetically-diverse group on Earth[2]. They can be broadly classified into multicellular macroalgae with sizes up to 60 m and unicellular microalgae being as small as 0.2 µm[3]. Microalgae constitute the majority of one to ten million algal species[4]. They arose through multiple endosymbiotic events forming eleven major phyla: Cyanophyta (blue-green algae), Chlorophyta (green algae), Rhodophyta (red algae), Glaucophyta, Euglenophyta, Chlorarachniophyta, Charophyta, Cryptophyta, Haptophyta, Heterokontophyta, and Dinophyta[2]. Microalgae are photosynthetic organisms occurring in freshwater, brackish, and marine environments[5], but also in moist terrestrial surroundings[6]. Microalgae account for 40-50 % of total atmospheric oxygen production by performing photosynthesis and simultaneously capturing massive amounts of CO_2[4]. Moreover, microalgae are considered the base of aquatic food chains making them crucial organisms for life on Earth[6].

The phylogenetic diversity of microalgae is joined with a variety in their biochemical composition[7]. Table 1 demonstrates the protein, carbohydrate, and lipid content of common microalgae under photoautotrophic conditions. However, it has to be considered that the chemical composition within species varies depending on environmental factors.

Table 1: Biochemical composition of some microalgae in percentage of dry algal biomass[3]

Species	Phylum	wt% Protein	wt% Carbs	wt% Fat
Arthrospira maxima	Cyanophyta	60-71	13-16	6-7
Spirulina platensis	Cyanophyta	46-63	8-14	4-9
Chlorella vulgaris	Chlorophyta	51-58	12-17	14-22
Chlorella pyrenoidosa	Chlorophyta	57	26	2
Dunaliella salina	Chlorophyta	57	32	6
Haematococcus pluvialis	Chlorophyta	48	27	15
Porphyridium cruentum	Rhodophyta	28-39	40-57	9-14

Apart from macronutrients, microalgae provide various bioactive compounds including vitamins, pigments such as chlorophylls, carotenoids (e.g., β-carotene, astaxanthin), or phycobilins (e.g., phycocyanin, phycoerythrin), omega-3 polyunsaturated fatty acids PUFAs (e.g., docosahexaenoic acid, eicospentaoinic acid), sulfated polysaccharides, and phenolics. The screening of bioactive metabolites derived from microalgae has been under active investigation due to their potential of fulfilling numerous global demands[2,8].

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Depending on the microalgal species and their valuable target compounds, microalgae find application in food and feed, cosmetic, pharmaceutical, or bioenergy industries[5]. More recently, microalgae are used for nutrient removal in wastewater and CO_2 mitigation[9,10]. Currently, the largest commercial application of microalgae is attributed to the functional food market[4].

1.2 Microalgae for food and feed purposes

The exponentially increasing population causes a strong demand for renewable nutrition sources. Since microalgae are rich in nutrients, they are considered a promising alternative. Microalgae contain vitamins (e.g., vitamin A, B1, B2, B6, B12, C, E), minerals (e.g., potassium, iron, magnesium, calcium, iodine), antioxidative pigments (β-carotenoids, astaxanthin), and PUFAs being required for a balanced and healthy diet[3]. Recently, the functional food market rapidly increased due to the raising awareness of healthy food and its benefits on human well-being[2]. The busy lifestyle in developed countries tempts the consumption of calorific food causing health issues such as obesity, high blood pressure, or diabetes. Functional foods are a perfect solution to prevent or even cure chronic diseases[3].

While macroalgae have been consumed in the human diet for hundreds of years, the commercial cultivation of microalgae began only about 60 years ago. In the 1960s, biomass production of green algae *Chlorella vulgaris* started in Japan and Taiwan. About twenty years later the mass cultivation of Cyanobacterium *Arthrospira* sp. and green algae *Dunaliella salina* and *Haematococcus pluvialis* followed in the USA, China, Thailand, Israel, and Australia[2]. According to the US Food and Drug Administration (FDA) and European Food Safety Authority (EFSA), these microalgae are classified as food sources belonging to the Generally Recognised As Safe (GRAS) category[11,12]. Algae-based supplements are either available as whole biomass (*C. vulgaris, Arthrospira* sp.) or as purified extracts containing high-value products, e.g., astaxanthin from *H. pluvialis* or β-carotene from *D. salina*[2]. The market values and production amounts are summarised in Table 2. Food supplements are obtained in the form of capsules, tablets, powders, and liquids being mixed with pastes, noodles, bread, yoghurt, snacks, cereals, biscuits, and drinks[3]. Besides the role of food additives, microalgal pigments are primarily used as natural food colourants[6]. Due to changes in market demands and legislation, the replacement of artificial colourants by natural pigments is preferred[13].

Table 2: Market values and world production of biomasses and derived products from microalgae[2]

Product	Price (USD/kg)	World production (tons/year)
Arthrospira sp. biomass	42	4100-6700
Chlorella sp. biomass	44	2000
β-Carotene	300-1500	1200
Astaxanthin	2500-7000	300

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Today, *Arthrospira* sp. and *Chlorella* sp. are dominating the microalgae market[11,14]. Its biomass is promoted as functional food with high-quality protein content and additional health benefits[2]. The protein content of *Chlorella* sp. and *Arthrospira* sp. exceeds conventional protein sources in terms of quantity (Table 3). Concerning its quality, their biomass shows a well-balanced amino acid profile for human consumption and provides all essential amino acids (EEAs), which cannot be synthesised by mammalians. Most plant-derived proteins do not include the complete EAA profile making microalgae more interesting for vegetarians and vegans[3].

Table 3: Protein content of various food sources[3]

Food origin	wt% Proteins
Beef	17.4
Fish, Chicken	19-24
Peanuts	26
Parmesan Cheese	36
Soybean flower	36
Beer yeast	45
Whole egg	47
Chlorella sp.	50-60
Spirulina sp.	60-70

Despite highly nutritious content, the use of microalgal biomass as a food source is still limited due to some obstacles. For instance, the powder-like consistency, the dark green colour, and the fishy smell affect consistency, colour, and taste of conventional food items. Moreover, large-scale cultivation of microalgae remains a major challenge[15]. Process optimisation in terms of enhanced growth rates and nutrient content is needed. Although major advances in genetic engineering were achieved in the last decades, the technology has not made satisfactory developments concerning microalgae yet[16]. *Chlamydomonas reinhardtii, Nannochloropsis* sp., or *Chlorella* sp. were genetically manipulated, but methods suffered from low efficacy of transformation and stability of exogenous transgenes[17]. However, the use of genetic engineering in the field of functional food is not well accepted by consumers, especially in Western countries[5]. At the moment, recombinant microalgae are not available at commercial phase for food and feed purposes[11].

In addition to human consumption, microalgal biomass is also applied as nutritious feed for animals such as fish, poultry, cows, bulls, horses, cats, or dogs[11]. About 30 % of the global microalgal production accounts for animal feed purposes[18], with aquaculture representing the main sector[12]. The biomass shows promoting effects on animal physiology e.g., enhanced immune response, disease resistance, and gut function. The usage of microalgae as animal feed improves the well-being of both animals and humans[19]. For instance, dairy cattle fed with *C. vulgaris* contained milk showing reduced proportions of saturated fatty acids and increased amounts of DHA[6].

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1.3 Microalgal cultivation

1.3.1 Cultivation systems

The first commercial cultivation of microalgae began about 60 years ago. From then on, different production strategies emerged based on their application. The two main types of large-scale cultivation are open-air systems and closed photobioreactors[2] (Figure 1). The choice of the cultivation system depends on numerous factors such as the microalgal species, target product, cost of land, climate, or the availability of sunlight, water, and nutrients[13].

Open-air systems are currently considered a more economically-viable system for the production of low-cost biomass because these systems are less complex and lower capital investment is required[2]. However, open-air systems show drawbacks such as higher risks of contamination, lower productivities (~ 0.5 g/L[20]), large areas of land, evaporative water losses, and inconstant parameters (seasonal dependence). Higher productivity can mainly be obtained by species that adapt to extremophile conditions (e.g., high salinity, pH, temperature) outcompeting contaminants. Common types of open-air systems are raceway ponds, big ponds, tanks, and circular ponds[13]. In particular, raceway ponds at a depth of 20-30 cm being unstirred or mixed by paddles are used[8].

Nowadays, research focuses on closed photobioreactors because these systems offer process control, higher biomass concentrations (5-25 g/L[20]), and lower contamination risks. Since the biochemical composition of microalgae varies according to environmental factors, controlled conditions in photobioreactors are favorable[8]. A controlled process is especially needed for the production of pharmaceuticals, nutraceuticals, and cosmetics[1]. However, closed photobioreactors are difficult to scale up due to light limitations[20] and are expensive due to higher building, sterilising, and energy costs (mixing, gas supply, gas exchange)[8]. Common closed reactor types are flat alveolar panels, tubular photobioreactors, or stirred fermenters with internal lights[13]. Photobioreactors can either be located indoors or outdoors with the use of artificial light sources or solar light[21]. Artificial lighting enables control of light intensity, frequency, and wavelength. Common electrical light sources are fluorescent lamps, high-intensity discharge lamps and light-emitting diodes (LEDs). LEDs recently gained more interest due to their compactness, longer lifetime, and lower power consumption[22].

Figure 1: Open raceway pond[23] (left) and closed tubular photobioreactor[24] (right)

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1.3.2 Photoautotrophy

Photoautotrophic cultivation is the most common form of microalgal biomass production and is typically performed in open raceway ponds[1,5]. Photoautotrophic microalgae rely on light energy, water, and inorganic substances such as CO_2, N, and P for growing[5]. CO_2 can directly be used from the atmosphere as well as from emissions of industrial power plants. However, only a few microalgae such as *Chlorella* sp. can efficiently grow on CO_2 concentrations higher than 5 %[25]. Nitrogen and phosphorous are usually assimilated in inorganic forms such as nitrates, nitrite, ammonium, and phosphate. Some species can also absorb organic nitrogen sources such as urea, glycine and yeast extract[26]. Micronutrients like Mo, K, Fe, Mg, Mn, Zn also show beneficial effects on microalgal growth due to their influence on enzymatic activities in microalgal cells[16].

Photosynthesis takes place in the thylakoid membranes of chloroplasts and is generally based on two main steps including light-dependent and dark reactions. In the presence of light, solar energy (photons) is converted into adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH). The energy can then be used for CO_2 fixation and synthesis of carbon skeletons during the dark period (Calvin-Benson cycle)[27]. The conversion rate from solar to chemical energy is called photosynthetic efficiency (PE) being a crucial parameter of photoautotrophic cultivation.

During the light-dependent reaction, light-harvesting chlorophyll antenna capture light energy and pass it to reaction centres; the excitation energy splits water molecules generating O_2 and chemical energy through an electron transport chain[25,26]. Chlorophyll is the most significant pigment for oxygenic photosynthesis, carotenoids or phycobilins are accessory pigments[27]. Chlorophyll absorbs light in the red (600-700 nm) and blue (400-500 nm) range, leaving a “green gap” (500-600 nm). While most higher plants are only able of using light in the spectrum of 400 nm to 700 nm, microalgae possess different chlorophyll types enabling the usage of light below 400 nm and above 700 nm. The variety of pigments in microalgae is one of the reasons for a higher PE than in plants[26]. Depending on the microalgal species and their pigments, certain wavelengths are effective for their photosynthesis. In terms of microalgal cultivation, spectral quality must be considered due to their impact on biomass productivity and composition. For instance, Blair et al.[28] found higher growth rates of *C. vulgaris* in white or blue light compared to red and green light.

Apart from spectral composition, PE is affected by light intensity and frequency. For most microalgae, optimal light intensity varies between 10 to 250 µmol/(m^2*s). In comparison, the intensity of sunlight reaches up to 2000 µmol/(m^2*s)[29]. Excess of irradiance can be harmful resulting in photosynthetic stress (photoinhibition) and the generation of toxic reactive oxygen species (ROS), which damage the photosynthetic machinery and reduce biomass yield. To

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react to this, microalgae activate the non-photochemical quenching (NPQ) mechanism inducing a de-excitation of chlorophylls[8]. Moreover, accessory pigments such as carotenoids or xanthophylls protect the photosynthetic machinery by emitting light energy as heat instead of passing it to reaction centres[26]. In terms of light frequency, specific durations of light and dark periods are required depending on the microalgal species and target product. The optimal photoperiod is also related to the applied light intensity: Longer light periods should be combined with lower light intensities, and shorter periods require higher intensities[27].

While light is the limiting factor of PE at low irradiance, the CO_2 fixation rate becomes the limiting parameter at higher irradiance. CO_2 is fixated by the enzyme Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO). However, RuBisCO also has an affinity to O_2 leading to ATP/NADPH consumption and loss of fixed carbon (photorespiration)[8]. The extent of photorespiration is primarily dependent on the O_2 concentration, which is why efficient gas exchange is required in photobioreactors to avoid assimilation of O_2 [26]. Effective mixing is also required to avoid the so called “self-shading effect”, which describes the issue that cells at lower layers are shaded from cells at upper layers. Uniform light distribution is a major problem in photoautotrophic cultivation for achieving high cell densities[16].

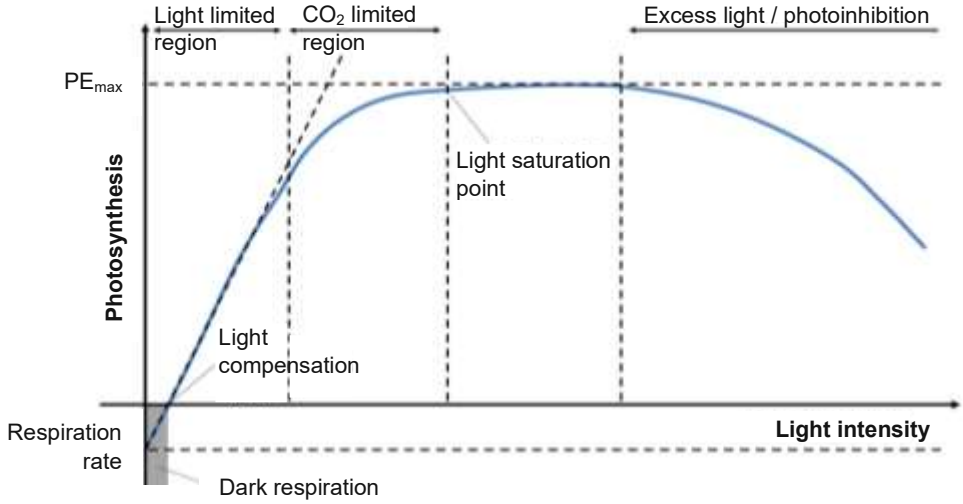

Figure 2: Light response for photosynthesis[8]

1.3.3 Heterotrophy

Most green microalgae are capable of assimilating organic carbon under aerobic respiration[30]. In contrast to photoautotrophic cultivation, energy is provided by organic carbon instead of light. The independence of light can remarkably reduce production costs and space when using low-cost carbon sources[5]. Heterotrophic regime overcomes light penetration challenges resulting in easier scale-up and higher biomass productivities (up to 100 g/L in scale-up systems[20]). Heterotrophic cultivation can be conducted in conventional closed fermenters[5] enabling controlled culture conditions. Since the carbon source is a major cost factor, several studies investigated the heterotrophic growth of various microalgal species using different low-

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cost carbon sources (Table 4). The usage of pure glucose as carbon source can comprise up to 60 % of the overall production costs[31]. The replacement of glucose by waste products can reduce the environmental impact up to 40 %[32]. Waste products such as glycerol from biodiesel production[33], whey or molasses from industrial wastewater[31], urban[34] and domestic[35] wastewater, food waste[36], or carbohydrates originated from agricultural waste[37] were tested as inexpensive substrate for microalgal cultivation. Table 4 summarises the potential of some waste substrates by comparing the biomass concentrations of *C. vulgaris* to the cultivation in glucose under heterotrophic conditions.

Table 4: Biomass concentrations of *C. vulgaris* under heterotrophic conditions in different media

Medium	Biomass (g/L)	Reference
Glucose (10 g/L)	1.2	38
Wheat bran	3.4	39
Sweet sorghum hydrolysate	2.7	37
Rice straw hydrolysate	3.1	37
Food waste	3.8	40
Soy whey	2.5	41
Ethanol Thin Stillage	5.3	41

Besides numerous advantages, the usage of organic carbon under aerobic respiration results in CO_2 generation instead of fixation[42] and contamination risks are higher when adding an organic substrate. Another issue is the biochemical composition of microalgae. In terms of the food and feed market, protein and pigment content are significant features. However, protein and pigment synthesis in heterotrophically grown microalgae is commonly lower compared to photoautotrophic or mixotrophic conditions[31,43].

1.3.4 Mixotrophy

Mixotrophic microalgae are able of simultaneously using inorganic carbon by photosynthesis and organic carbon through aerobic respiration (Figure 3)[30]. The mixotrophic regime should not be confused with species that are only able of switching between the photoautotrophic and heterotrophic metabolism. However, mixotrophy enables the flexibility of switching the cultivation mode to heterotrophy or photoautotrophy at will[25].

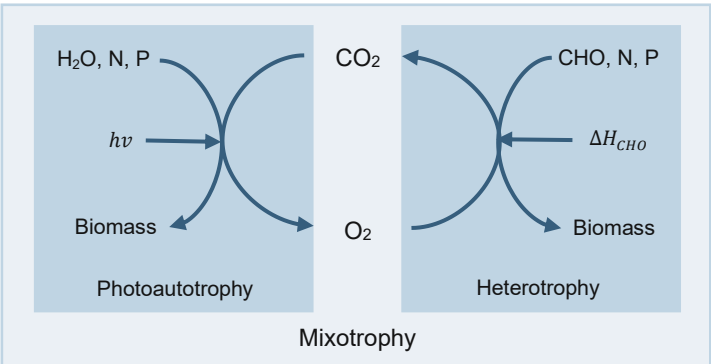

Figure 3: Schematic process of mixotrophy[30]

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Since mixotrophic microalgae are not strictly dependent on light or carbon sources for their growth, the metabolic flexibility offers advantages such as overcoming light limitation, prolonged exponential growth phase and increased biomass concentrations[25]. Compared to photoautotrophic cultivation, lower light intensities and shortage of photoperiod are sufficient reducing lighting energy costs[10]. Due to the consumption of O_2 in the heterotrophic metabolism, mixotrophic microalgae have a higher tolerance to photo-oxidative damage and the accumulation of toxic levels of O_2 is prevented[25].

Alike in heterotrophic cultivation, the carbon source can be provided by wastewater to increase the sustainability and minimise the need for freshwater, external nutrients, herbicides and pesticides[5,6]. Table 5 shows the potential of some waste products by comparing the biomass concentrations of mixotrophically grown *C. vulgaris* to the cultivation in glucose.

Table 5: Biomass concentrations of *C. vulgaris* under mixotrophic conditions in different media

Medium	Biomass (g/L)	Reference
Glucose (10 g/L)	1.4	44
Wheat bran hydrolysate	3.7	39
Corn steep liquor	2.1	45
Food waste hydrolysate	1.7	36
Dairy wastewater	1.5	46
Glycerol	1.7	33
Soy whey	5.0	41
Ethanol Thin Stillage	8.3	41

Compared to photoautotrophic conditions, the efficacy of nutrient removal from urban, agricultural or industrial wastewater by microalgae is enhanced under heterotrophic and mixotrophic regime[25]. Reduced amounts of substrate are sufficient in mixotrophic regime compared to heterotrophy[1]. While CO_2 is not fixated in heterotrophic mode, part of the CO_2 release can be compensated in mixotrophic cultivation by performing photosynthesis. However, only a limited number of microalgae are mixotrophic and their growth does not necessarily result in higher biomass concentrations than in photoautotrophic conditions. Examples of mixotrophic microalgae are the freshwater *Chlorella* spp., *Nannochloropsis* spp., *Chlorococcum* sp., or the cyanobacteria genera *Spirulina* and *Synechococcus*[25].

1.4 *Chlorella vulgaris*

The eukaryotic green algae *Chlorella vulgaris* evolved about 2.5 billion years ago and was discovered in 1890 by Martinus Willem Beijerinck. A decade later *C. vulgaris* gained attention due to its high protein content being considered a promising unconventional protein source[1]. Until today, *C. vulgaris* surpasses other microalgae in the food and feed market due to its highly nutritional biomass[6].

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1.4.1 Morphology and reproduction

C. vulgaris is a non-motile, spherical cell with a diameter of 2-10 µm naturally occurring in aquatic environments such as oceans, lakes, or ponds[47]. Cells reproduce asexually *via* auto-sporulation. Under favourable conditions, one mother cell can multiply into four daughter cells within a day. When the mother cell wall ruptures and releases daughter cells, the remaining debris serves as feed for the newly formed cells[1]. The schematic structure of a *C. vulgaris* cell is depicted in Figure 4. The gel-like cytoplasm covers internal organelles such as the nucleus, mitochondria, vacuoles, chloroplasts, or the Golgi body[1].

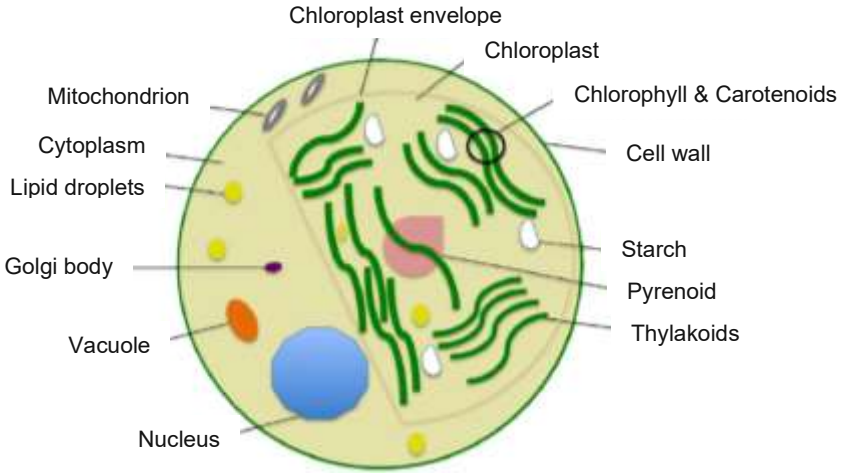

Figure 4: Schematic structure of *C. vulgaris*[1]

The chloroplast contains elements needed for the photosynthetic apparatus: The pyrenoid consists of the enzyme RuBisCO, being responsible for CO_2 fixation[1]. Chlorophyll is produced in thylakoids, typically in higher amounts compared to various plants[3]. Under stress conditions, chloroplasts store starch granules making it the most occurring carbohydrate[1] in *C. vulgaris* biomass.

The cell wall of a mature cell consists of a chitosan-like layer composed of glucosamine. The rigidity of the cell wall serves for the integrity of the cell and protection against harsh conditions. However, cell wall composition and thickness change according to the growth phase and environmental conditions[1].

1.4.2 Biochemical properties for the food and feed market

C. vulgaris is known for its high protein content reaching up to 60 % of DCW[2]. The amino acid profile is well balanced and even better compared to the standard profile for human nutrition recommended by World Health Organisation (WHO)[1]. In addition to the great protein content, *C. vulgaris* shows elevated chlorophyll amounts ranging from 1 to 4 % of DCW[48]. The pigment provides health benefits such as antioxidative, antimutagenic, and anticarcinogenic activities. Moreover, *C. vulgaris* contains essential polysaccharides such as α- and β-glucan showing

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antioxidative, anticarcinogenic, or immune-stimulating properties[1,8]. *C. vulgaris* exhibits small amounts of free fatty acids, but their profile can be rich in PUFAs being suitable for nutritional uses[1,12]. Generally, the biomass of *C. vulgaris* is considered functional food providing health benefits such as lowering blood pressure and cholesterol levels, enhancing the immune system and wound healing or tumour prevention[48].

1.4.3 Cultivation

C. vulgaris is a photoautotrophic, heterotrophic, and mixotrophic microalgae that has been cultivated in both open- and closed systems[1]. Different approaches have been studied to specifically target biomass, lipid, protein, carbohydrate, or pigment production. Also, two-stage cultivation processes were tested, where two cultivation modes are consecutively applied[47,49]. Depending on the cultivation environment, growth rates of *C. vulgaris* were higher under mixotrophic[41,50], or under heterotrophic conditions[43,51]. Carbon sources such as glucose, acetate, glycerol, and glutamate were studied, with glucose achieving highest growth rates[1].

1.5 Lignocellulosic biomass

The advancing depletion of the world's resources strongly demands the usage of renewable materials. Plant biomass represents the most abundantly renewable material on Earth[52]. Lignocellulosic waste offers chemicals such as carbohydrates, lignin, and carboxylic acids[53], which can be used as feedstock for cultivating bacteria, fungi, yeasts, and microalgae to produce renewable energies (bioethanol, biogas, biohydrogen) or other valuable products[54]. Various agricultural residues such as rice straw, sugar cane bagasse, sweet sorghum, or woody raw materials have been processed[55].

1.5.1 Composition of wheat straw

Among agricultural wastes, wheat straw has a remarkably high potential as raw material for future biorefineries due to its broad availability and low cost. Annually, around 680 million tons of wheat is produced, while the yield of the by-product wheat straw is around 1.35 kg/kg of wheat grain[52]. Wheat straw mainly consists of cellulose (35-45 %), hemicellulose (20-30 %), and lignin (~15 %)[55]. Cellulose is a non-branched, β-(1,6)-glycosidic linked polymer containing D-glucose units. Conversely, hemicellulose is a non-homogeneous polymer consisting of sugar units such as xylose, arabinose, mannose, glucose, and galactose, which are connected *via* β-(1,4)-glycosidic linkages[20]. In straw, the main component of hemicellulose is xylan[56]. Lignin is a complex polymer including phenylpropanoid units (hydroxyphenyl, guaiacyl, syringyl) bonded *via* ether and carbon-carbon linkages. Apart from these main components, wheat straw contains pectin, proteins, lipids, minerals, terpenoids, polyphenols, and alkaloids[20]. Variances of the composition are not only dependent on the agricultural residue itself but also on growth conditions (e.g., climate, seasonal changes, handling by the farmer)[57].

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1.5.2 Hydrolysation of lignocellulosic biomass

Lignocellulosic material comprises up to 75 % of sugars, but their polymerised state makes the direct use for most fermentation processes unfeasible[57]. To release sugars from cellulose and hemicellulose, a pre-treatment is required[58]. Firstly, lignocellulosic biomass is mechanically (mill, screw press) or physically (steam explosion) loosened to enhance subsequent hydrolysis. Since the pre-treatment constitutes between 20-40 % of the overall production costs, numerous chemical and physical hydrolysation techniques have been studied (e.g., dilute acid, alkaline, steam explosion, organic solvents)[59]. One cost-effective and economically friendly method is the so-called liquid hot water (LHW) treatment, where hydro-thermolysis is induced at elevated temperature (160-240 °C) and pressure to maintain water in the liquid state[59]. LHW extraction results in a solid stream and an aqueous side stream. While the solid stream primarily consists of cellulose and lignin, the aqueous side stream contains dissolved hemicelluloses and acetic acids being a potential feedstock for microbial fermentation processes. The severity of the hydrolysation is based on water temperature and time. Higher temperature and time enhance the efficiency, but also the formation of inhbitory degradation products[60]. Depending on the lignocellulosic biomass and the pre-treatment, typical by-products are aliphatic acids (formic-, acetic-, levulinic acid), furan derivates (5-hydroxymethylfurfural (HMF), furfural (FF)), and phenolic substances (phenol, vanillin, p-hydroxybenzoic acid (HBA))[61]. Acetic acid originates from hydrolysation of hemicellulose, furans are formed upon the degradation of sugars, and phenols upon degradation of lignin polymers[57]. A compromise has to be found, where hemicellulose is sufficiently dissolved and the formation of degradation products is minimised. Typically, hydrolysates are detoxified before microbial fermentation to avoid inhibitory effects by using different techniques such as solvent extraction, ion-exchange chromatography, enzymatic detoxification, or nanofiltrations. However, the removal of inhibitory by-products is another time-consuming and expensive process step[55]. After hydrolysis, enzymatic treatments (cellulases and hemicellulases derived from fungi and bacteria) can be applied to further increase saccharification efficiency by 65 % to 100 %[59]. To conclude, the conversion from lignocellulose into value-added products includes many steps starting from the pre-treatment, detoxification, enzymatic treatment, fermentation, and downstream processing (Figure 5).

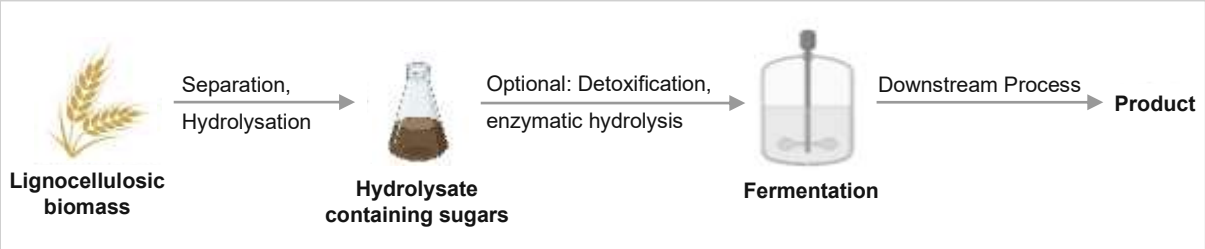

Figure 5: Conversion from lignocellulosic biomass to product

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1.5.3 Hydrolysates and microalgae

Although lignocellulosic hydrolysates are a promising feedstock for microorganisms, commercial usage is currently limited on yeast fermentation for bioethanol production. This can mainly be traced back to the lack of microorganisms, which are able of enduring inhibitors in the hydrolysate[62]. So far, the impact of numerous lignocellulosic-related compounds has been studied separately on microalgal cultivation. Positive effects of sugars and acetates on microalgal growth have been found[20]. Although xylose is one of the main sugars in lignocellulosic hydrolysates, the report on xylose uptake in microalgae is limited[63]. Only a few microalgal strains can utilise xylose, and is possibly limited on the genus *Chlorella*[25]. Phenolic compounds showed either stimulatory or inhibitory effects on microalgae depending on the structure and microalgal strain. For instance, o-HBA and p-HBA (13.8 mg/L) had a stimulating effect on *C. vulgaris* growth, while m-HBA resulted in growth inhibition[64]. Vanillic acid (16.8 mg/L) showed a promoting impact on growth of *C. pyrenoidosa*, while syringic acid (19.8 mg/L) was inhibitory[65]. Also, the effect of furans on microalgal growth was scarcely investigated[20]. Yu et al.[66] studied the impact of HMF and FF on photoautotrophic *Spirulina maxima* growth and discovered that full inhibition for FF was reached at 0.67 g/L and for HMF at 1.13 g/L.

Only a limited number reported about the cultivation of microalgae in lignocellulosic hydrolysates. However, lignocellulosic hydrolysates have the potential to stimulate microalgal growth under mixotrophic and heterotrophic conditions. The expedient combination of microalgal strain, cultivation conditions, agricultural waste product, and its pre-treatment technique has yet to be found[20].

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2 Objective

The increasing population and anthropogenic CO_2 emissions joined with the depletion of the world's resources strongly demand alternative food and feed sources and the usage of renewable materials. The green microalgae *Chlorella vulgaris* both fixates the greenhouse gas CO_2 through photosynthesis and simultaneously generates highly nutritional biomass. However, the realisation of its potential as a food and feed source requires a reduction of production costs. Since the organic carbon source can comprise up to 80 % of costs[40], reprocessing lignocellulosic waste streams into *C. vulgaris* biomass has the potential to reduce overall production costs and enhance sustainability.

In this thesis, a new approach of using wheat straw hydrolysates (WSH) as a renewable substrate for heterotrophic and mixotrophic cultivation of *C. vulgaris* is studied. Besides maximising the exploitation of wheat straw, the purpose of using WSH as a substrate for *C. vulgaris* cultivation is the increase economic feasibility and the minimisation of the environmental impact (waste-to-value). Thus, the aims of this thesis are:

- Find an appropriate dilution of WSH for *C. vulgaris* cultivation in terms of growth rates and biomass composition in shake flasks.
- Determine growth parameters under mixotrophic and heterotrophic conditions.
- Compare the obtained results in WSH to the cultivation in defined media and to a photoautotrophic control culture.
- Perform batch fermentation of *C. vulgaris* in a suitable concentration of WSH and compare to fermentation in defined media and to photoautotrophic fermentation.
- Analyse biomass composition for its protein, chlorophyll, and carbohydrate content to evaluate suitability for food and feed purposes.
- Investigate whether toxic compounds (HMF, FF) of WSH are taken up by *C. vulgaris* to determine suitability for nutritional use.

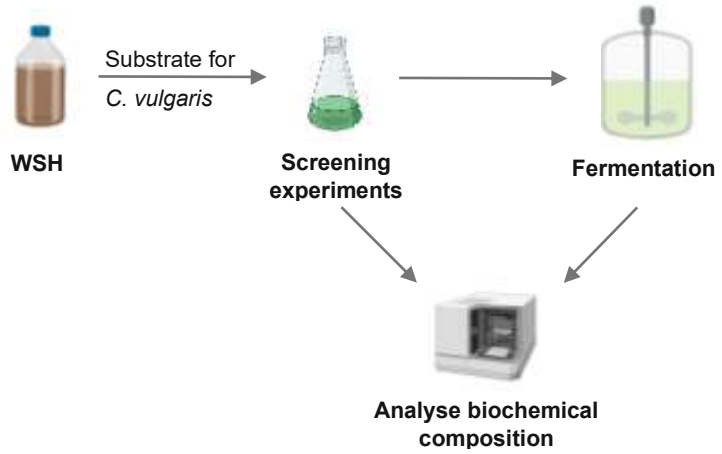

Figure 6: Objective of the thesis

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3 Materials and Methods

3.1 Chemicals and algal strain

Unless otherwise stated, chemicals for cultivation and biomass analysis were purchased from Carl Roth (Karlsruhe, Germany). The WSH was supplied from TU Vienna Institute of Chemical Engineering. The green algae *C. vulgaris* UTEX 2714 strain was obtained from Culture Collection of Algae at the University of Texas Austin.

3.1.1 BG-11 medium

Stock solutions for the BG-11 medium and the volume needed for preparing 1 L are listed in Table 6 and Table 7, respectively. All solutions were prepared with ultrapure water, which was provided by a Standard arium mini system from Sartorius (Goettingen, Germany). Before autoclaving, the pH was set to 8 for shake-flasks experiments and to 7.5 for batch fermentation. The pH was measured using a WTW SenTix 41 pH electrode from Thermo Fisher Scientific (Waltham, MA, USA). The medium was autoclaved for 20 min at 120 °C. Subsequently, Na_2CO_3 and Fe-Ammonium-Citrate were added by sterile filtration through a 0.22 µm filter.

Table 6: Stock solutions for BG-11 medium

Stock solution	Substance	Concentration (g/L)
BG-11 solution 100x	$MgSO_4$ x 7 H_2O	7.50
	$CaCl_2$ x 2 H_2O	3.60
	Citric Acid	0.60
Trace-Metal-Mix 1000x	H_3BO_3	2.86
	$MnCl_2$ x 4 H_2O	1.80
	$ZnSO_4$ x 7 H_2O	0.22
	Na_2MoO_4 x 2 H_2O	0.39
	$CuSO_4$ x 5 H_2O	0.08
	$Co(NO_3)_2$ x 6 H_2O	0.05
HEPES (pH 8)	HEPES	238
K_2HPO_4 solution	K_2HPO_4	40.0
EDTA solution	Na_2EDTA x 2 H_2O	0.87
Na_2CO_3 solution	Na_2CO_3	106
Fe-Ammonium-Citrate	Fe-Ammonium-Citrate	6.00

Table 7: Amounts of stock solution for preparing 1 L BG-11

Stock solution	Volume (mL)
BG-11 solution 100x	10
Trace-Metal-Mix 1000x	1
HEPES (pH 8)	10
K_2HPO_4	1
EDTA-solution	1
Na_2CO_3	1
Fe-Ammonium-Citrate	1

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3.1.2 Wheat straw hydrolysate

The wheat straw was hydrolysed by LHW extraction at 160 °C for 90 min. The obtained hydrolysate was concentrated, but neither detoxified nor enzymatically treated. The final concentration of sugars and degradation products of the concentrated WSH is listed in Table 8 and Table 9, respectively.

Table 8: Sugar concentration of WSH

	Sugar	Conc. (g/L)	SD (g/L)	Error (%)
Monomeric Sugar	Arabinose (C5)	1.63	0.06	3.64
	Glucose (C6)	0.05	0.01	24.76
	Xylose (C5)	1.30	0.06	4.61
	Summed C5-sugars	2.93	0.08	2.88
	Summed C6-sugars	0.05	0.01	24.76
Total Sugar	Arabinose (C5)	5.31	0.56	10.62
	Galactose (C6)	3.51	0.43	12.13
	Glucose (C6)	7.78	0.46	5.97
	Xylose (C5)	41.97	2.97	6.65
	Mannose (C6)	2.44	0.28	11.51
	Summed C5-sugars	47.28	2.85	6.02
	Summed C6-sugars	13.72	0.69	5.02

Table 9: Concentration of degradation products of WSH

Degradation product	Conc. (mg/L)	SD (mg/L)	Error (%)
Acetic Acid	4334	130	3.01
HMF	73.82	4.19	5.67
FF	10.82	3.88	35.86

3.1.3 Defined medium

The defined medium (DM) stock solution was based on the monomeric sugar and furan concentration of the WSH. The composition of the DM stock is presented in Table 10.

Table 10: DM stock solution

Sugar	Concentration (g/L)
Arabinose	1.63
Glucose	0.05
Xylose	1.30
HMF	0.07
FF	0.01

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3.2 Shake flask cultivation of *C. vulgaris*

Preparation of experiments and sampling was performed under the laminar hood. Shake flasks experiments in WSH and DM were performed in triplicates in 250 mL Erlenmeyer flasks containing 100 mL medium. Conversely, HMF/FF cultures were conducted in 100 mL Erlenmeyer flasks containing 20 mL medium.

3.2.1 Pre-culture

Shake flasks containing BG-11 medium (pH 8) were inoculated with *C. vulgaris* UTEX 2714 strain. Subsequently, the pre-cultures were incubated at 23 °C and 3 % (v/v) CO_2 supply. A 14/10 h light/dark period was set under exposure of 26.4 µmol/(m^2*s) light intensity.

3.2.2 Heterotrophic culture

Based on the total xylose content of the WSH, 1 g/L, 2 g/L, 5 g/L, and 7 g/L were tested, which corresponds to a 2.5 %, 5.0 %, 12.5 %, and 17.5 % (v/v) dilution of the hydrolysate (Table 11). The respective amount of concentrated WSH was sterile-filtered (0.4 µm pore size) into shake flasks containing BG-11 medium (pH 8). Since higher concentrations of the acidic WSH lowered the pH of the medium in a range that was not optimal for *C. vulgaris*, the pH of 12.5 % and 17.5 % WSH was adjusted to 8 prior to sterile filtration into shake flasks.

Subsequently, photoautotrophic *C. vulgaris* pre-culture was added achieving a starting OD_{600} value of 0.10. Triplicates were incubated in darkness at room temperature. 1 mL of sample was taken after 0, 4, 13, 20, 27, 37 and 48 days in terms of 2.5 % and 5.0 % WSH cultures, and after 0, 4, 10, 17, 24, 30, 38 and 46 days concerning 12.5 % and 17.5 % WSH. After finishing the heterotrophic cultivation, shake flasks were illuminated at 50 µmol/(m^2*s) for 10 more days to determine the impact of light on the biomass composition.

Table 11: Concentration of compounds of WSH in tested experiments

		2.5 %	5.0 %	12.5 %	17.5 %
Monomeric sugar (mg/L)	Arabinose	40.75	81.50	203.75	285.96
	Glucose	1.25	2.50	6.25	8.77
	Xylose	32.43	64.85	162.13	227.54
Total sugar (g/L)	Arabinose	0.13	0.27	0.66	0.93
	Galactose	0.09	0.18	0.44	0.62
	Glucose	0.19	0.39	0.97	1.36
	Xylose	1.05	2.10	5.25	7.36
	Mannose	0.06	0.12	0.30	0.43
Degradation product (mg/L)	Acetic acid	108.35	216.70	541.76	760.37
	HMF	1.85	3.69	9.23	12.95
	FF	0.27	0.54	1.35	1.90

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The same procedure was performed for the cultivation of *C. vulgaris* in the DM. Instead of adding WSH, the respective amount of the DM stock solution was used to achieve a dilution of 2.5 %, 5.0 %, 12.5 %, and 17.5 % (v/v) of the sugar concentrations. 1 mL of sample was transferred into a 1.5 mL Eppendorf tube after 0, 7, 14, 21, 29, 34 and 43 days of cultivation.

3.2.3 Mixotrophic culture

Based on the total xylose content of the WSH, 1 g/L and 2 g/L were tested, which corresponds to a 2.5 % and 5 % (v/v) dilution of the hydrolysate. The respective amount of the concentrated WSH was sterile-filtered (0.4 µm pore size) into shake flasks containing BG-11 (pH 8) medium. Then, photoautotrophic *C. vulgaris* pre-culture was added obtaining a starting OD_{600} value of 0.10. Triplicates were incubated at 23 °C, 3 % (v/v) of CO_2 supply, a 14/10 h light/dark cycle, and under a light intensity of 26.4 µmol/(m^2*s). 1 mL of sample was transferred into a 1.5 mL Eppendorf tube after 0, 7, 14, 22 and 29 days of cultivation.

The same procedure was applied for the cultivation of *C. vulgaris* in DM, using the respective amounts of the DM stock instead of the concentrated WSH.

3.2.4 Phototrophic culture

The respective amount of the photoautotrophic *C. vulgaris* pre-culture was transferred into BG-11 (pH 8) medium achieving a starting OD_{600} value of 0.10. Shake flasks were incubated at 23 °C and 3 % (v/v) of CO_2 supply. A 14/10 h light/dark cycle was set with exposure of 26.4 µmol/(m^2*s) light intensity. 1 mL of sample was transferred into a 1.5 mL Eppendorf tube after 0, 7, 14, 22, and 29 days of cultivation.

3.2.5 HMF/FF culture

C. vulgaris culture was tested in BG-11 (pH 8) medium modified with 1 g/L glucose, 1 g/L xylose, and 200 mg/L furans (HMF and FF). For control purposes, *C. vulgaris* was cultured in BG-11 containing 1 g/L glucose and 1 g/L xylose, without the addition of furans. Triplicates were incubated for two weeks under both heterotrophic (darkness, room temperature) and mixotrophic conditions (23 °C, 3 % (v/v) CO_2, 26.4 µmol/(m^2*s) light intensity). 1 mL of sample was transferred into a 1 mL Eppendorf tube at the beginning and after 2 weeks of cultivation.

3.3 Batch fermentation of *C. vulgaris*

3.3.1 Reactor setup

Batch fermentation was performed in a 2 L Minifors 2 bioreactor from Infors HT (Basel, Switzerland). The bioreactor showed multiple ports to control process parameters such as pH, temperature, agitation, dO_2, air inlet flow, CO_2 inlet flow, outlet CO_2 and O_2 mass flow. Fermentation parameters were set and monitored by the bioprocess platform software Eve from Infors HT (Basel, Switzerland).

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The autoclavable components of the BG-11 medium were transferred into the bioreactor. The pH electrode was calibrated by two-point calibration (pH 7, pH 4) and was then installed together with a dO_2 electrode, septum, condenser, inlet gas filter, and tubes for sampling, aeration, and base feed. The base used in this experiment was 1 M Na_2CO_3. The reactor and the base were autoclaved at 120 °C for 20 min.

After autoclaving, the gas condenser was connected to cooling water and to the previously calibrated mass flow controller (MFC). The connection between the MFC and the condenser included an off-gas filter and a Schott flask to prevent the entry of liquids or foam into the MFC. As soon as the medium cooled to 23 °C, the dO_2 electrode was calibrated by one-point calibration (100 %). Subsequently, 1M Na_2CO_3 and Fe-Ammonium-Citrate were added by sterile filtration (0.22 µm pore size) through the septum.

3.3.2 Cultivation conditions

Fermentation was conducted in mixotrophic, heterotrophic and photoautotrophic regime. The respective amount of the concentrated WSH or DM stock solution were added by sterile filtration (0.4 µm for WSH, 0.22 µm for DM) through the septum, achieving a dilution of 2.5 % (v/v). Subsequently, *C. vulgaris* pre-culture was inoculated through the septum reaching a starting OD_{600} value of 0.10. The parameters of the batch fermentation are summarised in Table 12. Light was supplied by a 0.25 m^2 LED panel supplying a light intensity of about 150 µmol/(m^2*s). 15 mL of sample was taken through a sampling port after 4, 7, 13, 17, 21, 25 and 28 days of inoculation using a 20 mL syringe. Samples were then transferred into 15 mL Falcon tubes.

Table 12: Parameters of batch fermentation of *C. vulgaris*

	Heterotroph	Mixotroph	Phototroph
Medium	BG-11	BG-11	BG-11
Carbon source	2.5 % WSH/DM	2.5 % WSH/DM	-
Starting OD_{600}	0.10	0.10	0.10
Reactor volume (L)	1	1	1.5
Starting pH	7.5	7.5	7.5
Temperature (°C)	23	23	23
Agitation speed (rpm)	300	300	300
Airflow (L/min)	0.5	0.5	0.5
CO_2 flow (L/min)	-	0.02	0.02
Light intensity (µmol/m^2*s)	-	150	150
Light/dark period (h)	-	14/10	14/10

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3.4 Sampling and harvest

First, optical density (OD_{600}) was photometrically measured at 600 nm using a NanoDrop One spectrophotometer from Thermo Fisher Scientific (Waltham, MA, USA). The OD_{600} of the sample was measured in a 1 mL cuvette against a blank solution, which contained the medium and the WSH in the appropriate concentration to account for the absorption of the WSH. Cultures showing an absorbance outside the linear range were diluted accordingly.

Subsequently, samples were centrifuged in 1.5 mL Eppendorf tubes at 16 000 rcf for 10 min at room temperature using an Eppendorf Centrifuge 5415 R (rotor: 85720). The supernatant was further analysed in terms of HPLC (chapter 3.5.3, 3.5.4) and pH measurement using a Bioblock scientific micro pH electrode from Thermo Fisher Scientific (Waltham, MA, USA). The remaining biomass pellet was stored at -20 °C until carbohydrate extraction was conducted. In terms of WSH samples, biomass was additionally washed three times with saline.

At the end of each experiment, the remaining culture was centrifuged in 50 mL Falcon tubes at 14 000 rcf for 10 min at 4 °C using a Sigma 3-30 K (rotor: 19776-H). While the supernatant was stored at -20 °C, the biomass pellet was frozen in liquid N_2 for subsequent lyophilisation at 0.02 mbar and -49 °C using a Freezone 2.5 Benchtop Freeze Dry System from Labconco (Kansas City, MO, USA). The freeze-dried biomass was then stored at -20 °C until protein and chlorophyll extraction were performed.

3.5 Growth parameters

3.5.1 Biomass concentrations

To determine the biomass concentration, a calibration was created between OD_{600} and the dry cell weight (Figure 7).

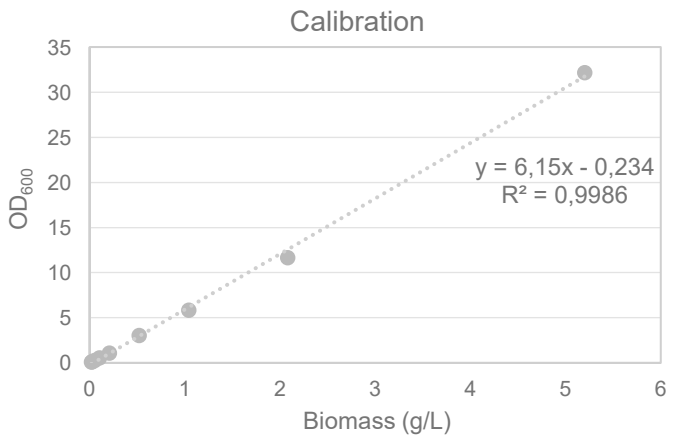

Figure 7: Dry cell weight calibration

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3.5.2 Specific growth rates

Specific growth rates were calculated using Equation 1:

$$\mu = \frac{1}{t} * \ln\left(\frac{c_{x,t}}{c_{x,0}}\right)$$

Equation 1

where μ represents the specific growth rate (day^{-1}), t is the cultivation time (day) and c_x the biomass concentration (g/L) at given time points (day).

3.5.3 Sugar concentrations

Sugars were quantified by high-performance liquid chromatography (HPLC) using a Vanquish Core system from Thermo Fisher Scientific (Waltham, MA, USA). Sugars were separated by an Aminex HPX-87H carbohydrate column and a guard column (Bio-Rad, Hercules, CA, USA) at a column temperature of 60 °C and pressure of ~65 bar. The mobile phase was 4 mM H_2SO_4 at a flow rate of 0.6 mL/min and elution was isocratic. The analyte was detected by a refractive index (RI) detector (Ercatech AG, Bern, Switzerland). Measurement was completed after 30 min and chromatograms were recorded and analysed using the software Chromeleon Studio 7.2.10 from Thermo Fisher Scientific (Waltham, MA, USA).

Calibration of standard series of sugars enabled the quantification of samples. However, the complex WSH medium resulted in shifted peaks. The largest peak of WSH chromatogram was attributed to xylose being the main component of the WSH, but other compounds were not separatable with the used method. Conversely, xylose and arabinose peaks in the DM chromatogram were determinable. Glucose and furan were not quantifiable due to their low concentrations (< 13 mg/L).

3.5.4 Furan concentrations

Furans (HMF and FF) were quantified by HPLC measurement. The same parameters as described in 3.5.3 were used, except for the fact that the analyte was detected by an ultraviolet detector at 277 nm (Thermo Fisher Scientific, Waltham, MA, USA) and that the measurement was completed after 50 min. Calibration of standard series of HMF and FF enabled the quantification of samples.

3.5.5 Off-gas analytics

While the O_2 production rate (OPR) and CO_2 uptake rate (CUR) were evaluated concerning mixotrophic and photoautotrophic conditions, the CO_2 evolution rate (CER) and O_2 uptake rate (OUR) were used for heterotrophic cultivation. Determination of these parameters is based on the general mass balance (Equation 2):

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$$\dot{V}_{i,in}\, c_{i,in} - \dot{V}_{i,out} c_{i,out} + V_R r_i = c_i \frac{\partial V_R}{\partial t} + V_R \frac{\partial c_i}{\partial t}$$

Equation 2

where $\dot{V}_i$ is the gas flow of component i (L/day) and c_i is the concentration of i (mol/L) inside or outside the reactor. V_R represents the reactor volume (L), r_i is the volumetric reaction rate of i (mol/(L*day)) and t is the processing time (day).

Although a batch fermentation is characterised by constant reactor volume, medium loss due to sampling and evaporation were taken into account. When assuming that the gas inflow rate is equal to the outflow rate, the general mass balance can be simplified to Equation 3:

$$r_i = \frac{\dot{V}_{i,in}\left(c_{i,in} - c_{i,out}\right)}{V_{R,t}}$$

Equation 3

Because the off-gas was measured in volumetric percentages and the airflow was supplemented with pure CO_2 gas, the following equations were used for calculating CUR (Equation 4) and OPR (Equation 5):

$$CUR = r_{CO2} = \frac{\dot{V}_{air,in} * vol\%_{CO2,in} + \dot{V}_{CO2,in} * 100 - (\dot{V}_{air,in} + \dot{V}_{CO2,in}) * \ vol\%_{CO2,out}}{V_{R,t} * V_M * 100}$$

Equation 4

$$OPR = r_{O2} = \frac{(\dot{V}_{air,in} + \dot{V}_{CO2,in}) * vol\%_{O2,out} - \dot{V}_{air,in} * vol\%_{O2,in}}{V_{R,t} * V_M * 100}$$

Equation 5

where CUR and OPR represent the volumetric CO_2 uptake rate and O_2 production rate (mol/(L*day)). $\dot{V}_{air}$ and $\dot{V}_{CO2}$ are the air and CO_2 gas flow rates (L/day), $vol\%_{CO2}$ and $vol\%_{O2}$ are the volumetric percentages of CO_2 and O_2 inside and outside the reactor (vol%). $V_{R,t}$ is the reactor volume (L) at process time t (day).

In terms of heterotrophic cultivation, the following equations were used for the calculation of CER (Equation 6) and OUR (Equation 7):

$$CER = r_{CO2} = \frac{\dot{V}_{air,in} * (vol\%_{CO2,out} - vol\%_{CO2,in})}{V_{R,t} * V_M * 100}$$

Equation 6

$$OUR = r_{O2} = \frac{\dot{V}_{air,in} * (vol\%_{O2,in} - vol\%_{O2,out})}{V_{R,t} * V_M * 100}$$

Equation 7

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Since the beginning, the CO_2 off-gas was lower and the O_2 off-gas was higher than expected. Thus, a correction factor was taken into account to obtain volumetric rates starting from zero. The respective factor (Equation 8, Equation 9) was added to the CO_2 off-gas and subtracted from the O_2 off-gas data.

$$F_{CO2} = vol\%_{CO2,in} - vol\%_{CO2,out}$$

Equation 8

$$F_{O2} = vol\%_{O2,out} - vol\%_{O2,in}$$

Equation 9

Finally, the division of the production rate by the uptake rate delivers the respiratory quotient (RQ):

$$RQ = \frac{OPR}{CUR} = \frac{CER}{OUR}$$

Equation 10

3.6 Biomass analytics

3.6.1 Protein content

Protein extraction of microalgae is mostly performed by solubilisation in alkaline conditions followed by precipitation with trichloroacetic acid (TCA)[1]. In this study, protein extraction was conducted according to the protocol of Slocombe et al.[67]. Firstly, 5 mg ± 1 mg of freeze-dried *C. vulgaris* biomass was weighed into a 1.5 mL Eppendorf tube. The biomass was then mixed with 200 µL of 24 % (w/v) TCA and incubated at 95 °C for 15 min using a TS basic thermo-shaker from CellMedia (Zeitz, Germany). Subsequently, the sample was diluted to 6 % (w/v) TCA by adding 600 µL of ultrapure water and was centrifuged at 15 000 g for 20 min at 4 °C using a Sigma 1-15 PK (rotor: 12132).

Meanwhile, the following solutions were freshly mixed in a ratio of 48:1:1:

- 2 % (w/v) Na_2CO_3 (anhydrous) in 0.1 M NaOH
- 1 % (w/v) NaK tartrate tetrahydrate in ultrapure water
- 0.5 % (w/v) $CuSO_4.5H_2O$

While the supernatant was discarded, the remaining pellet was resuspended in 500 µL of the prepared mixture. The samples were again incubated at 55 °C for 3 h using a TS basic thermo-shaker (CellMedia, Zeitz, Germany). After centrifugation at 15 000 g for 20 min at room temperature (Sigma 1-15 PK, rotor: 12132), the supernatant was frozen at -20 °C until bicinchoninic acid (BCA) assay was conducted.

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Different to Slocombe et al.[67], a BCA assay was conducted instead of a Lowry assay. Since BCA is stable under alkaline conditions, the BCA assay can be performed in a single step compared to the two-step process of Lowry assay. Both assays are based on the reduction of Cu^{2+} to Cu^{1+} by proteins under alkaline conditions (biuret reaction). Subsequently, BCA forms a stable complex with Cu^{1+} resulting in an intense purple colour being detectable at an absorbance maximum of 562 nm[68].

Before measurement, 50 parts of bicinchoninic acid solution (Sigma-Aldrich, St. Louis, MO, USA) were mixed with 1 part of copper(II) sulfate solution (Sigma-Aldrich, St. Louis, MO, USA) resulting in a light green-coloured solution. Protein standard series were prepared using a 2 mg/mL BSA analytical standard (Sigma-Aldrich, St. Louis, MO, USA). Subsequently, 190 µL of the green-coloured mixture and 10 µL of the sample or standard solution were transferred into a 96-well plate. One well was used as blank, where ultrapure water was mixed with the green-coloured solution. The absorbance was measured at 562 nm using a Spark multimode microplate reader from Tecan (Männedorf, Switzerland). The program of the microplate reader can be found in Table 13.

Table 13: Program of the microplate reader for BCA assay

Reaching Temperature (°C)	37
Shaking (s, rpm)	30, 1440
Waiting (min)	14
Shaking (s, rpm)	30, 1440
Waiting (min)	14
Shaking (s, rpm)	30, 1440
Measure Absorbance (nm)	562

The protein concentration of the sample was determined by calibration of the BSA protein standard (Sigma-Aldrich, St. Louis, MO, USA). The protein content of *C. vulgaris* was determined by Equation 11:

$$Y_{Protein} = \frac{c_{Protein}}{c_x}$$

Equation 11

where $Y_{Protein}$ represents the protein yield ($\mu g_{Protein}/mg_x$), $c_{Protein}$ is the protein concentration (µg/mL) and c_x is the biomass concentration (mg/mL).

3.6.2 Chlorophyll content

To determine the chlorophyll content, 5-10 mg of freeze-dried biomass was weighed into 2 mL microtubes containing screw caps. Subsequently, one 5 mm glass bead and 0.29 g of 1 mm glass beads were added. After subjecting the samples to liquid N_2, cell disruption was achieved

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by a FastPrep-24 instrument from MP Biomedicals (Santa Ana, CA, USA). The frozen samples were fixed into the homogeniser and were disrupted three times for 30 s at 4 m/s.

Before extracting chlorophylls, the light was dimmed to avoid light-associated degradation of chlorophylls. The disrupted samples were mixed with 1 mL acetone and were then centrifuged at 16 000 rcf for 5 min at room temperature using an Eppendorf Centrifuge 5415 R (rotor: 85720). Subsequently, the green-coloured supernatant was collected in a 10 mL volumetric flask. The procedure of adding acetone, centrifuging, and collecting the supernatant was repeated until the supernatant was colourless. Finally, the volumetric flasks were filled up with acetone to 10 mL.

Chlorophyll determination was based on the method from Porra et al.[69]. To reduce the conversion of chlorophylls, 2.5 mM sodium phosphate buffer (pH 7.8) was added to each sample achieving an 80 % (v/v) aqueous acetone solution. Subsequently, absorption of the samples was measured at 664 nm, 647 nm and 750 nm in a 1 mL quartz glass cuvette using a NanoDrop One spectrophotometer from Thermo Fisher Scientific (Waltham, MA, USA). In order to determine the concentration of chlorophylls *a* and *b*, Equation 12 was adopted from Porra et al.[69]:

$$c_{Chl} = 17.76 * (A^{647\,nm} - A^{750\,nm}) + 7.34 * (A^{664\,nm} - A^{750\,nm})$$

Equation 12

where c_{Chl} is the concentration of chlorophyll *a* and *b* (µg/mL) and A is the absorption at a respective wavelength. The chlorophyll *a* and *b* content of *C. vulgaris* was determined using Equation 14:

$$Y_{Chl} = \frac{c_{Chl}}{c_x}$$

Equation 13

where Y_{Chl} represents the chlorophyll yield ($\mu g_{Chl}/mg_x$), c_{Chl} is the concentration of chlorophyll *a* and *b* (µg/mL) and c_x is the biomass concentration (mg/mL).

3.6.3 Carbohydrate content

The frozen biomass pellets were dried at 70 °C for 24 h. The next day, the biomass was hydrolysed by adding 1 mL 7.5 % H_2SO_4 and incubation at 95 °C for 2 h under shaking (900 rpm) using a TS basic thermo-shaker from CellMedia (Zeitz, Germany). The samples were then centrifuged at 16 000 rcf for 10 min at room temperature using an Eppendorf Centrifuge 5415 R (rotor: 85720). Subsequently 100 µL sample and 400 µL ultrapure water were transferred into a 0.5 mL ion chromatography (IC) vial. Carbohydrates were measured by Dionex™ Ion Chromatography from Thermo Fisher Scientific (Waltham, MA, USA). The

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analyte was separated by a CarboPac™ PA1 (4x250 mm) connected to a guard column (Thermo Fisher Scientific, Waltham, MA, USA) at a column temperature of 30 °C and pressure of ~100 bar. Carbohydrates were detected by an ICS-6000 conductivity detector from Thermo Fisher Scientific(Waltham, MA, USA). The mobile phase was (A) ultrapure water and (B) 200 mM NaOH (Sigma-Aldrich, St. Louis, MO, USA) with a flow rate of 1 mL/min. Table 14 shows the time-dependent gradient profile of the mobile phase.

Table 14: Gradient profile of IC measurement

Time (min)	Eluent A (%)	Eluent B (%)
0-16	91	9
16-21.5	0	100
21.5-55	91	9

Measurement was completed after 55 min and the chromatograms were recorded and analysed by the software Chromeleon Studio 7.2.10 from Thermo Fisher Scientific (Waltham, MA, USA). Calibration with standard series of sugars enabled carbohydrate quantification of the samples. The carbohydrate content of *C. vulgaris* was then calculated using Equation 14:

$$Y_{Carb} = \frac{\sum c_{sugars}}{c_x}$$

Equation 14

where Y_{Carb} represents the carbohydrate yield ($\mu g_{Carb}/mg_x$), Σc_{sugars} shows the sum of detected sugars (µg/mL) and c_x is the biomass concentration (mg/mL).

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4 Results

4.1 Heterotrophic cultivation of *C. vulgaris*

First of all, heterotrophic shake flask cultivation of *C. vulgaris* in different concentrated WSH was investigated and compared to cultivation in DM. To avoid growth inhibition by high nutrient content, the WSH was diluted to 2.5 %, 5.0 %, 12.5 %, and 17.5 % (v/v).

4.1.1 Biomass concentrations

Biomass concentrations of *C. vulgaris* in different concentrated WSH and DM are depicted in Figure 8 and Figure 9, respectively. In the WSH, biomass concentration was similar between the tested concentrations indicating that growth was not inhibited by high nutrient content in the higher studied concentrations. In the beginning, 2.5 % WSH resulted in highest biomass concentration (0.13 g/L), and was then surpassed by 5.0 % WSH after two weeks (0.22 g/L). While growth stagnated after two weeks at around 0.2 g/L in the lower concentrated WSH (2.5 %, 5.0 %), the biomass concentration further increased to 0.3 g/L in higher the concentrated WSH (12.5 %, 17.5 %). After 37 days, another rise in growth occurred in all tested concentrations reaching 0.3 - 0.45 g/L. This increase might be traced back to light assimilation during sampling or to cell lysis and thus the providence of nutrients for living *C. vulgaris* cells.

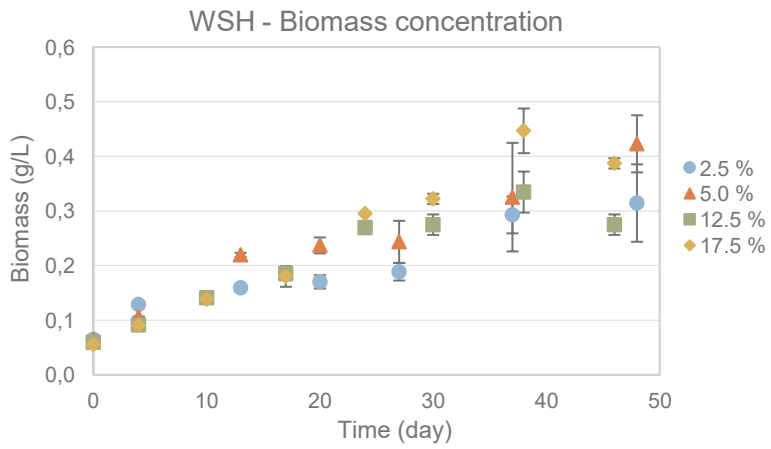

Figure 8: Biomass concentrations in different concentrated WSH in heterotrophic conditions

When comparing the biomass concentration between the cultivation in WSH and DM, it can be observed that the growth of *C. vulgaris* was around twice as high in the WSH (0.3 g/L) than in the DM (0.15 g/L). The biomass concentration in the DM almost stayed constant at 0.06 g/L during the first two weeks and then rose to 0.15 g/L the following four weeks. Since the DM only contained monomeric sugars and furans of the WSH, it can be concluded that other substances of the hydrolysate such as trace elements, vitamins, organic acids, fatty acids, or amino acids promoted the growth of *C. vulgaris*. For instance, it has been reported that *C. vulgaris* metabolises acetic acid[70], being the fourth most abundant component of the WSH.

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Enhanced growth of *C. vulgaris* in hydrolysates compared to the DM under heterotrophic conditions was also stated by Lau et al.[40] in food waste hydrolysates or by Cai et al.[71] using broken rice hydrolysates.

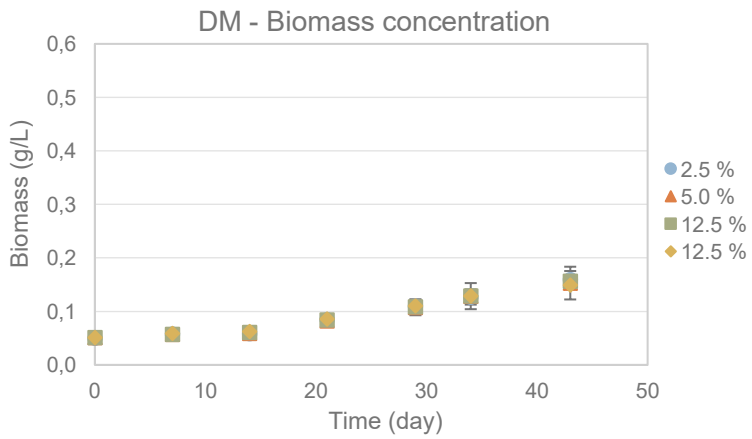

Figure 9: Biomass concentrations in different concentrated DM in heterotrophic conditions

4.1.2 Specific growth rates

Specific growth rates of *C. vulgaris* in different concentrated WSH and DM are depicted in Figure 10 and Figure 11. In the WSH, growth rates showed a decreasing development from 0.17 day^{-1} to 0.04 day^{-1} in the course of cultivation time, which can be attributed to the linear growth behaviour of *C. vulgaris* in WSH (Figure 8). While 2.5 % WSH resulted in the highest growth rate (0.17 day^{-1}) after four days, the highest growth rate was obtained in 5.0 % WSH after 13 days. All tested concentrations ended at a growth rate of 0.04 day^{-1}. Conversely, *C. vulgaris* showed a long lag phase in the DM (Figure 9), which is why growth rates increased from 0.014 day^{-1} to 0.025 day^{-1} after three weeks of cultivation in the DM. Growth rates in the DM were almost the same between the tested concentrations.

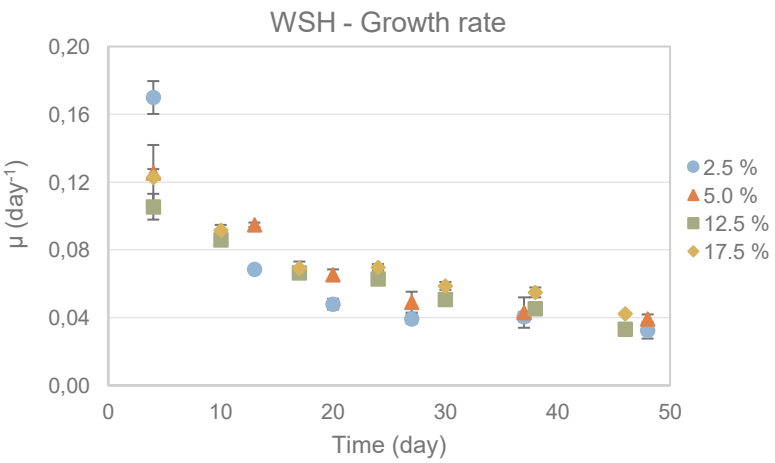

Figure 10: Growth rates in different concentrated WSH in heterotrophic conditions

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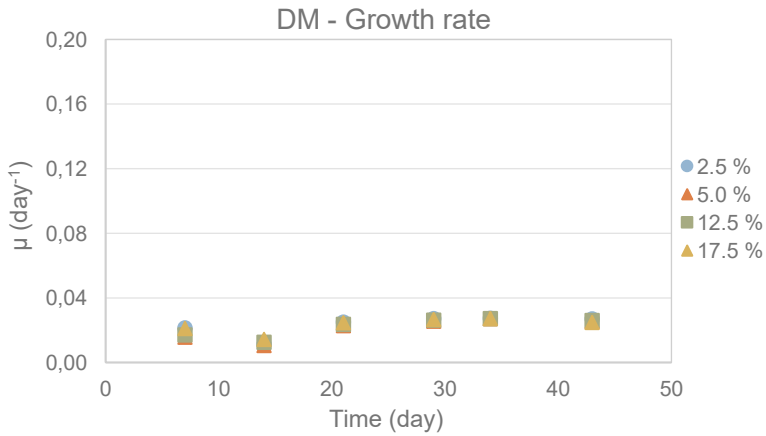

Figure 11: Growth rates in different concentrated DM in heterotrophic conditions

4.1.3 pH

The pH values during heterotrophic cultivation of *C. vulgaris* in different concentrated WSH and DM are depicted in Figure 12 and Figure 13. Generally, the optimal pH for heterotrophic growth of *C. vulgaris* is between 7.5 and 8[72]. While the pH showed no significant changes in terms of 2.5 % and 5.0 % WSH, the higher concentrated experiments (12.5 %, 17.5 %) showed a declining pH over time. Generally, an increase of the pH during sugar assimilation was expected due to an inducible hexose/H^+ symporter of *C. vulgaris*[73,74]. This observation could be found in the DM after three weeks, being in accordance with the increase of the biomass concentration (Figure 8). However, the reduction of the pH in the higher concentrated WSH might be attributed to the consumption of other compounds of the WSH. Cai et al.[71] investigated the cultivation of *C. vulgaris* in rice straw hydrolysate and observed a decline of the pH suggesting an uneven consumption of ions and other components. Since cells must achieve a balance of total positive and negative charges, the absorption of a positive ion leads to a discharge of a proton reducing the pH of the medium. More precisely, Cai et al.[71] proposed that the decrease of the pH in the hydrolysate was mainly caused by ammonium consumption.

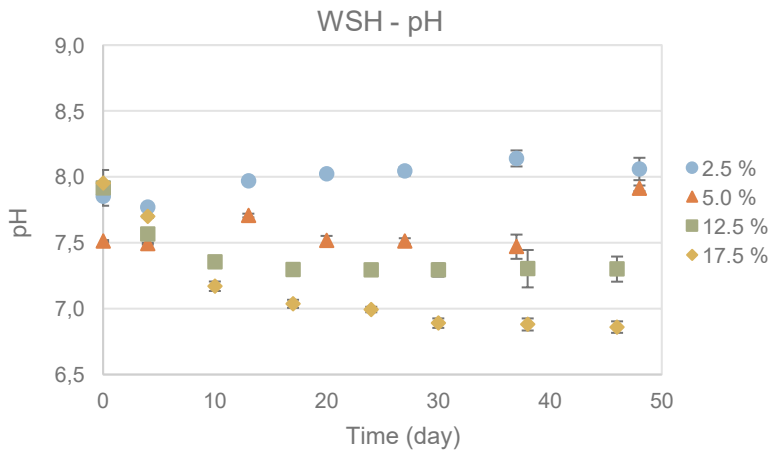

Figure 12: pH in different concentrated WSH in heterotrophic conditions

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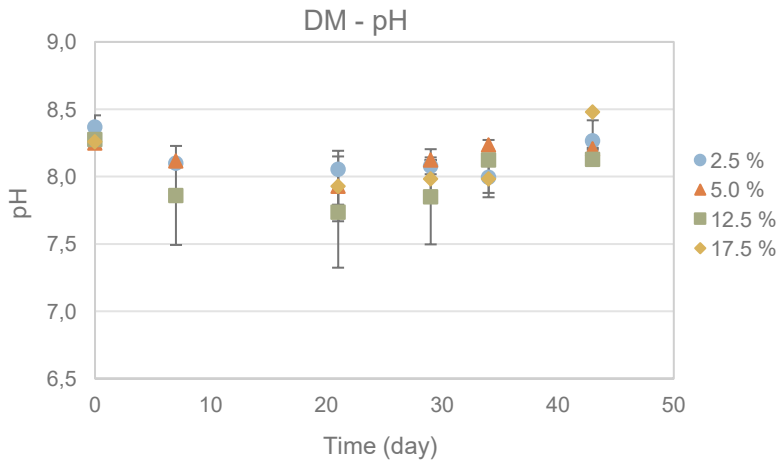

Figure 13: pH in different concentrated DM in heterotrophic conditions

4.1.4 Sugar concentrations

Xylose concentration of the different concentrated WSH can be found in Figure 14. Since other peaks of the WSH chromatogram were not separatable with the used method, only xylose was determined. It has to be considered that only 3 % of total xylose content in the WSH were monomeric xylose units. Figure 14 shows that the xylose concentration showed no significant changes during the whole cultivation process. Former studies reported that *C. vulgaris* is not capable of utilising xylose in the absence of light[38,75]. This was attributed to the fact that the light-dependent NADPH production contributes to xylose catabolism[76]. In fact, NADPH is needed as a cofactor for the reduction of xylose to xylitol catalysed by xylose reductase[63].

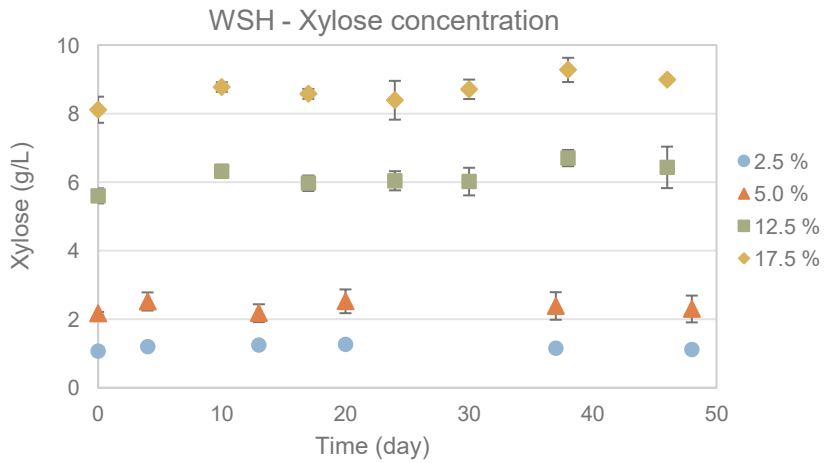

Figure 14: Xylose concentrations in different concentrated WSH in heterotrophic conditions

Xylose and arabinose concentrations of the DM can be found in Figure 15 and Figure 16. Since the DM was based on the monomeric sugar composition of the WSH, xylose concentrations were lower than in the complex WSH (Figure 14). A decrease of the xylose and arabinose content could be observed in the DM, especially after three weeks of cultivation. This observation matches the obtained biomass concentrations (Figure 8) and pH values (Figure 13), which increased after three weeks of cultivation. While xylose and arabinose were

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completely taken up after 29 days in 2.5 % DM, sugars were consumed after 43 days in 5.0 % DM. In higher concentrated DM (12.5 %, 17.5 %) the xylose concentration was reduced by 38 % and 32 %, and arabinose concentration by 59 % and 42 %.

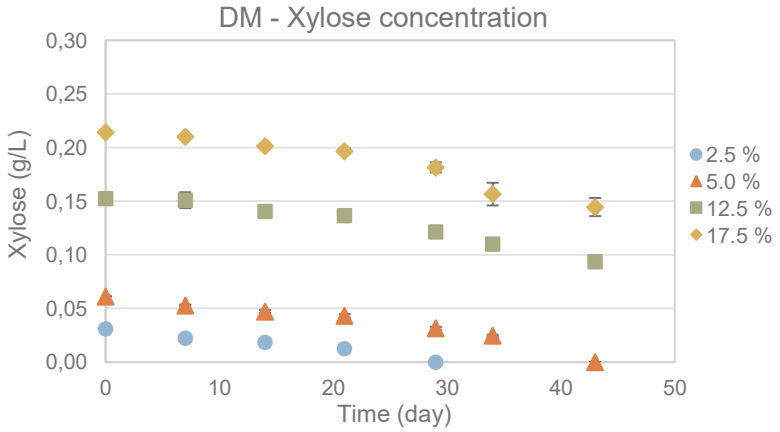

Figure 15: Xylose concentrations in different concentrated DM in heterotrophic conditions

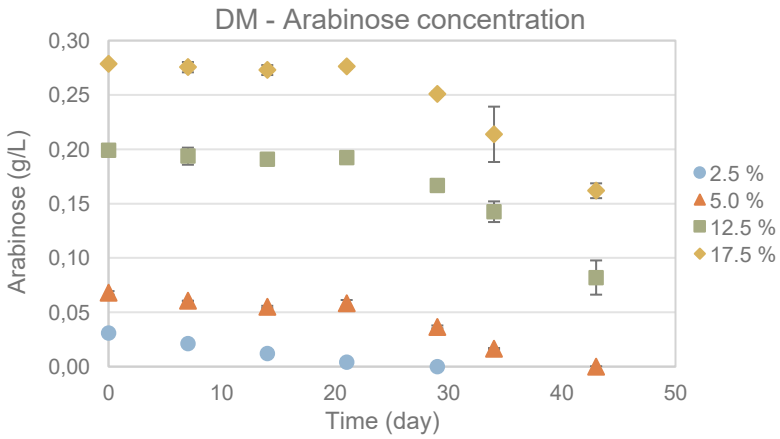

Figure 16: Arabinose concentrations in different concentrated DM in heterotrophic conditions

While many studies reported that microalgae are not able of using pentoses as a carbon source, only a few stated the opposite: Zheng et al.[63] reported slow xylose uptake by *C. sorokiniana* even under dark conditions. Also, xylose uptake was suppressed by the presence of glucose, galactose, and fructose, but neither by arabinose or ribose. This observation might be attributed to different affinities of sugars passing through the inducible hexose/H^+ symporter. Generally, the symporter shows a broad uptake specificity including all aldo- and ketohexoses and two pentoses, xylose and arabinose[74]. Since the DM primarily consisted of xylose and arabinose, assimilation was not suppressed by other substances. However, pentoses show lower affinities against hexose/H^+ symporter, which might be the cause for the long lag phase in the DM. Conversely, *C. vulgaris* seemed to prefer other components of the WSH, which might be the reason why xylose was not utilised in the WSH. For instance, Weibao et al.[38] studied growth of *C. vulgaris* using different carbon sources and obtained 2.5-fold higher growth rates in acetic acid than in xylose in heterotrophic conditions.

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4.1.5 Protein contents

Since the biomass composition of microalgae is influenced by environmental conditions, protein content was determined. Generally, protein production is expected to be higher under photoautotrophic and mixotrophic conditions compared to heterotrophic regime[31]. Thus, heterotrophic cultivations were illuminated for ten more days to stimulate protein synthesis. The results of the protein contents in different concentrated WSH and DM before and after illumination are depicted in Figure 17 and Figure 18. *C. vulgaris* biomass consisted of 15 - 20 % of proteins in the WSH and 13 - 21 % in the DM. While the exposure to light increased protein content from 154 µg/mg to 196 µg/mg in 2.5 % and 5.0 % WSH, the protein production was less stimulated in 12.5 % and 17.5 % WSH. In the DM, protein content only increased from 153 µg/mg to 167 µg/mg in 2.5 % DM after illumination. However, standard deviations are comparably high in the DM making statements about the protein content pre and post illumination less reliable. The reason for the high standard deviations might be traced back to different lighting between the samples due to different location in the incubator or to measurement imperfections during protein extraction procedure (including weighing, cell lysis, extraction, and photometrical measurement).

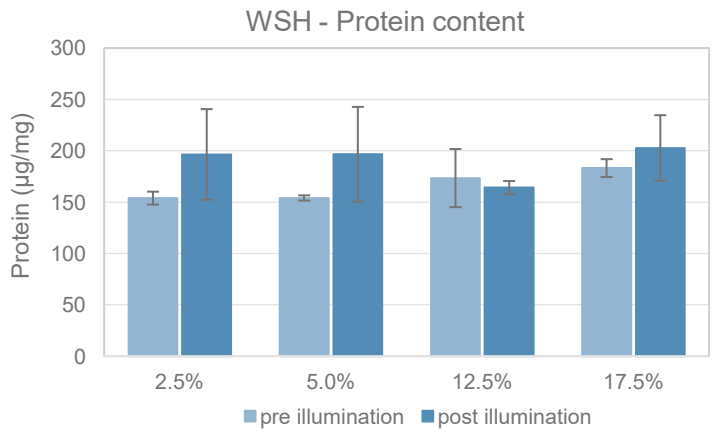

Figure 17: Protein contents in different concentrated WSH pre and post illumination

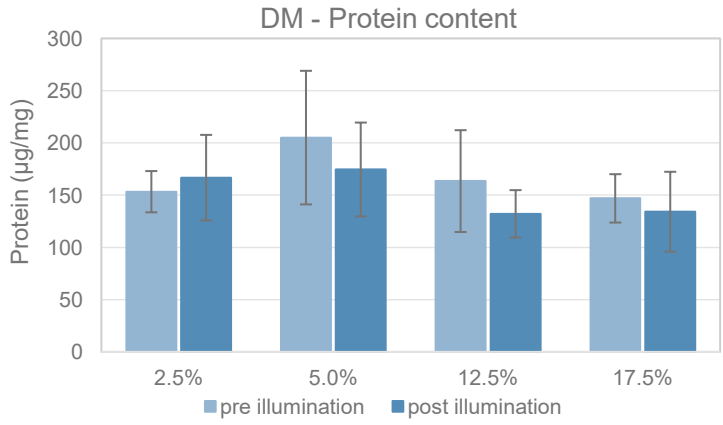

Figure 18: Protein contents in different concentrated DM pre and post illumination

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4.1.6 Chlorophyll contents

Chlorophyll *a* and *b* content was determined at the end of the cultivation in the WSH and DM (Figure 19, Figure 20). Alike protein content, chlorophyll production is known to be higher under the presence of light[43]. Hence, chlorophyll content was determined before and after an illumination period of ten days. Contrary to the protein outcome, chlorophyll content varied depending on the medium, concentration, and illumination. Concerning the cultivation in lower concentrated media (2.5 %, 5.0 %), pigment production was higher in the WSH (22 µg/mg, 14 µg/mg) compared to the DM (10 µg/mg, 7 µg/mg) indicating that other compounds of the WSH had a stimulating effect on chlorophyll synthesis. Conversely, chlorophyll content reduced to 3 µg/mg in the higher concentrated WSH (12.5 %, 17.5 %) being lower than in the DM (7 µg/mg, 11 µg/mg). This implies that higher nutrient content of the WSH inhibited chlorophyll production[38,44]. Moreover, chlorophyll formation was promoted by light exposure in terms of lower concentrated WSH (2.5 %, 5.0 %), but not in higher concentrated WSH (12.5%, 17.5 %). In the DM, all samples showed higher chlorophyll content after illumination. This again indicates that higher concentration of carbon sources might have inhibited chlorophyll production[38,44], or light penetration was not efficient due to the darker colour of the WSH[36].

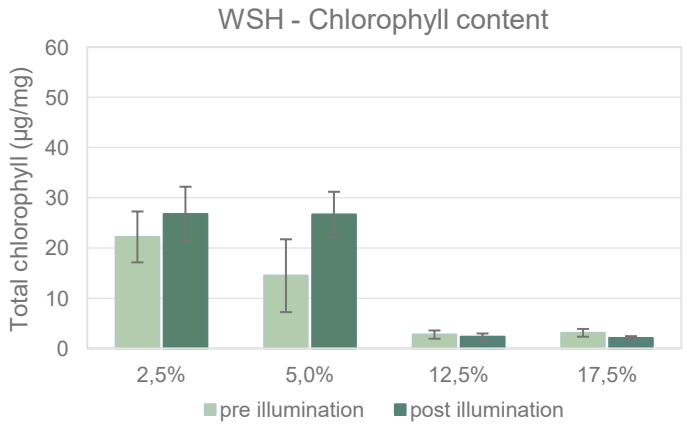

Figure 19: Chlorophyll contents in different concentrated WSH pre and post illumination

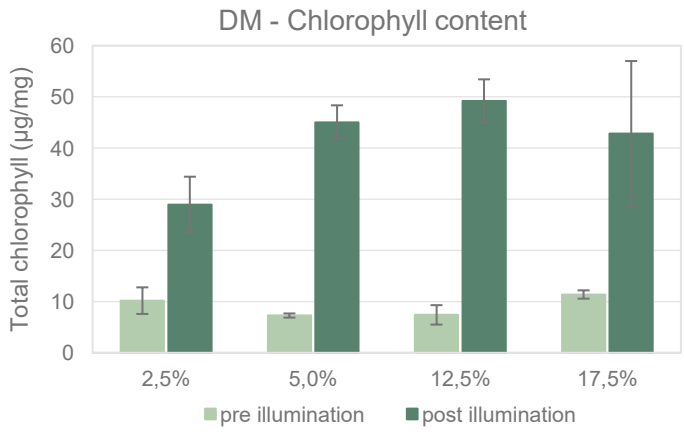

Figure 20: Chlorophyll contents in different concentrated DM pre and post illumination

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4.2 Mixotrophic cultivation of *C. vulgaris*

Lower concentrations (2.5 %, 5.0 %) of WSH and DM were investigated under mixotrophic conditions due to the following results of heterotrophic cultivation: *C. vulgaris* showed similar growth behaviour and protein contents between the studied concentrations. Pigment formation was not stimulated by illumination in higher concentrated WSH indicating that light penetration was insufficient due to its darker colour.

4.2.1 Biomass concentrations

Biomass concentrations of *C. vulgaris* in different concentrated WSH and DM can be found in Figure 21. Contrary to heterotrophic conditions, biomass concentration increased similarly between the WSH and DM. This can be attributed to the fact that mixotrophically grown *C. vulgaris* was not strictly dependent on organic sources.

After one week of cultivation, 2.5 % WSH showed higher biomass concentration (0.5 g/L) than the other experiments (0.3 - 0.4 g/L). From then on, the biomass concentration in 5 % WSH surpassed the other experiments reaching 1.3 g/L after 3 weeks of cultivation. While growth stagnated after two weeks in 2.5 % WSH at 0.9 g/L, stagnation occurred after three weeks in 5.0 % WSH at 1.3 g/L. In the first two weeks, lower biomass concentrations (0.8 g/L in 2.5 %, 0.7 g/L in 5.0 %) were obtained in the DM compared to the WSH (0.9 g/L in 2.5 %, 1.0 g/L in 5.0 %). After four weeks of cultivation, DM showed higher biomass concentration (1.1 g/L) than 2.5 % WSH (0.9 g/L).

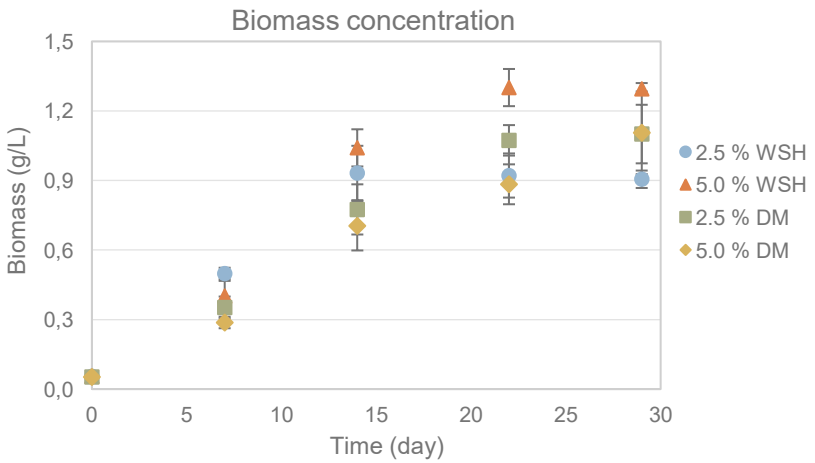

Figure 21: Biomass concentrations in different concentrated WSH and DM in mixotrophic conditions

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4.2.2 Specific growth rates

Figure 22 shows that the specific growth rates of *C. vulgaris* were similar between the different concentrated WSH and DM. The decreasing development of specific growth rates from 0.3 day^{-1} to 0.1 day^{-1} can be attributed to the linear growth of *C. vulgaris* (Figure 21).

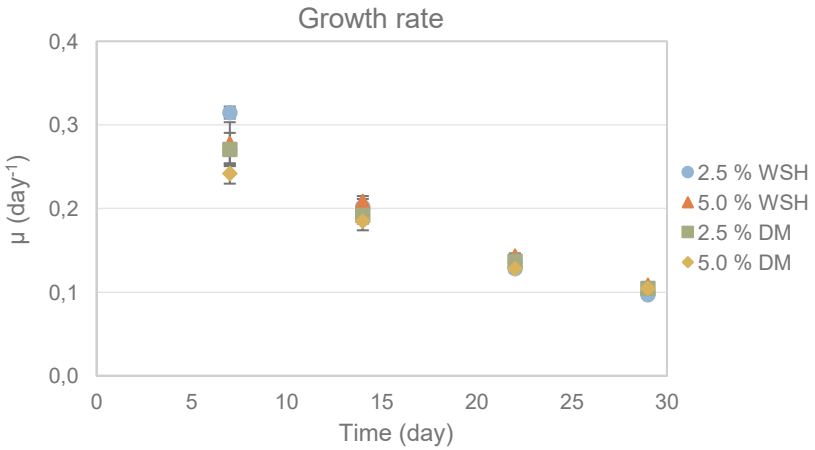

Figure 22: Growth rates in different concentrated WSH and DM in mixotrophic conditions

4.2.3 pH

The pH values during mixotrophic cultivation of *C. vulgaris* in different concentrated WSH and DM are summarised in Figure 23. It can be observed that the pH declined from 7.9 to 7.6 after one week followed by an increase (7.7 - 7.9). In terms of 5.0 % WSH, the pH started from 7.6 and reduced to 7.5 after one week. As mentioned in chapter 4.1.3, an increase of the pH after sugar uptake is expected due to an inducible hexose/H^+ symporter. The decrease of the pH after one week might be attributed to the consumption of other ions and components[71]. Also, the pH is dependent on the light/dark period in mixotrophic conditions, because CO_2 is assimilated during the day (photosynthesis) and released during the night (dark respiration)[2]. Hence, pH values are higher in the presence of light and lower during the night meaning that the measured pH values are dependent on the sampling time.

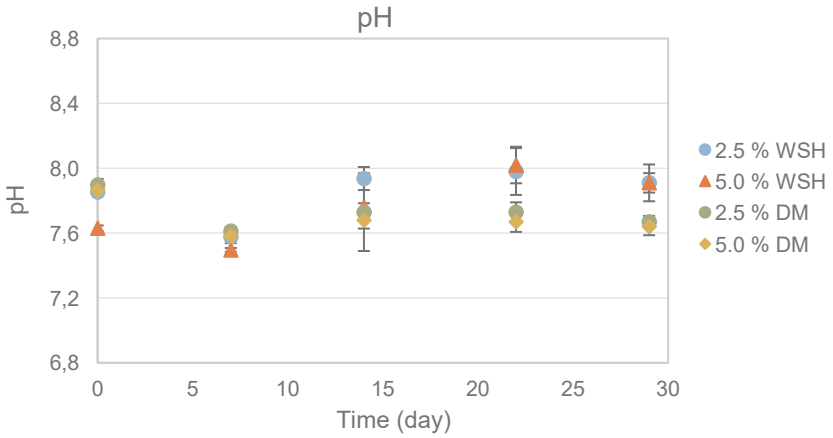

Figure 23: pH in different concentrated WSH and DM in mixotrophic conditions

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4.2.4 Sugar concentrations

Xylose concentrations of the different concentrated WSH are depicted in Figure 24. In contrast to heterotrophic cultivation, the xylose content decreased from 0.9 g/L to 0.8 g/L in 2.5 % WSH and from 1.7 g/L to 1.5 g/L in 5.0 % WSH after one week. However, the xylose concentration again increased from then on. The changes of the xylose concentration might be attributed to the imprecise evaluation of the complex WSH chromatogram; the used method does not allow reliable statements about the xylose concentration in the complex WSH medium.

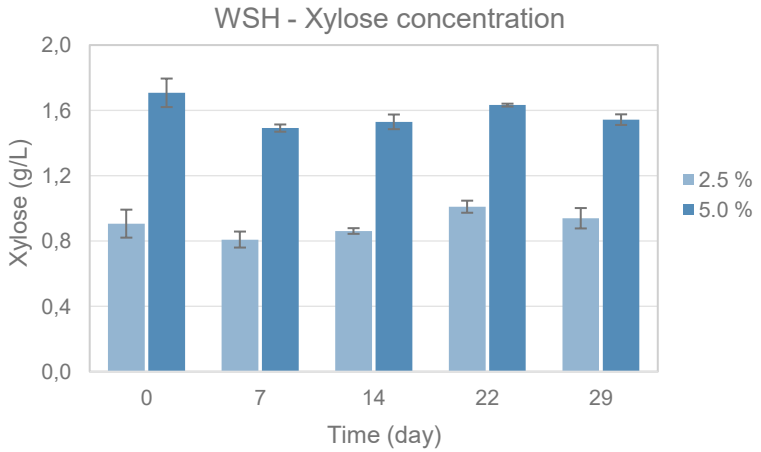

Figure 24: Xylose concentrations in different concentrated WSH in mixotrophic conditions

Conversely, xylose and arabinose were quantifiable in the DM (Figure 25, Figure 26). While xylose was completely taken up within three weeks, arabinose concentration reduced to approximately 30 mg/L for both tested DM concentrations. In comparison, heterotrophic grown *C. vulgaris* took 29 days in 2.5 % and 43 days in 5.0 % DM until xylose and arabinose were completely consumed. Hence, xylose was faster assimilated in mixotrophic conditions, but arabinose was not completely used compared to heterotrophic regime.

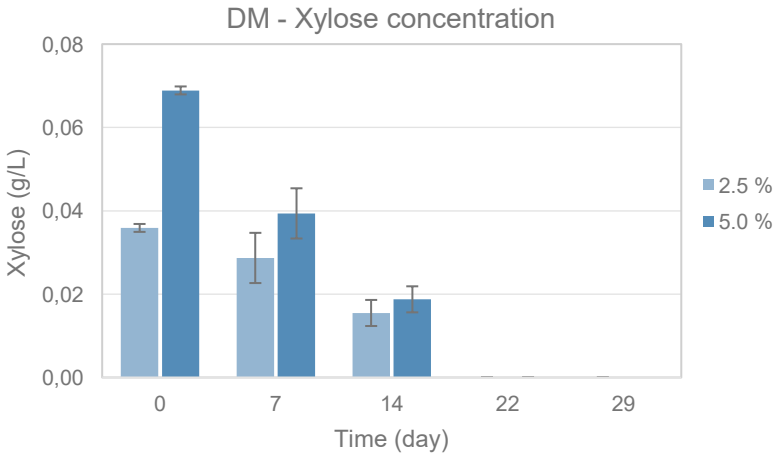

Figure 25: Xylose concentrations in different concentrated DM in mixotrophic conditions

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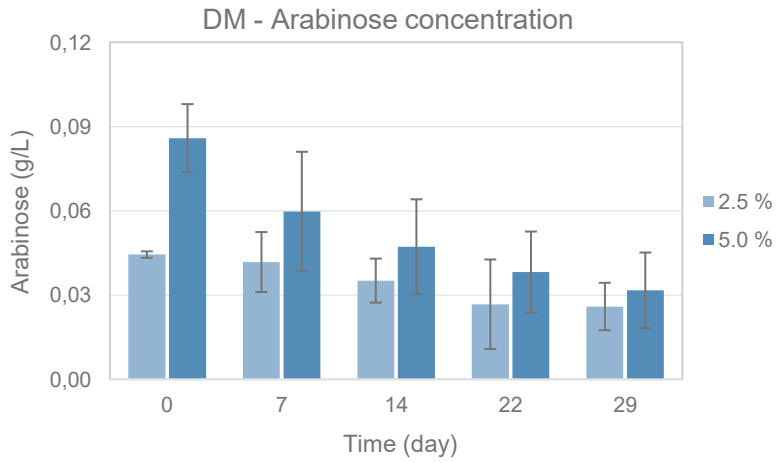

Figure 26: Arabinose concentrations in different concentrated DM in mixotrophic conditions

4.2.5 Protein contents

Protein content was determined at the end of the cultivation and is illustrated in Figure 27. It can be observed that the biomass consisted of similar protein content (150 - 170 µg/mg) between the DM and WSH. However, a higher protein content than 17 % of DCW was expected under mixotrophic conditions. The reason for the reduced protein production might be nitrogen starvation. While *C. vulgaris* is known for protein content up to 60 % of DCW under nitrogen-rich conditions, nitrogen limitation can reduce protein production to 20 % of DCW[12].

The comparison to the photoautotrophic control will demonstrate whether protein production was negatively affected by sugars and furans. Since the protein content was similar between the WSH and DM, it can be assumed that other compounds of the WSH showed no adverse effect on the protein production in *C. vulgaris*.

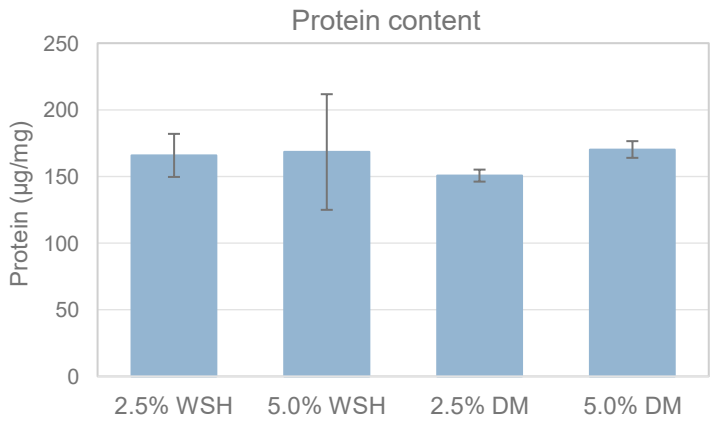

Figure 27: Protein contents in different concentrated WSH and DM in mixotrophic conditions

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4.2.6 Chlorophyll contents

Chlorophyll *a* and *b* content was determined at the end of the mixotrophic cultivation and is shown in Figure 28. Alike the protein outcome, no significant changes of chlorophyll production between the tested experiments can be observed indicating that light penetration was efficient in terms of the studied WSH concentrations. *C. vulgaris* biomass contained 32 - 34 µg/mg chlorophyll *a* and *b* under mixotrophic conditions.

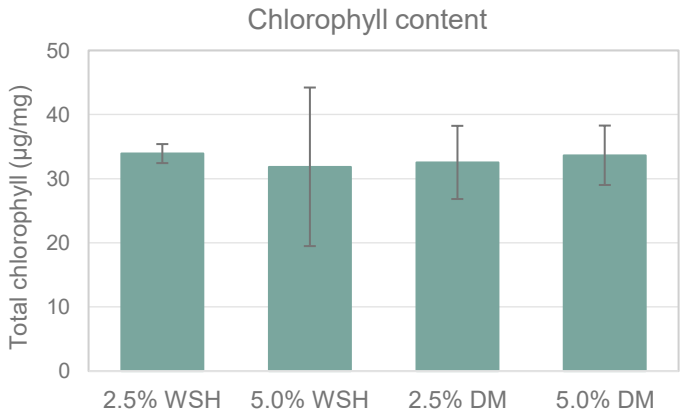

Figure 28: Chlorophyll contents in different concentrated WSH and DM in mixotrophic conditions

4.3 Comparison of cultivation modes

In this section, growth behaviour and biomass composition of *C. vulgaris* cultivated in WSH (2.5 %, 5.0 %) under heterotrophic and mixotrophic conditions were compared to a photoautotrophic culture.

4.3.1 Biomass concentrations

Biomass concentrations of *C. vulgaris* grown in WSH under mixotrophic and heterotrophic conditions are compared to photoautotrophic cultivation in Figure 29. It can be seen that the biomass concentration in heterotrophic conditions (0.2 g/L) was less than a quarter of the photoautotrophic cultivation (0.9 g/L). Conversely, mixotrophic and photoautotrophic cultivation exhibited similar biomass concentration (0.4-0.5 g/L) after one week. From then on, higher biomass concentration in mixotrophic conditions (0.9 g/L in 2.5 % WSH, 1.0 g/L in 5.0 % WSH) than in photoautotrophic cultivation (0.7 g/L) was obtained. The biomass concentration in 2.5 % WSH stagnated abruptly after two weeks and finally resulted in the same concentration (0.9 g/L) as derived by the photoautotrophic culture. Conversely, biomass concentration further increased in 5.0 % WSH to 1.3 g/L achieving the best growth performance.

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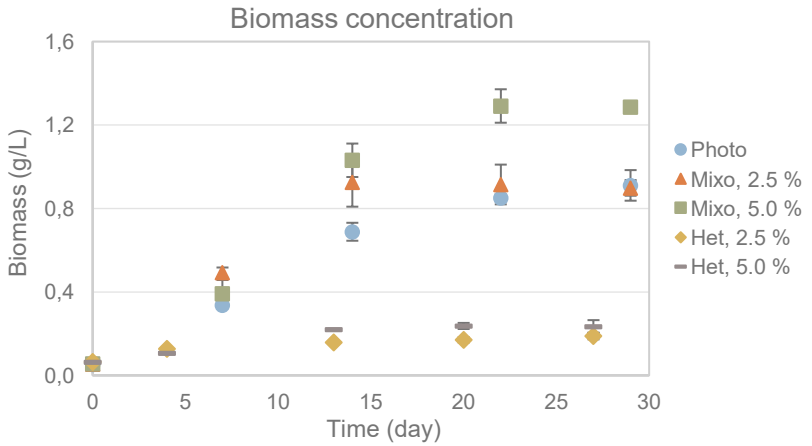

Figure 29: Biomass concentrations in photoautotrophic, mixotrophic, and heterotrophic conditions

4.3.2 Specific growth rates

Figure 30 depicts the comparison of specific growth rates of *C. vulgaris* grown in different cultivation modes. As can be seen, growth rates under mixotrophic and photoautotrophic conditions (~0.2 day^{-1}) were twice as high as in heterotrophic regime (~0.1 day^{-1}). All growth rates showed a decreasing development over time, which can be attributed to the linear increase of biomass concentration (Figure 29).

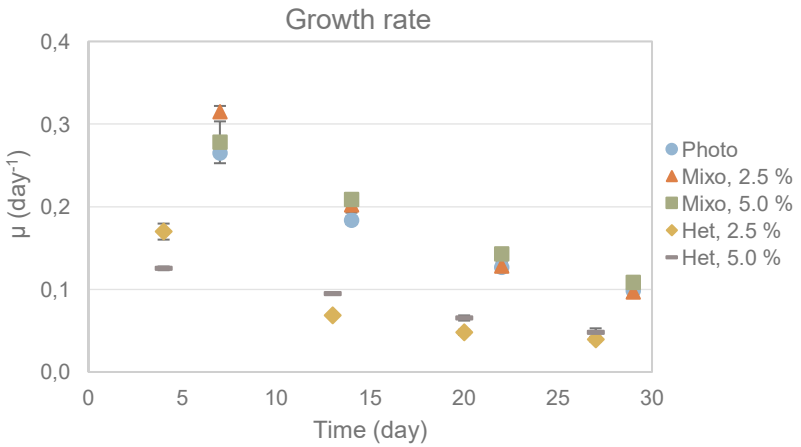

Figure 30: Growth rates in photoautotrophic, mixotrophic, and heterotrophic conditions

4.3.3 pH

The pH of photoautotrophic, mixotrophic, and heterotrophic cultivation can be found in Figure 31. The pH under mixotrophic and heterotrophic conditions decreased in the first week followed by an increase in the second week. A decline in the pH can be attributed to the assimilation of ammonium or other compounds of the WSH. Conversely, sugar uptake leads to an increase in the pH value. In terms of photoautotrophic (and mixotrophic) conditions, pH varies according to the light/dark cycle and is thus dependent on sampling time. In general, pH values are higher during the day (assimilation of CO_2) and lower pH during the night (release of CO_2).

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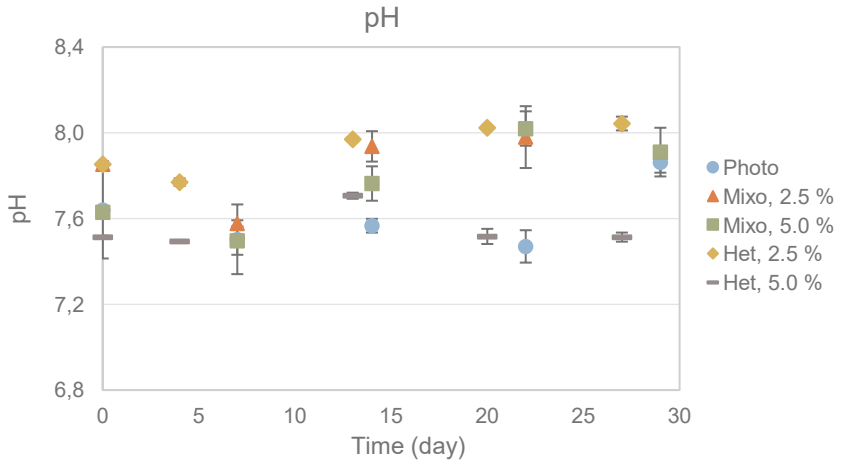

Figure 31: pH in photoautotrophic, mixotrophic, and heterotrophic conditions

4.3.4 Protein contents

Protein contents at the end of cultivation are compared between the different cultivation modes in Figure 32. As mentioned before, protein production was expected to be higher in the presence of light. The protein content was ranging between 166 - 168 µg/mg in photoautotrophic and mixotrophic conditions being slightly higher than in heterotrophic mode (154 µg/mg). As mentioned in chapter 4.2.5, the reason for the low protein production might be nitrogen starvation at the end of cultivation. However, it can be concluded that the WSH had no negative impact on protein formation because the photoautotrophic control also showed a reduced protein content.

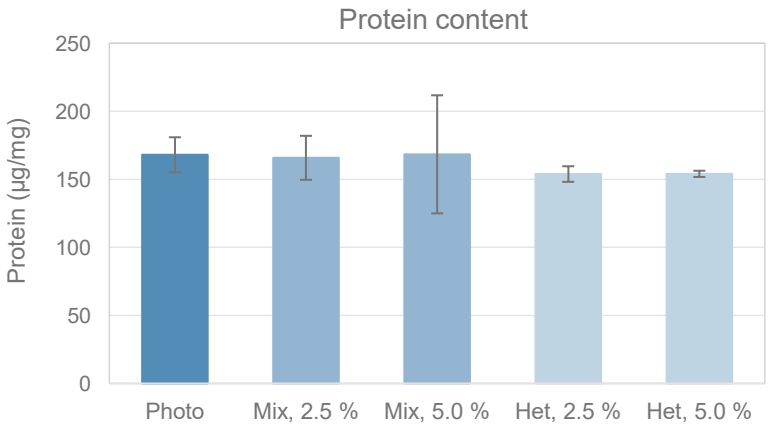

Figure 32: Protein contents in photoautotrophic, mixotrophic, and heterotrophic conditions

4.4.5 Chlorophyll contents

The impact of the cultivation regime on chlorophyll production is illustrated in Figure 33. Chlorophyll content was similar between photoautotrophic (36 µg/mg) and mixotrophic (34 µg/mg in 2.5 % WSH, 32 µg/mg in 5.0 % WSH) conditions indicating that light penetration was not affected by the darker colour of the WSH. Pigment formation was higher under the presence of light compared to heterotrophic conditions (22 µg/mg in 2.5 % WSH, 14 µg/mg in

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5.0 % WSH). This can be traced back to the absence of light resulting in degeneration of chloroplasts in heterotrophic metabolism[25].

The reason for the high standard deviation in terms of Mix, 5.0 % might be attributed to different lighting between the samples due to different location in the incubator, or to measurement imperfections during chlorophyll extraction procedure (including weighing, cell disruption, extraction, photometrical measurement).

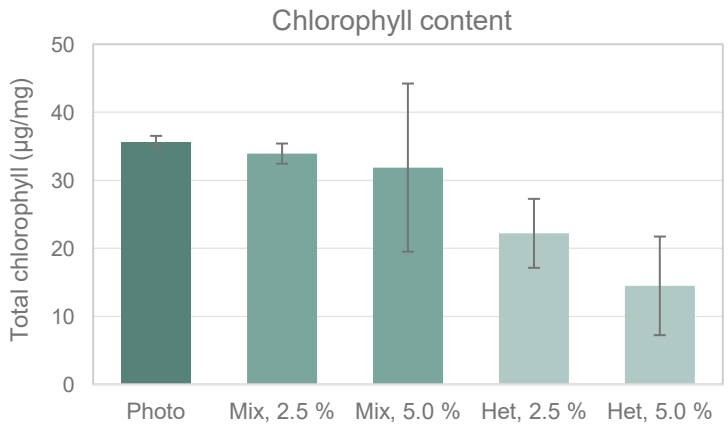

Figure 33: Chlorophyll contents in photoautotrophic, mixotrophic, and heterotrophic conditions

4.4 Batch fermentation of *C. vulgaris*

Batch fermentation of *C. vulgaris* was performed to study its performance in WSH at a larger scale offering more control and information of the process (pH, T, mixing, gas analysis). Batch mode was preferred to fed-batch to avoid growth inhibition by high concentrations of nutrients and toxic compounds. Fermentation was conducted in 2.5 % WSH and 2.5 % DM under mixotrophic and heterotrophic conditions. For comparison reasons, photoautotrophic fermentation was performed in BG-11 medium. Fermentations were not carried out in triplicates due to time limitations meaning that standard deviations could not be determined.

4.4.1 Biomass concentrations

Biomass concentrations of *C. vulgaris* fermented in different media and regimes can be found in Figure 34. It can be observed that biomass concentration increased faster in the presence of light and was only slightly changing in heterotrophic conditions. In fact, biomass concentration only increased from 0.05 g/L to 0.13 g/L in the WSH and to 0.80 g/L in the DM after two weeks of heterotrophic cultivation. Conversely, light-dependent fermentations resulted in more than ten times higher biomass concentrations than heterotrophic conditions. During the first two weeks, a similar development in growth was observed in mixotrophic and photoautotrophic cultivation. From then on, mixotrophically grown *C. vulgaris* in the DM surpassed the other fermentations reaching the highest biomass concentration (2.4 g/L), whereas photoautotrophic cultivation ended at 1.9 g/L and mixotrophic fermentation in WSH

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at 1.4 g/L. One reason for the lower performance in the WSH compared to the DM might be the fact that the airflow was not enriched with pure CO_2 during the first four days.

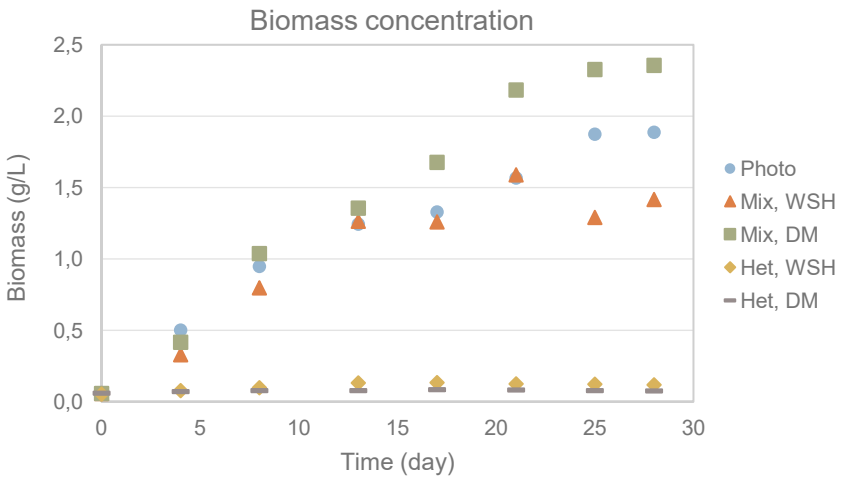

Figure 34: Biomass concentrations during fermentation in different media and cultivation modes

4.4.2 Specific growth rates

Specific growth rates of fermented *C. vulgaris* are summarised in Figure 35. The decreasing development of the growth rates over fermentation time can be attributed to the linear increase of biomass concentration (Figure 34). Growth rates were similar between mixotrophic (0.33 day^{-1} in WSH, 0.36 day^{-1} in DM) and photoautotrophic (0.36 day^{-1}) fermentation and were higher compared to the heterotrophic regime (0.07 day^{-1} in WSH, 0.03 day^{-1} in DM). Contrary to the mixotrophic cultivation, heterotrophic fermentation in the WSH resulted in twice as high growth rates than in the DM indicating that other compounds of the WSH had a promoting effect on *C. vulgaris*.

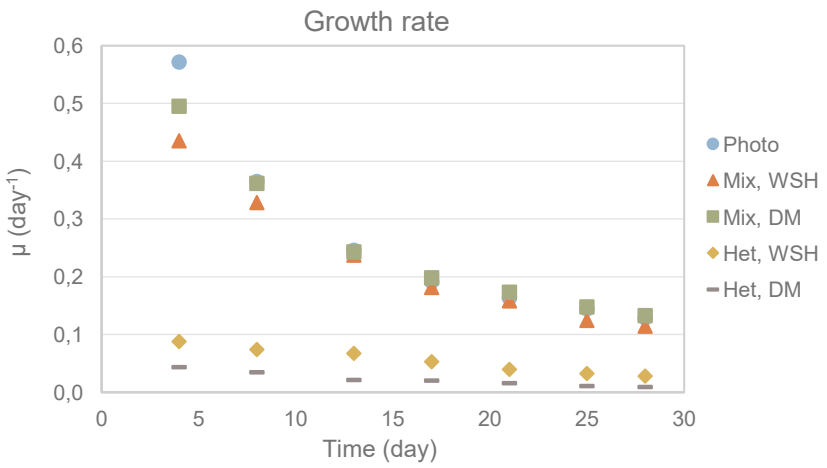

Figure 35: Growth rates during fermentation in different media and cultivation modes

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4.4.3 pH

The pH in the course of the fermentation process is depicted in Figure 36. In terms of light-dependent fermentations, fluctuation of the pH according to the light/dark period can be observed due to the assimilation of CO_2 during the day (pH increase) and release during darkness (pH decrease)[2]. Since the pH stayed in a range being suitable for *C. vulgaris* (7.2 - 8.5) and changes of the pH occur according to the photosynthetic metabolism, the pH was not externally regulated during fermentation.

The pH increased strongest in the first and second week compared to the time onwards, which can be related to faster growth of *C. vulgaris* (Figure 35). Since the airflow was not enriched with pure CO_2 for the first four days during the mixotrophic fermentation in WSH, the pH increased faster in this period (7.5 to 8.5). The decline of the pH from 7.9 to 7.7 after ten days of photoautotrophic cultivation can be attributed to a decrease in the airflow (appendix) joined with an increase of the CO_2 input (Figure 44). In mixotrophic fermentations, the pH showed a raising development over the whole process, whereas the pH only increased in the first week during heterotrophic cultivation. This can be attributed to earlier stagnation of growth in heterotrophic conditions (Figure 34). However, the pH increased stronger in the WSH compared to the DM, which can be traced back to the consumption of other compounds of the WSH such as acetate.

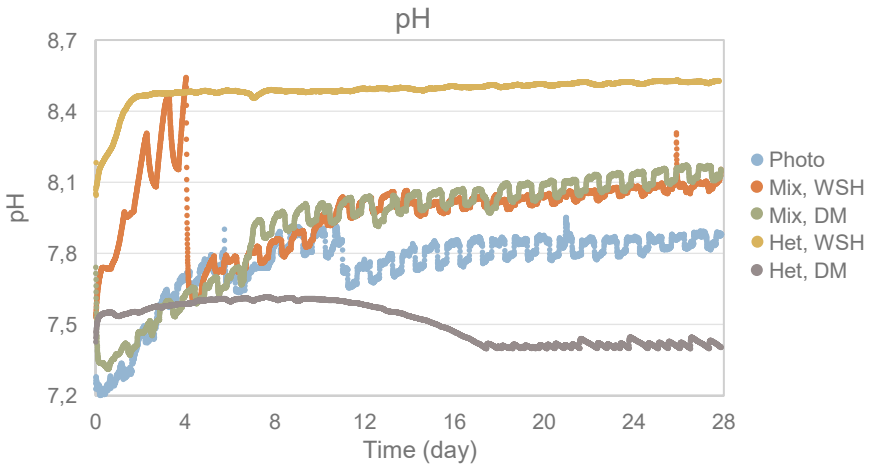

Figure 36: pH during fermentation in different media and cultivation modes

4.4.4 Sugar concentrations

Xylose concentrations of the WSH were determined during fermentation (Figure 37). The xylose assigned peak of the chromatogram was reduced by about 30 % after 17 and 21 days in mixotrophic and heterotrophic conditions, respectively. This decrease in xylose concentration corresponds to a higher amount than monomeric xylose units were available. In addition, the xylose concentration again increased up to 0.73 g/L in mixotrophic regime after 21 days indicating that evaluation of the WSH chromatogram was not reliable. As already

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described in chapter 4.2.4, the used method does not allow statements about the xylose concentration due to imprecise evaluation of the WSH chromatogram.

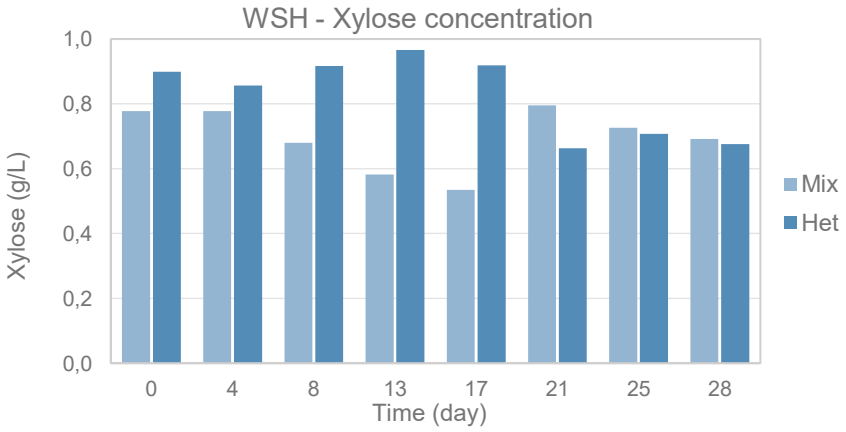

Figure 37: Xylose concentrations during mixotrophic and heterotrophic fermentation in WSH

Xylose and arabinose concentrations in the DM during *C. vulgaris* fermentation are plotted against process time in Figure 38. Both sugars were taken up within four days in mixotrophic fermentation and within eight days in heterotrophic fermentation. Xylose was faster assimilated than arabinose concerning heterotrophic cultivation. While sugars were taken up within eight days during fermentation, *C. vulgaris* needed three to four weeks in shake flasks experiments implying that mixing and aerating showed a significant impact on sugar uptake.

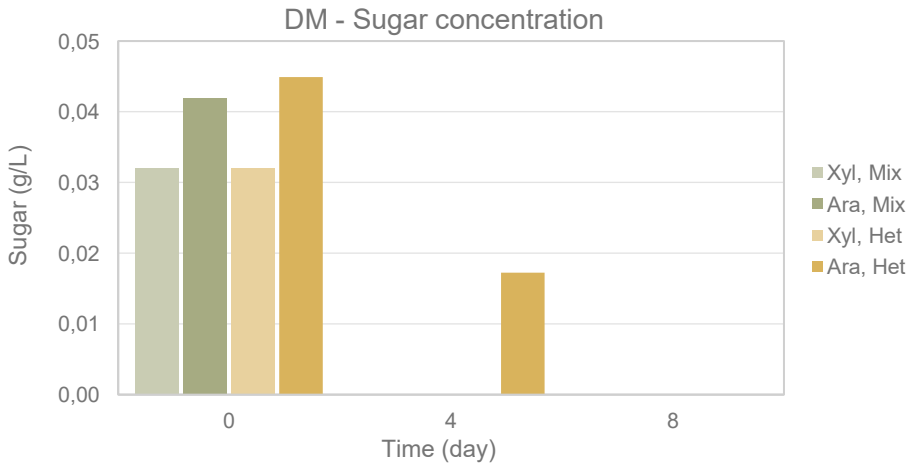

Figure 38: Sugar concentrations during mixotrophic and heterotrophic fermentation in DM

4.4.5 pO2

Figure 39 - Figure 42 show the dissolved O_2 concentration during fermentation in different media and cultivation modes. In terms of light-dependent fermentation, fluctuations of the dissolved O_2 can be observed according to the light and dark periods. CO_2 is assimilated and O_2 is produced during the day resulting in higher dissolved O_2 concentrations. Conversely, O_2 is used during dark respiration leading to decreased pO_2 values. It can be observed that the pO_2 signal had a decreasing development over time indicating that the photosynthetic

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efficiency (PE) reduced. Also, the differences of the pO_2 signal between day and night (ΔpO_2) narrowed at the end of the cultivation (Figure 40). The reducing PE over the cultivation process can be attributed to insufficient light penetration due to increasing cell densities or to inhibiting substances of the WSH medium. It has to be mentioned that evaporative water losses minimised the medium volume to around 60 % (appendix) resulting in a higher concentrated medium and higher cell densities.

In terms of the mixotrophic fermentation in WSH, ΔpO_2 was around 17 % after one week, 10 % after two weeks and reduced to 5 % after three weeks. The minimising PE over fermentation time is in accordance with the biomass stagnation after two weeks (Figure 35). Conversely, ΔpO_2 was highest (22 %) in photoautotrophic conditions after 12 days and was still twice as high as in the WSH at the end of the fermentation (Figure 39). In terms of the DM, ΔpO_2 was highest after three days at around 19 % and stayed constant at around 12 % until the end. An explanation for pO_2 values higher than 100 % might be the abrupt changes of the pO_2 signal between the night and day making it error-prone.

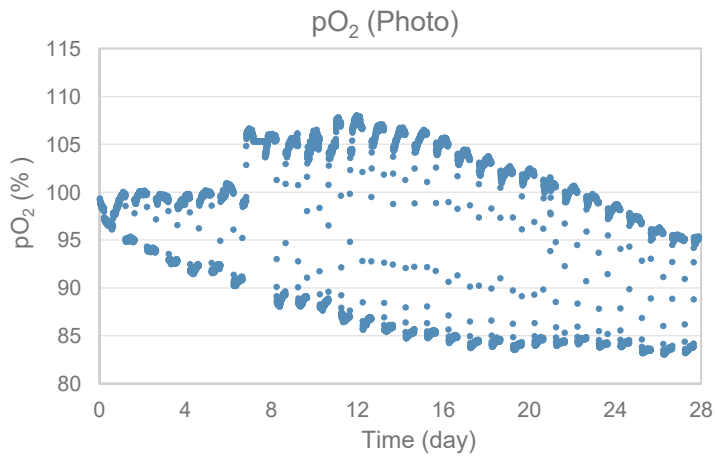

Figure 39: pO_2 of photoautotrophic fermentation

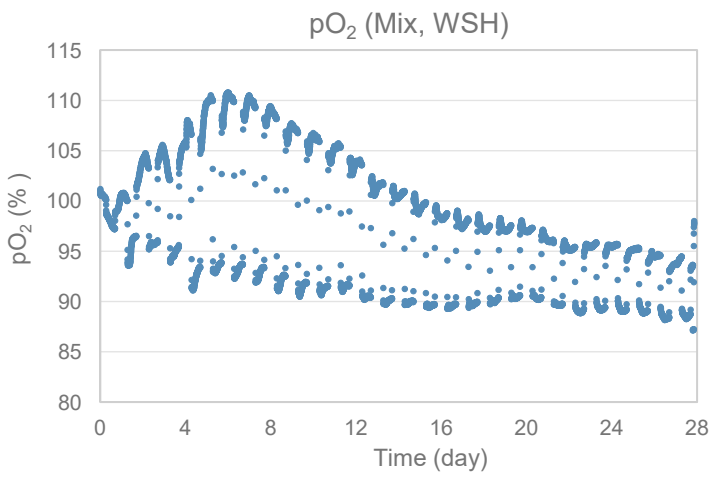

Figure 40: pO_2 of mixotrophic fermentation in WSH

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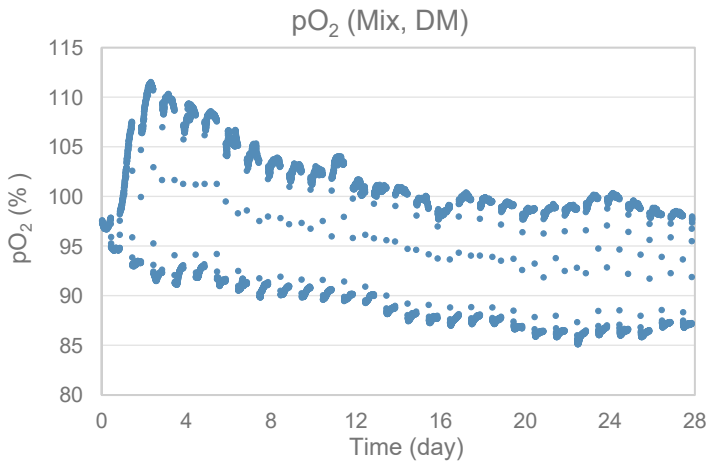

Figure 41: pO_2 of mixotrophic fermentation in DM

In contrast to light-dependent fermentations, heterotrophic microalgae are not capable of producing O_2. Thus, the pO_2 signal showed no fluctuations and decreased from 100 % to around 90 – 92 % in the first two weeks meaning that O_2 was assimilated under aerobic respiration (Figure 42). From then on, the pO_2 signal stayed constant being in accordance with the biomass concentrations (Figure 34).

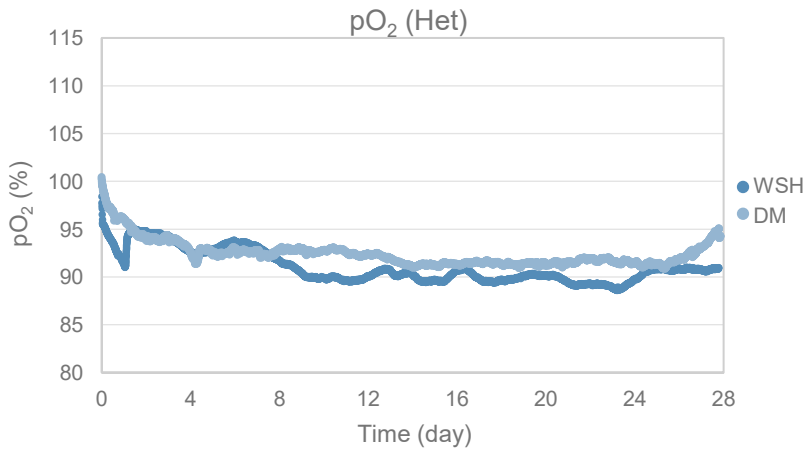

Figure 42: pO_2 of heterotrophic fermentation in WSH and DM

4.4.6 Off-gas analytics

Figure 43 and Figure 44 depict the volumetric percentages of O_2 and CO_2 in the off-gas. Alike in the pO_2 and pH signal (chapter 4.4.3 and 4.4.5), fluctuations in the off-gas signal can be observed according to the light/dark period in light-dependent fermentations. The O_2 off-gas showed a decreasing development especially after one week of fermentation meaning that more O_2 was assimilated during the night than O_2 was produced during the day. A reducing PE over cultivation time was also found in the pO_2 data. The strongest decrease of the O_2 off-gas signal can be found during the mixotrophic fermentation in WSH indicating that photosynthesis was stronger inhibited. This might be traced back to the consumption of organic compounds of the WSH performing aerobic respiration or to inhibition by other substances.

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Since the WSH medium is rich in nutrients, it is more prone to inhibition of photosynthesis compared to BG-11 medium and to the DM.

In terms of heterotrophic fermentation, the O_2 off-gas declined since the beginning because photosynthesis and O_2 production cannot be performed in the absence of light. The O_2 off-gas decreased stronger during the first weeks compared to the end of the fermentation, which can be attributed to growth stagnation of *C. vulgaris* after two weeks in heterotrophic cultivation. This observation was also found in the pO_2 signal (Figure 42).

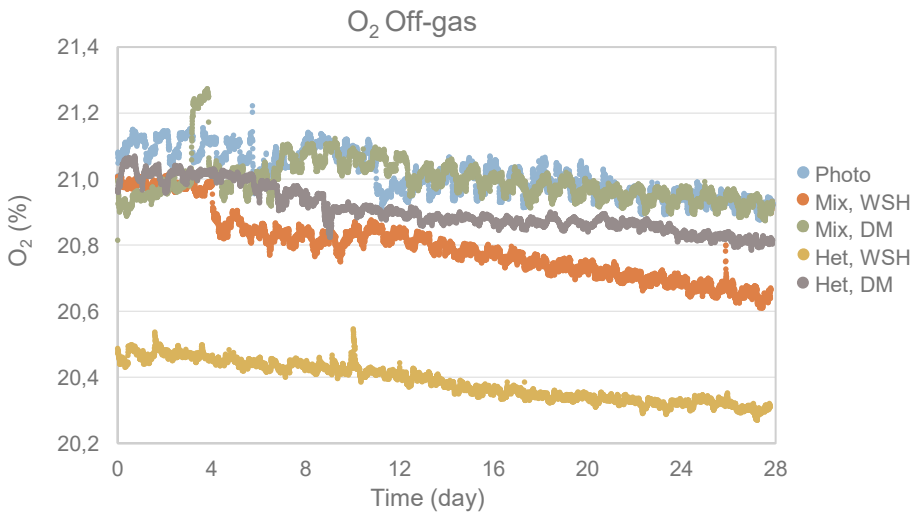

Figure 43: O_2 off-gas during fermentation in different media and cultivation modes

Figure 44 shows that the CO_2 off-gas was constant (0.04 % in WSH, 0.02 % in DM) during heterotrophic fermentation of *C. vulgaris*. Conversely, air was enriched with pure CO_2 gas setting a gas flow of 4 % CO_2 in light-dependent fermentations. However, only 0.8 - 1.2 % CO_2 was detected (Figure 44), which might be attributed to the low flow rate (0.02 L/min) resulting in imprecise measurements. Concerning the fermentation in WSH, the CO_2 off-gas increased and O_2 decreased after four days, because the airflow was not enriched with pure CO_2 gas in this period. In the DM, the CO_2 off-gas abruptly decreased from 1.2 % to 0.8 % after one week joined with an increase of O_2 from 21.0 % to 21.1 %. However, both the airflow and CO_2 flow rate showed no differences over the whole process (appendix). Also, the CO_2 off-gas showed some abrupt changes after six, eight and 12 days in photoautotrophic fermentation. While the increase after 12 days from 0.8 % to 1.2 % can be attributed to a reduced airflow rate, the other abrupt changes in the CO_2 off-gas might be explained by the low CO_2 flow rate leading to imprecise measurements.

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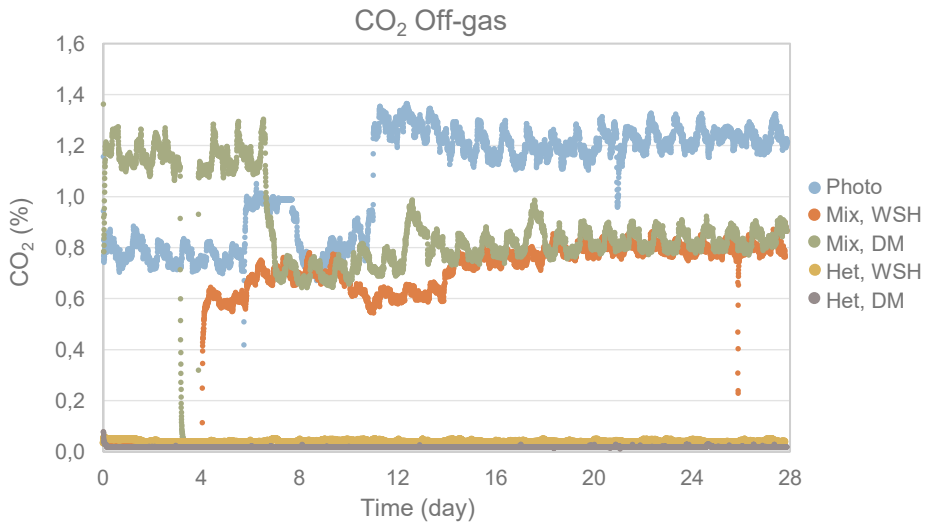

Figure 44: CO_2 off-gas during fermentation in different media and modes

4.4.7 OPR, CUR

The following diagrams (Figure 45 - Figure 50) depict the oxygen production rate (OPR) and carbon uptake rate (CUR). Since the off-gas data showed some drops during fermentation influencing the OPR and CUR, the off-gas data was included in the diagrams. As can be seen, the OPR and CUR reached negative values due to the decreasing development of the O_2 off-gas signal and drops of the CO_2 off-gas. While a decreasing O_2 off-gas signal is accompanied by reduced OPR, a declining CO_2 off-gas results in higher CUR. Since dark respiration leads to O_2 uptake and CO_2 release, it has to be considered that the increasing signal during light exposure represents the actual OPR and CUR values.

Concerning photoautotrophic fermentation (Figure 45, Figure 46), the OPR reached around 0.02 mol/(L*day) during light exposure, while the CUR showed values around 0.05 mol/(L*day). Hence, a RQ of 0.4 can be estimated meaning that more CO_2 was fixated than O_2 was produced during the day.

Figure 47 and Figure 48 show the OPR and CUR of mixotrophic fermentation in the WSH. Since airflow was not enriched with pure CO_2 gas during the first four days, lower OPR and CUR values can be found during this period. From then on, the OPR and CUR also reached values around 0.02 mol/(L*day) and 0.05 mol/(L*day), respectively.

The OPR and CUR of the mixotrophic fermentation in the DM are illustrated in Figure 49 and Figure 50. The OPR had lower values (0.02 mol/(L*day)) for the first four days compared to the time onwards (0.04 mol/(L*day)). This observation matches the fact that sugars were used up within four days (Figure 38) meaning that aerobic respiration was simultaneously performed. However, CUR was constant around 0.05 mol/(L*day).

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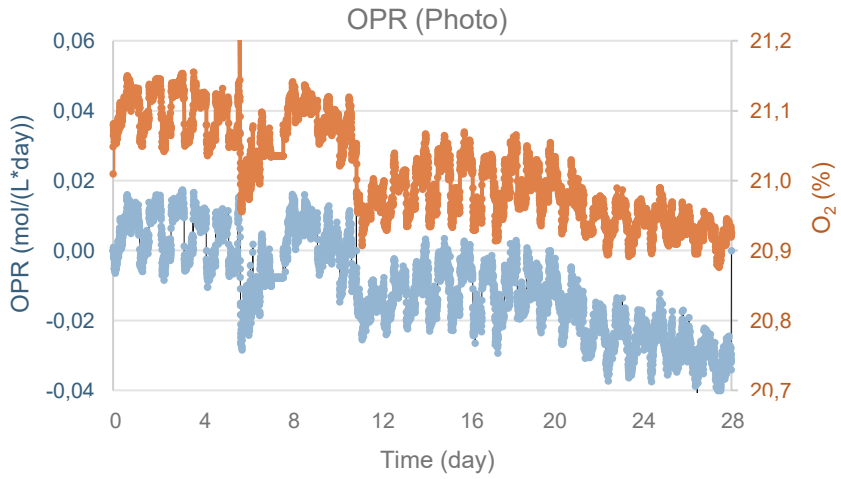

Figure 45: OPR and O_2 off-gas of photoautotrophic fermentation

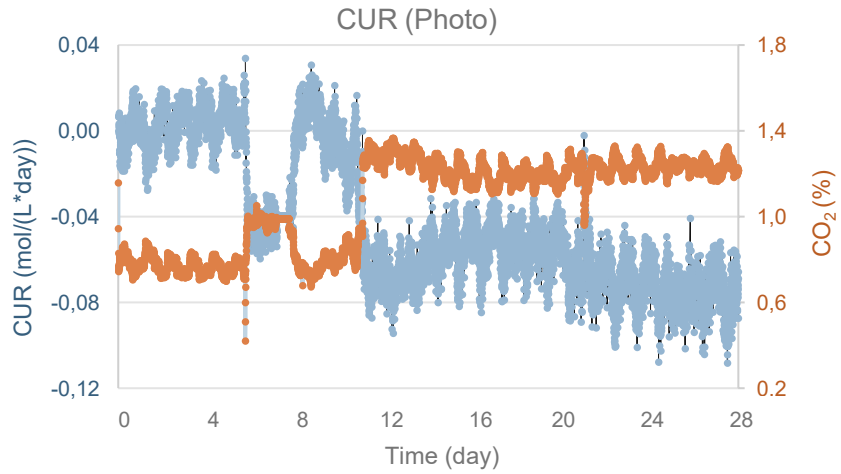

Figure 46: CUR and CO_2 off-gas of photoautotrophic fermentation

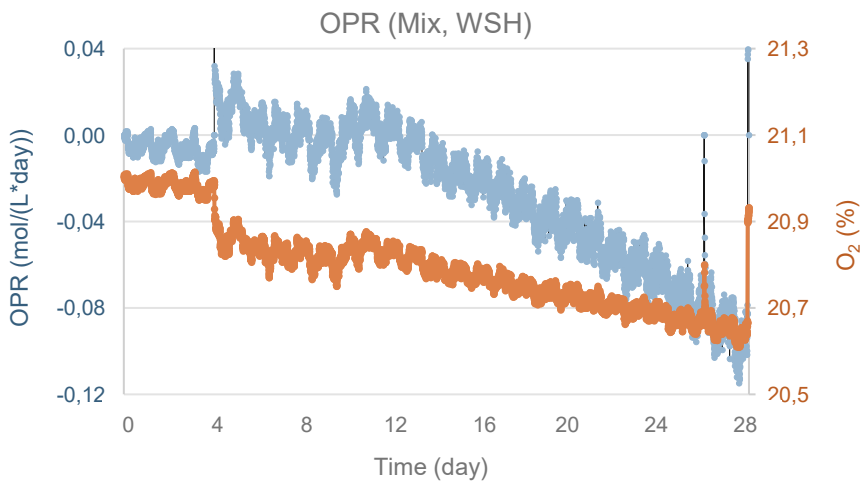

Figure 47: OPR and O_2 off-gas of mixotrophic fermentation in WSH

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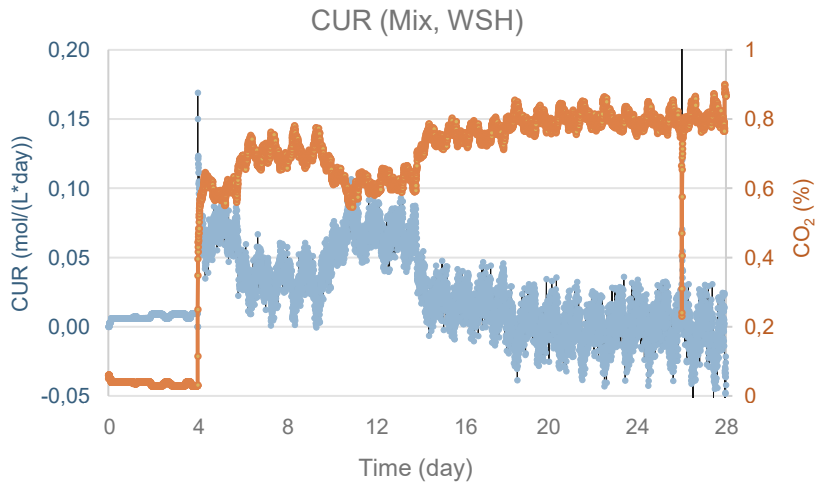

Figure 48: CUR and CO_2 off-gas of mixotrophic fermentation in WSH

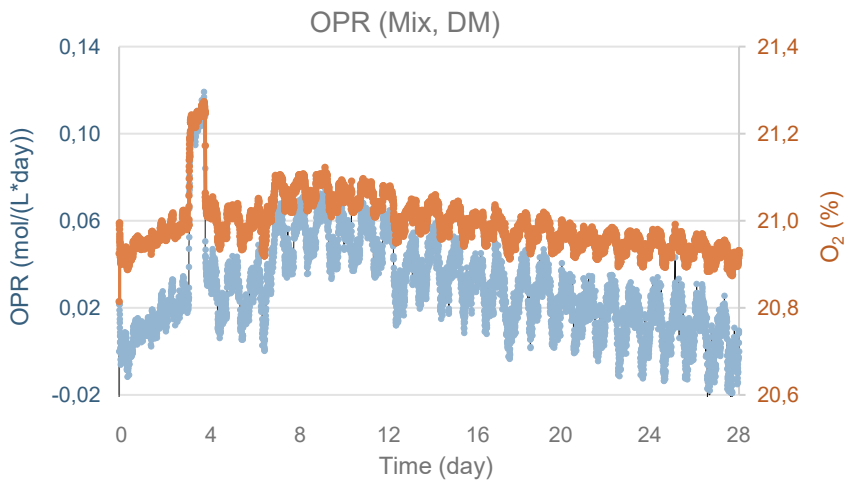

Figure 49: OPR and O_2 off-gas of mixotrophic fermentation in DM

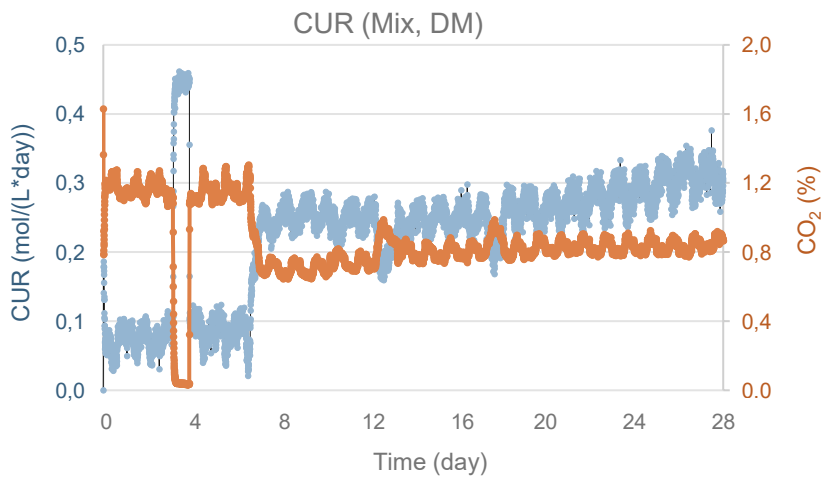

Figure 50: CUR and CO_2 off-gas of mixotrophic fermentation in DM

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4.4.8 OUR, CER

Oxygen uptake rate (OUR) and carbon evolution rate (CER) were calculated during heterotrophic fermentation in WSH (Figure 51, Figure 52) and in the DM (Figure 53, Figure 54). OUR increased with decreasing O_2 off-gas values implying that O_2 was taken up. However, the O_2 off-gas showed fluctuations during cultivation, which was neither observable in the pO_2 (Figure 42) nor in the pH (Figure 36) indicating that the O_2 off-gas and therefore the calculated OUR were not reliable. Conversely, the CO_2 off-gas was rather constant leading to CER values of zero.

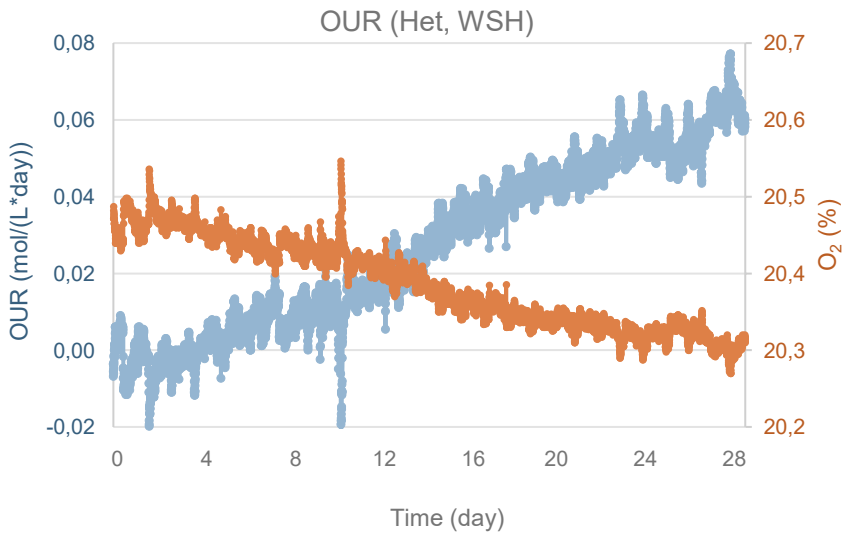

Figure 51: OUR and O_2 off-gas of heterotrophic fermentation in WSH

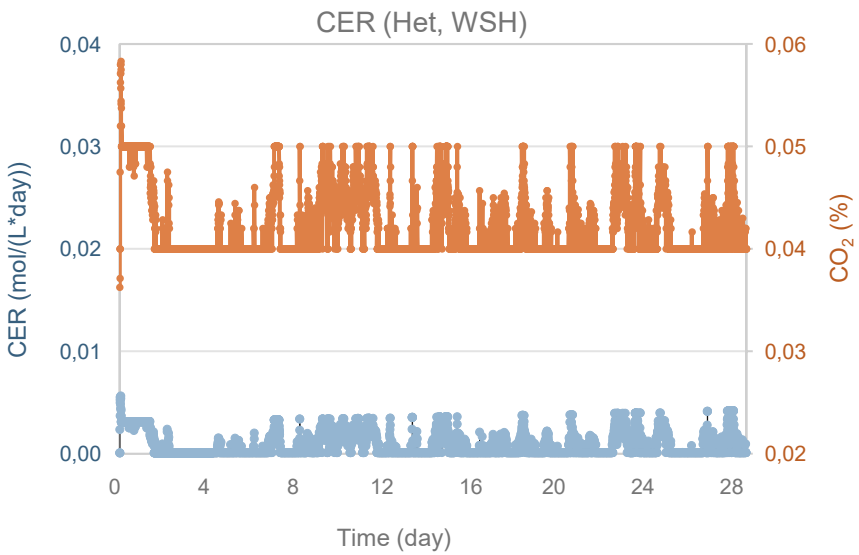

Figure 52: CER and CO_2 off-gas of heterotrophic fermentation in WSH

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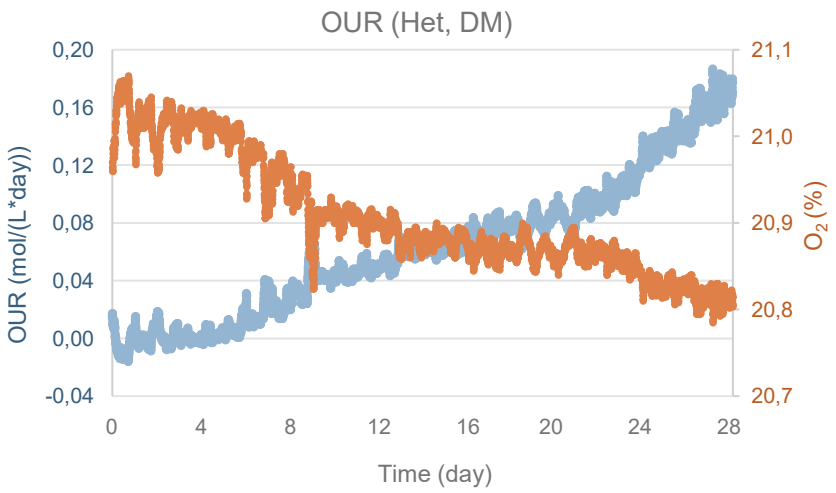

Figure 53: OUR and O_2 off-gas of heterotrophic fermentation in DM

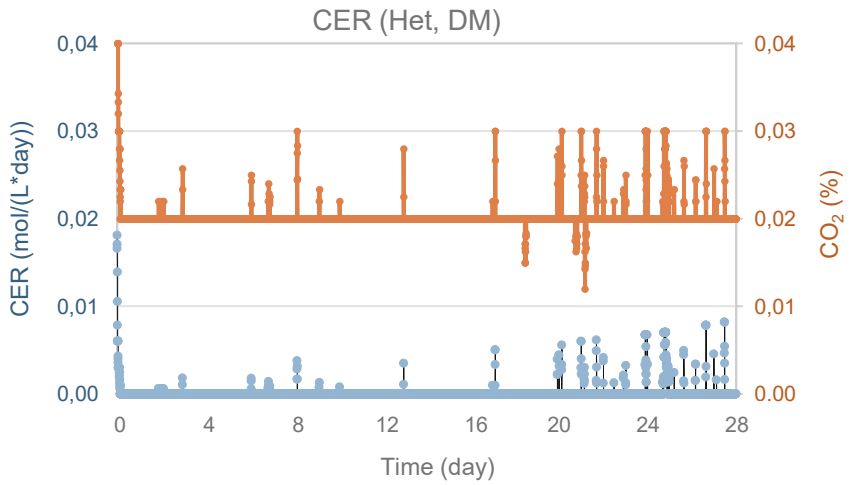

Figure 54: CER and CO_2 off-gas of heterotrophic fermentation in DM

4.4.9 Protein contents

The protein contents of *C. vulgaris* after fermentation in different cultivation conditions are summarised in Figure 55. The protein content was around 10 % of DCW in the DM and reached 15 - 18 % in the WSH. Photoautotrophic fermentation resulted in a protein content of 12 % of DCW. It can again be observed that the protein formation was not promoted by light exposure and was not negatively affected by the WSH.

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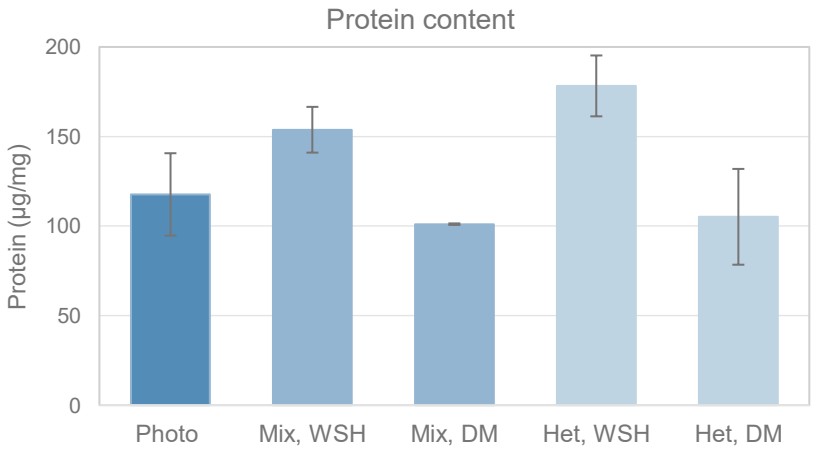

Figure 55: Protein contents after fermentation in different media and cultivation modes

4.4.10 Chlorophyll contents

The sum of chlorophyll *a* and *b* content was determined at the end of fermentation (Figure 56). Chlorophyll formation was higher under photoautotrophic (28 µg/mg) and mixotrophic (42 µg/mg in WSH, 31 µg/mg in DM) cultivation compared to heterotrophic conditions (19 µg/mg in WSH, 7 µg/mg in DM). Since the fermentation in the DM resulted in lower pigment content than in the WSH, it can be assumed that other compounds of the WSH had a promoting effect on chlorophyll synthesis.

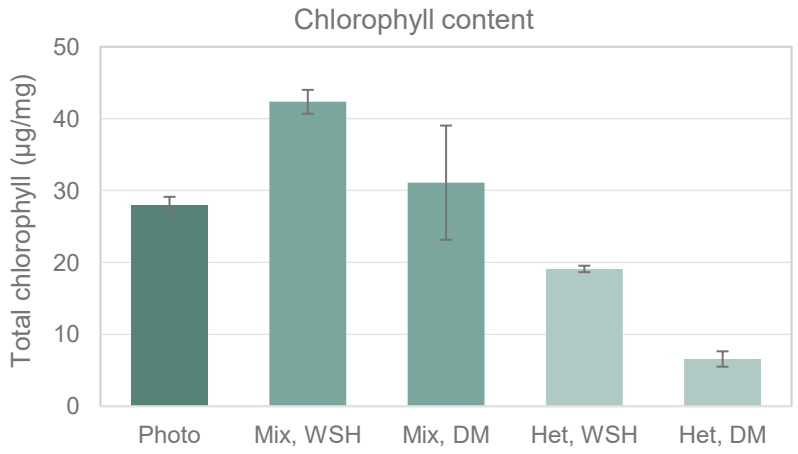

Figure 56: Chlorophyll contents after fermentation in different media and cultivation modes

4.4.11 Carbohydrate contents

Carbohydrate contents of *C. vulgaris* during fermentation are depicted in Figure 57. In addition, carbohydrate composition is illustrated in Figure 58 - Figure 60. Unfortunately, biomass concentrations during heterotrophic fermentation were too low for reliable determination of carbohydrate content and composition. Hence, only mixotrophic and photoautotrophic results are presented in the following diagrams.

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Generally, glucose, galactose, xylose, mannose, rhamnose, arabinose, and fructose were found in *C. vulgaris* biomass. However, xylose and mannose were combined in the following diagrams due to their overlapping in the IC-chromatogram. Standard deviation is based on the used analytical method, but not on triplicates.

C. vulgaris pre-culture (inoculum of fermentation) exhibited a carbohydrate content between 13 - 17 % of DCW (Figure 57). After four days of cultivation, only minor changes could be observed. After two weeks of cultivation, the carbohydrate content of *C. vulgaris* increased in the DM under mixotrophic conditions reaching 23 % of DCW. Similarly, the carbohydrate content started to increase after three weeks of cultivation in photoautotrophic conditions achieving 35 % of DCW at the end of the fermentation. The increase might be attributed to starch production during photosynthesis, which can also be observed in the carbohydrate composition by an enhanced glucose content (Figure 58, Figure 60). Conversely, the carbohydrate content of *C. vulgaris* grown in WSH decreased to 7 % of DCW. In addition, its carbohydrate composition (Figure 59) distinguished from the other compositions: While the major carbohydrate of *C. vulgaris* was glucose, the most abundant component during the cultivation in WSH was xylose/mannose. In fact, xylose/mannose content raised from 10 % to 55 % within four days indicating that *C. vulgaris* assimilated xylose.

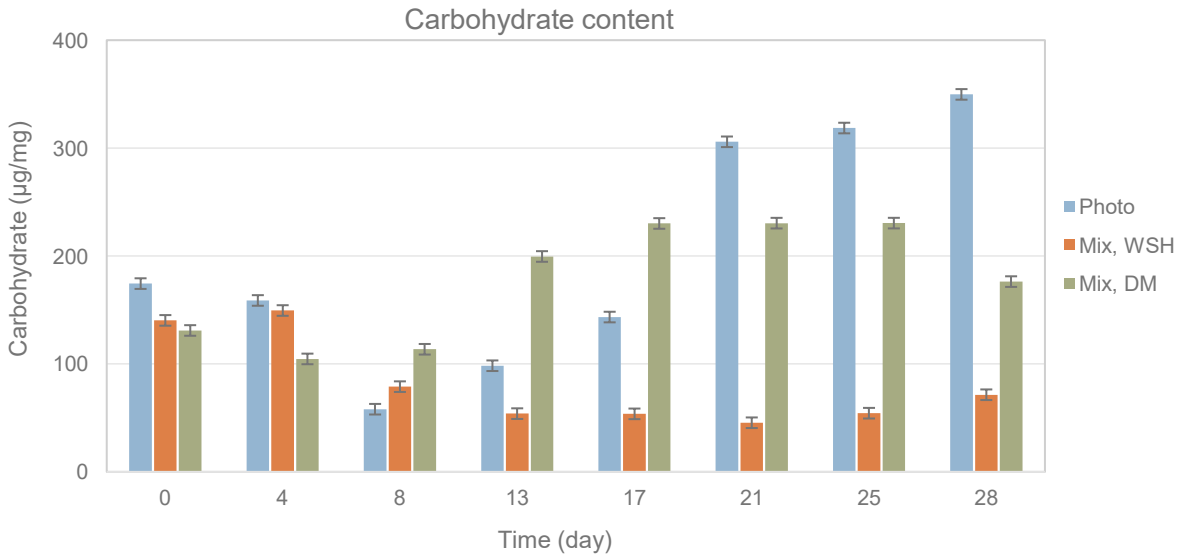

Figure 57: Carbohydrate contents during photoautotrophic and mixotrophic fermentation

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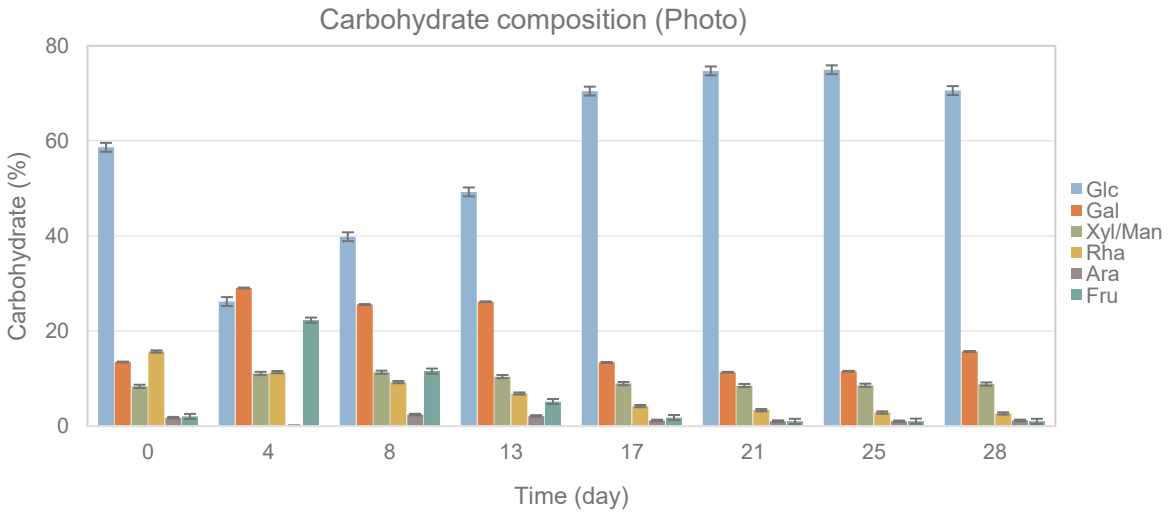

Figure 58: Carbohydrate composition during photoautotrophic fermentation

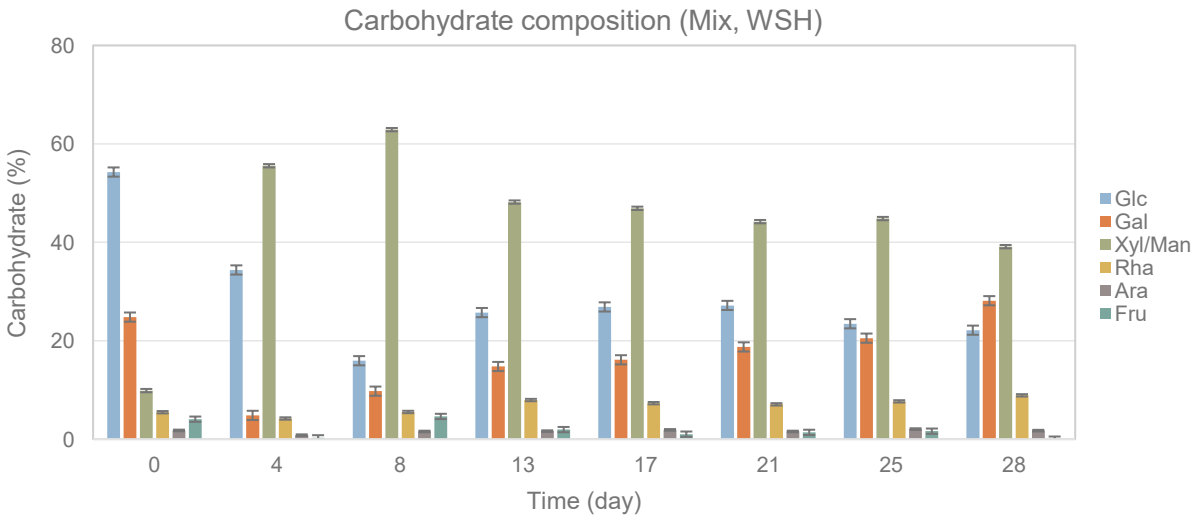

Figure 59: Carbohydrate composition during mixotrophic fermentation in WSH

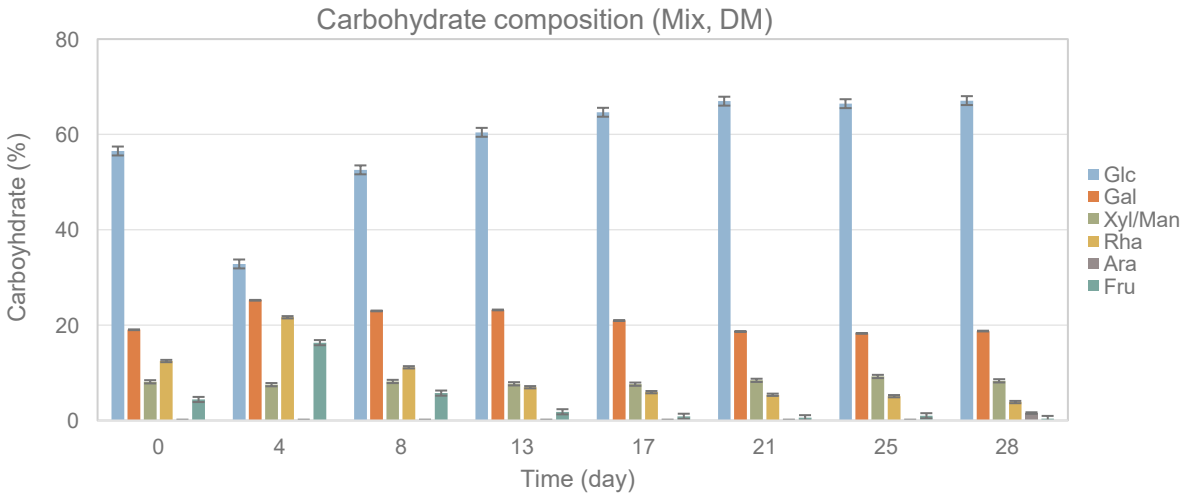

Figure 60: Carbohydrate composition during mixotrophic fermentation in DM

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To compare the carbohydrate composition of *C. vulgaris* (Figure 58 - Figure 60), the three most occurring carbohydrates are summarised in Table 15.

Table 15: Order of most occurring carbohydrates in *C. vulgaris* performed in different fermentations

Fermentation	Carbohydrates
Photoautotroph	glc > gal > xyl
Mixotroph, WSH	xyl > glc > gal
Mixotroph, DM	glc > gal > xyl

C. vulgaris biomass exhibited similar carbohydrate composition between photoautotrophic conditions and the cultivation in the DM. This might be traced back to the fact that all sugars were taken up within four days in the DM. From then on, cultivation conditions were comparable between mixotrophic cultivation in DM to photoautotrophic regime. In terms of the cultivation in WSH, xylose was the most abundant carbohydrate in *C. vulgaris* implying that xylose was assimilated. Since the carbohydrate composition of *C. vulgaris* was different between the cultivation in DM and WSH, it can be concluded that other nutrients of the WSH had an impact on *C. vulgaris* carbohydrate content.

Generally, the cell wall of *C. vulgaris* consists of rhamnose, galactose, xylose, arabinose, mannose and glucose[77]. Ho et al.[78] stated that *C. vulgaris* carbohydrate composition mainly consisted of glucose (~90 %) under photoautotrophic conditions. The remaining sugars of the carbohydrate composition were galactose, xylose, arabinose and rhamnose, which is in accordance with the data obtained in this study.

4.5 HMF/FF cultivation

In terms of the food and feed market, it is of interest whether toxic compounds of the WSH (HMF and FF) are assimilated by *C. vulgaris* or not. Generally, the impact of furans on microalgae is only scarcely known. Thus, cultivation of *C. vulgaris* in higher furan concentrations (200 mg/L) was investigated and compared to a control cultivation excluding toxic compounds. Since HMF and FF originate from glucose and xylose[57], respectively, these sugars were used as carbon sources (1 g/L). The supernatant of the cultivation was analysed in terms of glucose, xylose, FF, and HMF concentration by HPLC measurement and was compared before and after incubation of two weeks.

While furans were completely taken up after two weeks in mixotrophic cultivation (Figure 61), HMF concentration reduced by 50 % and FF by 91 % in heterotrophic conditions (Figure 62). Thus, it can be assumed that FF is faster assimilated than HMF. Figure 63 shows that xylose was slower taken up in the presence of furans in mixotrophic cultivation. In fact, xylose concentration reduced by 60 % in the absence of furans, and by 80 % in the presence of furans. Conversely, xylose concentration only decreased by 8 % in heterotrophic conditions

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excluding furans, and was not used in the presence of furans (Figure 64). While glucose was almost completely used up in heterotrophic cultivation, glucose concentration only reduced by 34 % in the presence of furans.

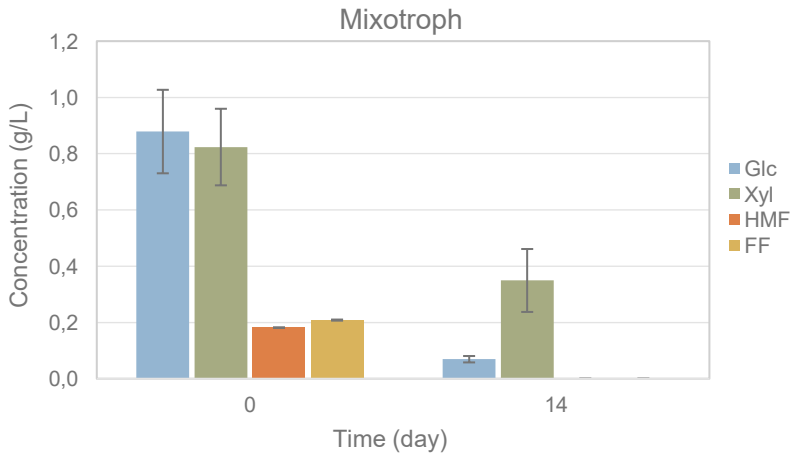

Figure 61: Concentration of sugars and furans in mixotrophic cultivation

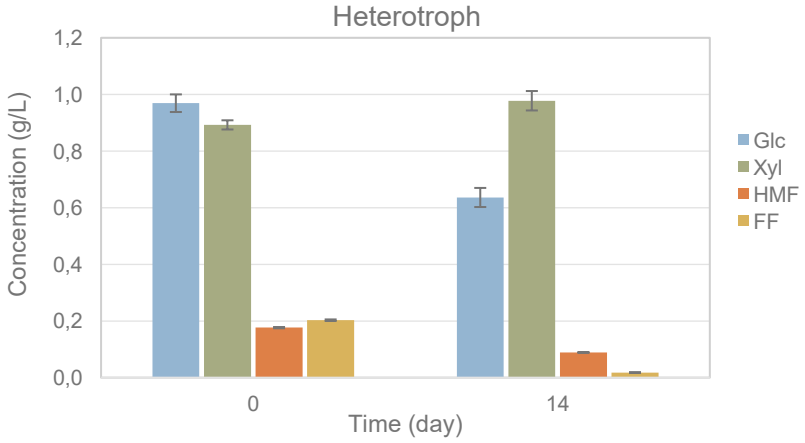

Figure 62: Concentration of sugars and furans in heterotrophic cultivation

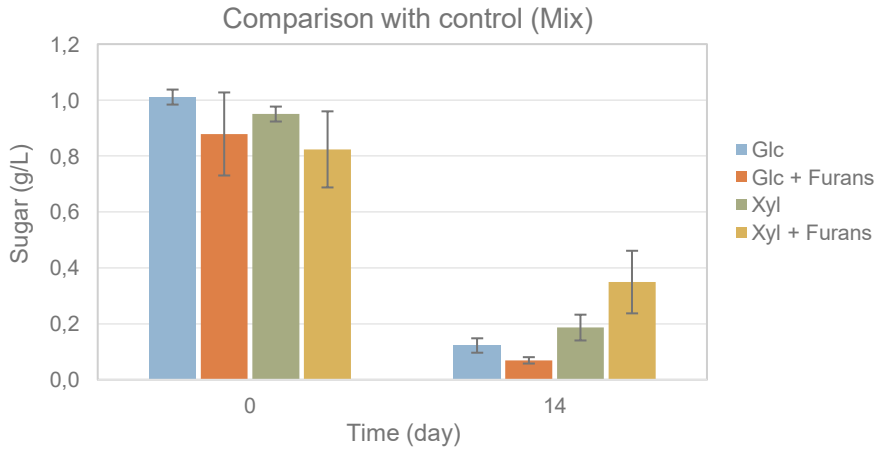

Figure 63: Sugar concentration in mixotrophic cultivation with and without furans

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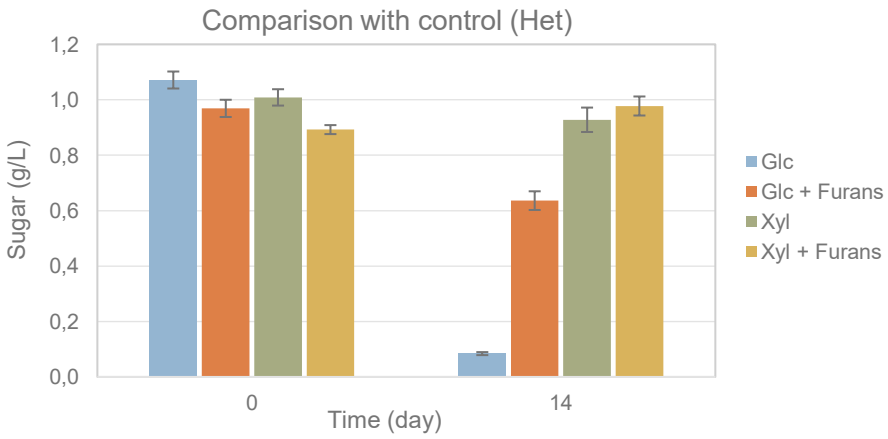

Figure 64: Sugar concentration in heterotrophic cultivation with and without furans

The chromatograms showed a disappearing HMF and FF peak (retention time 30 and 46) after two weeks of cultivation and new peaks at a retention time of 19 and 29 arose (Figure 65). Since the control cultivation showed no arising peaks at a retention time of 19 and 29, the upcoming peaks must be linked to FF and HMF. *C. vulgaris* might be able to degrade or even detoxify HMF and FF making *C. vulgaris* a potential detoxification treatment for lignocellulosic hydrolysates. Some microorganisms such as yeasts (*Saccharomyces cerevisiae, Pichia stipitis*) and bacteria (*Escherichia coli*) were reported to be able of reducing FF and HMF to less toxic alcohol derivates in NADPH-dependent reactions[57]. However, degradation of furans by microalgae is still unknown and must be analysed. In terms of the food and feed market, the question of whether toxic compounds remain in *C. vulgaris* must be clarified to determine the suitability for nutritional purposes.

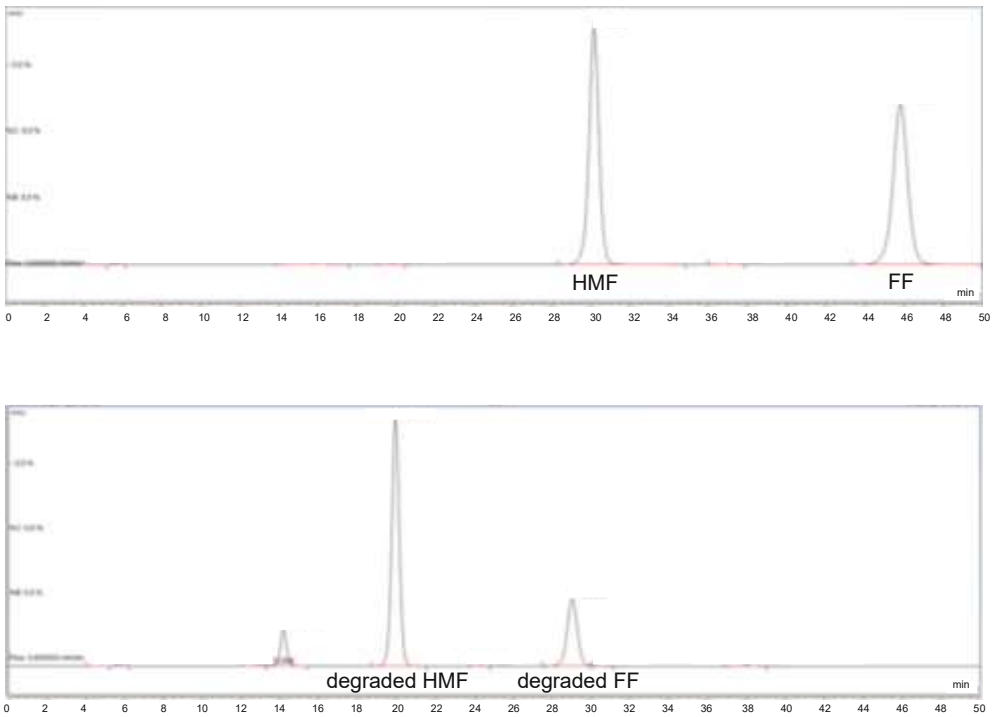

Figure 65: Chromatograms measured at UV/VIS 277 nm pre incubation (above) and after two weeks (below)

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5 Discussion

In the last decades, the threats of human overpopulation and the progressive depletion of the world's resources became ever more evident. Alternative food and feed sources based on sustainable resources are strongly needed. Microalgae such as *C. vulgaris* are able of fixating greenhouse CO_2 and provide highly nutritional biomass making them a promising candidate. Moreover, non-arable land can be used for their cultivation, which avoids competition of resources for traditional food and feed production. However, optimisation of microalgal mass cultivation in terms of processing costs and sustainability is required. Hence, the usage of WSH as a renewable substrate for *C. vulgaris* cultivation has been investigated.

5.1 Summary of results

First of all, shake flasks experiments were performed to find an appropriate dilution of WSH for *C. vulgaris* cultivation. Both heterotrophic and mixotrophic conditions were studied to find the ideal cultivation mode. In addition, the outcome was compared to the cultivation in a defined medium and to a state of the art photoautotrophic cultivation. Table 16 gives an overview of results of shake flasks cultivations.

Table 16: Summary of shake flask cultivations

Medium		DCW (g/L)		μ (day^{-1})		Protein (μg/mg)		Total Chl (μg/mg)	
	(%)	Het	Mix	Het	Mix	Het	Mix	Het	Mix
WSH	2.5	0.18 ± 0.01	0.92 ± 0.10	0.069 ± 0.000	0.203 ± 0.009	154 ± 6	166 ± 16	22.2 ± 5.0	33.9 ± 1.5
	5.0	0.25 ± 0.03	1.30 ± 0.08	0.095 ± 0.001	0.209 ± 0.006	154 ± 2	168 ± 43	14.9 ± 7.3	31.9 ±12.4
	12.5	0.27 ± 0.01		0.086 ± 0.003		173 ± 28		2.8 ± 0.8	
	17.5	0.29 ± 0.00		0.092 ± 0.003		183 ± 8		3.1 ± 0.8	
DM	2.5	0.11 ± 0.00	1.07 ± 0.07	0.025 ± 0.001	0.192 ± 0.010	153 ± 20	151 ± 5	10.2 ± 2.6	32.5 ± 5.7
	5.0	0.11 ± 0.00	0.88 ± 0.09	0.023 ± 0.001	0.185 ± 0.011	205 ± 64	170 ± 6	7.3 ± 0.4	33.7 ± 4.6
	12.5	0.11 ± 0.00		0.024 ± 0.001		205 ± 49		7.4 ± 1.9	
	17.5	0.11 ± 0.00		0.025 ± 0.001		147 ± 23		11.4 ± 0.8	
					Photo				
BG-11		0.85 ± 0.02		0.183 ± 0.005		168 ± 13		35.7 ± 0.9	

Biomass is based on data after 24 days (het) and 22 days (mix) of cultivation; growth rate on the second data point.

The next step was the investigation of *C. vulgaris* cultivation in WSH at larger scale. In this study, the most diluted WSH (2.5 %) was tested under heterotrophic and mixotrophic conditions. As comparison, fermentation was also conducted in 2.5 % DM and under photoautotrophic conditions. Table 17 summarises the major results of batch fermentation.

Table 17: Summary of batch fermentation

Condition	Medium	DCW (g/L)	μ (day^{-1})	Protein (μg/mg)	Total Chl (μg/mg)	Carbs (μg/mg)
Phototroph	BG-11	1.89	0.365	118 ± 23	28.0 ± 1.2	350 ± 5
Mixotroph	WSH	1.42	0.329	154 ± 13	42.3 ± 1.7	72 ± 5
	DM	2.36	0.362	101 ± 0.4	31.1 ± 7.9	176 ± 5
Heterotroph	WSH	0.13	0.074	178 ± 17	19.6 ± 0.4	-
	DM	0.08	0.037	105 ± 27	6.6 ± 1.1	-

No triplicates - given standard deviation is based on the used method. Growth rates are based on the second data point (after 8 days).

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5.2 Growth parameters

In terms of shake flasks, heterotrophically grown *C. vulgaris* resulted in lower biomass concentrations and growth rates (0.2 - 0.3 g/L, 0.07 - 0.10 day^{-1}) than under photoautotrophic (0.85 g/L, 0.18 day^{-1}) and mixotrophic conditions (0.9 - 1.3 g/L, 0.20 - 0.21 day^{-1}). This can be attributed to the fact that heterotrophic growth is strictly dependent on organic carbon sources. Since hexoses are preferred carbon sources compared to pentoses, heterotrophic cultivation of *C. vulgaris* in WSH (being primarily based on xylose and arabinose) was not efficient. An increase of monomeric glucose concentration by modifying LHW hydrolysation parameters or by including an enzymatic treatment could significantly enhance the outcome. Besides glucose being the best C-source in terms of microalgal growth, xylose uptake is also supported by the presence of glucose[75]. Cai et al.[71] investigated heterotrophic growth of *C. vulgaris* in enzymatically pre-treated broken rice straw and evaluated that despite additional costs of ∝-amylase and glucoamylase, the culture medium cost per 1 kg biomass was 90 % less than that in glucose.

Lower concentrations of WSH (2.5 %, 5.0 %) were tested under mixotrophic conditions because heterotrophic cultivation showed reduced pigment formation in 12.5 % and 17.5 % WSH. However, higher concentrations of WSH under mixotrophic conditions should also be studied in the future, as *C. vulgaris* performance was higher at 5.0 % WSH compared to 2.5 % WSH. In fact, *C. vulgaris* showed 1.5-fold higher growth under mixotrophic conditions compared to the photoautotrophic control. A similar outcome was obtained by Salati et al.[33], who mixotrophically cultivated *C. vulgaris* in glycerol and in white wine lees and achieved 1.4-fold higher growth compared to the photoautotrophic culture.

The mixotrophic mode may require additional light energy costs compared to heterotrophic cultivation, but it is more sustainable in terms of CO_2 fixation. Moreover, the light period can be shortened compared to photoautotrophic mode reducing energy costs. Further research is needed on *C. vulgaris* cultivation in WSH in terms of optimising photoperiod, light intensity or spectral composition.

C. vulgaris growth was promoted in the bioreactor compared to shake flasks under mixotrophic and photoautotrophic conditions, but not in the heterotrophic regime. The reason for the lower performance in the bioreactor under heterotrophic conditions might be attributed to the maintenance of darkness over the whole process, which was not possible in shake flasks during sampling. Conversely, higher biomass concentrations and growth rates in light-dependent fermentations can be traced back to mixing and aeration providing uniform distribution of light, nutrients and gases[16]. In addition, higher light intensity during fermentation (~150 µmol/(m^2*s)) compared to shake flasks (26.4 µmol/(m^2*s)) might have had a positive impact. However, evaporative water losses reduced the volume of the medium by

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approximately 40 % resulting in higher inhibition of photosynthetic efficiency due to higher cell densities and concentrating of the WSH. Also, the air was not enriched with additional CO_2 gas for the first four days. Hence, potential for growth improvement of *C. vulgaris* in WSH is given. To provide statistically relevant information, fermentations should to be repeated in triplicates to obtain more reliable statements about growth performance.

To compare growth parameters with literature, Table 18 gives an overview of biomass concentrations and specific growth rates of *C. vulgaris* in different media and conditions. It has to be noted that hydrolysates were enzymatically treated resulting in higher monomeric glucose content. However, this step was not performed in this study to avoid additional costs and to investigate growth behaviour based on pentoses.

Table 18: Biomass concentration and growth rates of *C. vulgaris* in different media and conditions

Condition	Carbon source	Biomass (g/L)	μ_{max} (day^{-1})	Reference
Het	Broken Rice hydrolysate	4.0	1.38	71
	Wheat bran hydrolysate	3.4	-	39
	Sweet sorghum hydrolysate	2.7	0.21	37
	Rice straw hydrolysate	3.1	0.25	37
Mix	Wheat bran hydrolysate	3.7	-	39
	Vinasse	0.8	0.34	45
	Cheese whey	1.6	0.29	45
	Corn steep liquor	2.1	0.38	45
	Food waste (from cafeteria)	1.7	0.34	36
Photo	-	0.9	0.29	45
	-	0.4	0.23	38

As can be observed, heterotrophic growth of *C. vulgaris* in WSH (0.1 - 0.3 g/L, 0.07 - 0.09 day^{-1}) could not compete with growth performances obtained in enzymatically treated hydrolysates. Thus, it can be concluded that an increase of monomeric glucose content is needed in terms of heterotrophic regime. Conversely, growth performance of mixotrophically grown *C. vulgaris* in WSH (1.3 - 1.4 g/L, 0.20 - 0.33 day^{-1}) is comparably high considering the fact that monomeric glucose was not available.

De Freitas et al.[79] mixotrophically cultivated *C. vulgaris* in a synthetic mixture of xylose and arabinose. Similar xylose concentrations (38 mg/L, 77 mg/L) were studied resulting in growth rates of 0.13 day^{-1} and 0.19 day^{-1}, which are in accordance with the growth rate obtained in the DM (0.19 day^{-1}).

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5.3 Biomass composition

One major parameter for the food and feed industry is the protein content. While *C. vulgaris* is reported to contain up to 60 % protein content under nitrogen-rich conditions[12], only between 15 - 21 % of DCW were obtained in shake flasks experiments and 10 – 18 % of DCW after batch fermentation. Since the photoautotrophic control also resulted in lower protein contents, it can be concluded that the WSH had no negative impact on protein formation.

Generally, increased protein production under mixotrophic and photoautotrophic conditions was expected[39]. However, reduced protein contents were also obtained by Melo et al.[45], who studied mixotrophic cultivation of *C. vulgaris* in vinasse and cheese whey. The protein content was between 22 - 23 % of DCW such as the photoautotrophic culture (22 % of DCW)[45]. Chew et al.[36] mixotrophically cultivated *C. vulgaris* in different concentrated food waste mixtures (from a cafeteria of Malaysia) showing a protein content of 70 - 128 mg/g.

The reduced protein content of *C. vulgaris* might be attributed to the complete consumption of nitrogen sources at the end of cultivation[80]. Thus, protein content and nitrogen concentration should be determined during the whole cultivation process to confirm this assumption. Optimisation of parameters (e.g., light intensity, photoperiod, spectral composition, CO_2 concentration, nutrients) regarding protein content is required to make the biomass more attractive for the food and feed industry. For instance, Xie et al.[81] stimulated protein formation of heterotrophically grown *C. vulgaris* from 38 % to 44 % of DCW by applying a nitrogen-deficient period (48 h) followed by a nitrogen-rich phase. The increase of the protein content had no adverse impact on growth behaviour. Also, Zhang et al.[76] cultivated *C. pyrenoidosa* in straw hydrolysates and found higher protein content in rising nitrate concentration.

Another important parameter for the food and feed market is the chlorophyll content. Since chlorophyll plays a significant role during photosynthesis, chlorophyll formation is expected to be higher in the presence of light compared to the heterotrophic regime[25]. Weibao et al.[38] stated the following decreasing order of chlorophyll formation in *C. vulgaris*: Photoautotrophy (45 µg/mg) > mixotrophy > heterotrophy, 2 g/L carbon source > 10 g/L carbon source. The finding is in accordance with the data obtained in shake flasks experiments. While photoautotrophic conditions resulted in chlorophyll content of 36 µg/mg, mixotrophic and heterotrophic cultivation in the WSH reached a chlorophyll content of 34 µg/mg and 22 µg/mg, respectively. After batch fermentation, pigment content was even higher in mixotrophic conditions in the WSH (42 µg/mg) compared to photoautotrophic (28 µg/mg) and heterotrophic (20 µg/mg) fermentation. Thus, it can again be highlighted that the WSH showed no adverse effect on chlorophyll synthesis in *C. vulgaris*.

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Moreover, the carbohydrate content of *C. vulgaris* was analysed during mixotrophic and photoautotrophic batch fermentation. While *C. vulgaris* showed higher carbohydrate production (35 % of DCW) in photoautotrophic conditions, mixotrophic fermentation resulted in lower carbohydrate content (7 - 18 % of DCW). Since *C. vulgaris* biomass cultivated in WSH contained both reduced protein and carbohydrate content, it can be assumed that lipid production was stimulated. Other studies reported that mixotrophic conditions resulted in enhanced lipid content in *C. vulgaris* compared to heterotrophic and photoautotrophic mode[38,41,82]. Shen et al.[83] found that the activity of the tricarboxylic acid cycle and therefore fatty acid synthesis in *C. vulgaris* was higher under mixotrophic conditions using acetate as an organic carbon source. In fact, *C. vulgaris* reached 63 % fatty acid content after nine days in 5 g/L sodium acetate[83]. Hence, lipid content and composition should to be determined in the future to evaluate suitability for the food and feed industry. Former studies reported that *C. vulgaris* lipid profile can be rich in PUFAs being desirable for nutritional purposes[12,84].

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6 Outlook

To conclude, a new approach of using WSH as a renewable substrate for the cultivation of *C. vulgaris* was studied. While mixotrophic cultivation resulted in 1.5-fold higher biomass concentration compared to photoautotrophic cultivation, heterotrophic cultivation conditions showed 4-times lower biomass concentration. The usage of WSH has the potential to enable an economically and environmentally-friendly cultivation process, but optimisation is still required starting from the pre-treatment and hydrolysis of the WSH to the microalgal cultivation parameters. Concerning the cultivation of *C. vulgaris* in WSH, the following proposals for the future are given:

- Investigate higher concentrations of WSH under mixotrophic conditions to test whether biomass yield can be further increased. Find the inhibiting concentration of WSH of *C. vulgaris* cultivation.
- Optimise light and dark period to reduce light energy costs in the mixotrophic regime.
- Cultivation of *C. vulgaris* in WSH without the supply of minerals and vitamins by BG-11 to reduce medium costs.
- Alter LHW hydrolysation parameters to increase monomeric hexose content. Higher glucose concentration will enhance growth rates under heterotrophic and mixotrophic conditions.
- Investigate the cultivation of *C. vulgaris* in enzymatically pre-treated WSH and evaluate whether higher growth rates compensate for additional costs of enzymes.
- Optimise cultivation parameters (e.g., light intensity, spectral composition, photoperiod, CO_2 and nutrient concentration) in terms of protein content.
- Determine lipid content and composition to fully understand the primary biomass composition of *C. vulgaris* in WSH. High content of PUFAs would be desirable concerning the food and feed market.
- Investigate the impact of FF and HMF on *C. vulgaris* growth:
    - Find inhibiting furan concentration.
    - Investigate the degradation pathway of furans.
    - Find out whether toxic compounds remain in *C. vulgaris* biomass.
- Perform a life cycle assessment and cost evaluation based on the usage of WSH to evaluate economic feasibility and sustainability of the cultivation process.

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7 Abbreviations

Ara	Arabinose
ATP	Adenosine triphosphate
BCA	2 2'-Biquinoline-4 4'-dicarboxylic acid
CER	Carbon dioxide evolution rate
Chl	Chlorophyll
CUR	Carbon dioxide uptake rate
DM	Defined medium
DCW	Dry cell weight
EDTA	2,2',2",2'"-(Ethane-1,2-diyldinitrilo)tetraacetic acid
FF	Furfural
Fru	Fructose
Gal	Galactose
Glc	Glucose
Het	Heterotroph
HEPES	2-(4-(2-Hydroxyethyl)piperazin-1-yl)ethanesulfonic acid
HMF	5-(Hydroxymethyl)furfural
HPLC	High-Performance Liquid Chromatography
IC	Ion chromatography
LHW	Liquid hot water
LCA	Life cycle assessment
Man	Mannose
Mix	Mixotroph
NADPH	Nicotinamide adenine dinucleotide phosphate
OD_{600}	Optical density at 600 nm
OPR	Oxygen production rate
OUR	Oxygen uptake rate
PE	Photosynthetic efficiency
Photo	Photoautotroph
pO2	Partial pressure of oxygen
PUFA	Polyunsaturated fatty acid
Rha	Rhamnose
rcf	Relative centrifugal force
rpm	Round per minute
RuBisCO	Ribulose-1,5-bisphosphate carboxylase oxygenase
RQ	Respiratory quotient

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T	Temperature
TCA	Trichloroacetic acid
WSH	Wheat straw hydrolysate
Xyl	Xylose
μ	Specific growth rate

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8 References

(1) Safi, C.; Zebib, B.; Merah, O.; Pontalier, P.-Y.; Vaca-Garcia, C. Morphology, composition, production, processing and applications of Chlorella vulgaris: A review. *Renewable and Sustainable Energy Reviews* **2014,** *35*, 265.

(2) Barkia, I.; Saari, N.; Manning, S. R. J. M. d. Microalgae for high-value products towards human health and nutrition. **2019,** *17* (5), 304.

(3) Koyande, A. K.; Chew, K. W.; Rambabu, K.; Tao, Y.; Chu, D.-T.; Show, P.-L. Microalgae: A potential alternative to health supplementation for humans. *Food Science and Human Wellness* **2019,** *8* (1), 16.

(4) Metting, F. B. Biodiversity and application of microalgae. *Journal of Industrial Microbiology & Biotechnology* **1996,** *17* (5-6), 477.

(5) Buono, S.; Langellotti, A. L.; Martello, A.; Rinna, F.; Fogliano, V. J. F.; function. Functional ingredients from microalgae. **2014,** *5* (8), 1669.

(6) Camacho, F.; Macedo, A.; Malcata, F. J. M. d. Potential industrial applications and commercialization of microalgae in the functional food and feed industries: A short review. **2019,** *17* (6), 312.

(7) Borowitzka, M. A. J. J. o. a. p. High-value products from microalgae—their development and commercialisation. **2013,** *25* (3), 743.

(8) Benedetti, M.; Vecchi, V.; Barera, S.; Dall'Osto, L. J. M. c. f. Biomass from microalgae: the potential of domestication towards sustainable biofactories. **2018,** *17* (1), 1.

(9) Znad, H.; Al Ketife, A. M. D.; Judd, S.; Almomani, F.; Vuthaluru, H. B. Bioremediation and nutrient removal from wastewater by Chlorella vulgaris. *Ecological Engineering* **2018,** *110*, 1.

(10) Abreu, A. P.; Fernandes, B.; Vicente, A. A.; Teixeira, J.; Dragone, G. J. B. t. Mixotrophic cultivation of Chlorella vulgaris using industrial dairy waste as organic carbon source. **2012,** *118*, 61.

(11) García, J. L.; de Vicente, M.; Galán, B. J. M. B. Microalgae, old sustainable food and fashion nutraceuticals. **2017,** *10* (5), 1017.

(12) Molino, A.; Iovine, A.; Casella, P.; Mehariya, S.; Chianese, S.; Cerbone, A.; Rimauro, J.; Musmarra, D. J. I. j. o. e. r.; health, p. Microalgae characterization for consolidated and new application in human food, animal feed and nutraceuticals. **2018,** *15* (11), 2436.

(13) Hosikian, A.; Lim, S.; Halim, R.; Danquah, M. K. Chlorophyll Extraction from Microalgae: A Review on the Process Engineering Aspects. *International Journal of Chemical Engineering* **2010,** *2010*, 1.

(14) Pulz, O.; Gross, W. J. A. m.; biotechnology. Valuable products from biotechnology of microalgae. **2004,** *65* (6), 635.

(15) Becker, E. W. Micro-algae as a source of protein. *Biotechnology Advances* **2007,** *25* (2), 207.

(16) Khan, M. I.; Shin, J. H.; Kim, J. D. J. M. c. f. The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. **2018,** *17* (1), 36.

(17) Yang, B.; Liu, J.; Jiang, Y.; Chen, F. J. B. j. Chlorella species as hosts for genetic engineering and expression of heterologous proteins: Progress, challenge and perspective. **2016,** *11* (10), 1244.

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(18) Spolaore, P.; Joannis-Cassan, C.; Duran, E.; Isambert, A. Commercial applications of microalgae. *Journal of Bioscience and Bioengineering* **2006,** *101* (2), 87.
(19) Lum, K. K.; Kim, J.; Lei, X. G. J. J. o. a. s.; biotechnology. Dual potential of microalgae as a sustainable biofuel feedstock and animal feed. **2013,** *4* (1), 53.
(20) Miazek, K.; Remacle, C.; Richel, A.; Goffin, D. J. E. Effect of lignocellulose related compounds on microalgae growth and product biosynthesis: a review. **2014,** *7* (7), 4446.
(21) Pawar, S. J. R.; Reviews, S. E. Effectiveness mapping of open raceway pond and tubular photobioreactors for sustainable production of microalgae biofuel. **2016,** *62*, 640.
(22) Loganathan, B. G.; Orsat, V.; Lefsrud, M.; Wu, B. S. J. B.; engineering, b. A comprehensive study on the effect of light quality imparted by light-emitting diodes (LEDs) on the physiological and biochemical properties of the microalgal consortia of Chlorella variabilis and Scenedesmus obliquus cultivated in dairy wastewater. **2020,** *43* (8), 1445.
(23) https://www.aquaculturealliance.org/advocate/pond-cultivated-algae-slimy-superhero-aquafeeds/. **, 17.11.2021**.
(24) https://www.wikiwand.com/en/Photobioreactor. **, 17.11.2021**.
(25) Prokop, A.; Bajpai, R.; Zappi, M.; Springer, Cham, 2017.
(26) Kuenz, A.; Grimm, D.; Rahmann, G. J. O. A. Versatility of algae—exploring the potential of algae for nutrient circulation. **2021,** *11*, 251.
(27) Wang, S.-K.; Stiles, A. R.; Guo, C.; Liu, C.-Z. Microalgae cultivation in photobioreactors: An overview of light characteristics. *Engineering in Life Sciences* **2014,** *14* (6), 550.
(28) Blair, M. F.; Kokabian, B.; Gude, V. G. J. J. o. e. c. e. Light and growth medium effect on Chlorella vulgaris biomass production. **2014,** *2* (1), 665.
(29) Posten, C.; Chen, S. F. Microalgae biotechnology. **2016**.
(30) Nirmalakhandan, N.; Selvaratnam, T.; Henkanatte-Gedera, S.; Tchinda, D.; Abeysiriwardana-Arachchige, I.; Delanka-Pedige, H.; Munasinghe-Arachchige, S.; Zhang, Y.; Holguin, F.; Lammers, P. J. A. R. Algal wastewater treatment: Photoautotrophic vs. mixotrophic processes. **2019,** *41*, 101569.
(31) Ende, S. S. W.; Noke, A. Heterotrophic microalgae production on food waste and by-products. *Journal of Applied Phycology* **2019,** *31* (3), 1565.
(32) Smetana, S.; Sandmann, M.; Rohn, S.; Pleissner, D.; Heinz, V. J. B. T. Autotrophic and heterotrophic microalgae and cyanobacteria cultivation for food and feed: life cycle assessment. **2017,** *245*, 162.
(33) Salati, S.; D'Imporzano, G.; Menin, B.; Veronesi, D.; Scaglia, B.; Abbruscato, P.; Mariani, P.; Adani, F. J. B. t. Mixotrophic cultivation of Chlorella for local protein production using agro-food by-products. **2017,** *230*, 82.
(34) Singh, R.; Birru, R.; Sibi, G. J. J. o. E. P. Nutrient removal efficiencies of Chlorella vulgaris from urban wastewater for reduced eutrophication. **2017,** *8* (01), 1.
(35) Miao, M.-S.; Yao, X.-D.; Shu, L.; Yan, Y.-J.; Wang, Z.; Li, N.; Cui, X.-T.; Lin, Y.-M.; Kong, Q. Mixotrophic growth and biochemical analysis of Chlorella vulgaris cultivated with synthetic domestic wastewater. *International Biodeterioration & Biodegradation* **2016,** *113*, 120.
(36) Chew, K. W.; Chia, S. R.; Show, P. L.; Ling, T. C.; Arya, S. S.; Chang, J.-S. Food waste compost as an organic nutrient source for the cultivation of Chlorella vulgaris. *Bioresource Technology* **2018,** *267*, 356.

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(37) Sibi, G. J. J. o. E. S.; Technology. Low cost carbon and nitrogen sources for higher microalgal biomass and lipid production using agricultural wastes. **2015,** *8* (3), 113.

(38) Weibao, K.; Yang, S.; Wang, H.; Huanran, H.; Baomin, G.; Liu, N.; Zhang, A.; Shiquan, N. J. J. o. A. P. Regulation of biomass, pigments, and lipid production by Chlorella vulgaris 31 through controlling trophic modes and carbon sources. **2020,** *32* (3), 1569.

(39) El-sheekh, M. M.; Bedaiwy, M. Y.; Osman, M. E.; Ismail, M. M. J. I. j. o. r. o. o. w. i. a. Mixotrophic and heterotrophic growth of some microalgae using extract of fungal-treated wheat bran. **2012,** *1* (1), 1.

(40) Lau, K. Y.; Pleissner, D.; Lin, C. S. K. J. B. t. Recycling of food waste as nutrients in Chlorella vulgaris cultivation. **2014,** *170*, 144.

(41) Mitra, D.; Van Leeuwen, J.; Lamsal, B. Heterotrophic/mixotrophic cultivation of oleaginous Chlorella vulgaris on industrial co-products. *Algal Research* **2012,** *1* (1), 40.

(42) Hu, J.; Nagarajan, D.; Zhang, Q.; Chang, J.-S.; Lee, D.-J. J. B. a. Heterotrophic cultivation of microalgae for pigment production: A review. **2018,** *36* (1), 54.

(43) Barros, A.; Pereira, H.; Campos, J.; Marques, A.; Varela, J.; Silva, J. J. S. r. Heterotrophy as a tool to overcome the long and costly autotrophic scale-up process for large scale production of microalgae. **2019,** *9* (1), 1.

(44) Kong, W.; Song, H.; Cao, Y.; Yang, H.; Hua, S.; Xia, C. J. A. J. o. B. The characteristics of biomass production, lipid accumulation and chlorophyll biosynthesis of Chlorella vulgaris under mixotrophic cultivation. **2011,** *10* (55), 11620.

(45) Melo, R. G. D.; Andrade, A. F. D.; Bezerra, R. P.; Correia, D. S.; Souza, V. C. D.; Brasileiro-Vidal, A. C.; Viana Marques, D. D. A.; Porto, A. L. F. Chlorella vulgaris mixotrophic growth enhanced biomass productivity and reduced toxicity from agro-industrial by-products. *Chemosphere* **2018,** *204*, 344.

(46) Gramegna, G.; Scortica, A.; Scafati, V.; Ferella, F.; Gurrieri, L.; Giovannoni, M.; Bassi, R.; Sparla, F.; Mattei, B.; Benedetti, M. J. B. T. R. Exploring the potential of microalgae in the recycling of dairy wastes. **2020,** *12*, 100604.

(47) Yu, Q.; Wang, H.; Li, X.; Yin, Y.; Qin, S.; Ge, B. Enhanced biomass and CO2 sequestration of Chlorella vulgaris using a new mixotrophic cultivation method. *Process Biochemistry* **2020,** *90*, 168.

(48) Matos, J.; Cardoso, C.; Bandarra, N.; Afonso, C. J. F.; function. Microalgae as healthy ingredients for functional food: a review. **2017,** *8* (8), 2672.

(49) Cui, H.; Meng, F.; Li, F.; Wang, Y.; Duan, W.; Lin, Y. Two-stage mixotrophic cultivation for enhancing the biomass and lipid productivity of Chlorella vulgaris. *AMB Express* **2017,** *7* (1).

(50) Yen, H.-W.; Chang, J.-T. J. B.; engineering, b. A two-stage cultivation process for the growth enhancement of Chlorella vulgaris. **2013,** *36* (11), 1797.

(51) Perez-Garcia, O.; De-Bashan, L. E.; Hernandez, J. P.; Bashan, Y. J. J. o. P. Efficiency of growth and nutrient uptake from wastewater by heterotrophic, autotrophic, and mixotrophic cultivation of Chlorella vulgaris immobilized with Azospirillum brasilense 1. **2010,** *46* (4), 800.

(52) Del Río, J. C.; Prinsen, P.; Gutiérrez, A. A Comprehensive Characterization of Lipids in Wheat Straw. *Journal of Agricultural and Food Chemistry* **2013,** *61* (8), 1904.

(53) Heinonen, J.; Tamper, J.; Laatikainen, M.; Sainio, T. J. C. e.; technology. Chromatographic recovery of monosaccharides and lignin from lignocellulosic hydrolysates. **2018,** *41* (12), 2402.

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(54) Ishaq, A. R.; Jabeen, F.; Manzoor, M.; Younis, T.; Noor, A.; Noor, F. Current status of microalgae to produce biogas through pretreatment of lignocellulosic waste in the era of 21-Century. **2020**.

(55) Ghazali, N. F.; Razak, N. D. A. J. F.; Processing, B. Recovery of saccharides from lignocellulosic hydrolysates using nanofiltration membranes: A review. **2021**.

(56) Hendriks, A.; Zeeman, G. J. B. t. Pretreatments to enhance the digestibility of lignocellulosic biomass. **2009,** *100* (1), 10.

(57) van der Pol, E. C.; Bakker, R. R.; Baets, P.; Eggink, G. J. A. m.; biotechnology. By-products resulting from lignocellulose pretreatment and their inhibitory effect on fermentations for (bio) chemicals and fuels. **2014,** *98* (23), 9579.

(58) Pleissner, D.; Rumpold, B. A. J. W. M. Utilization of organic residues using heterotrophic microalgae and insects. **2018,** *72*, 227.

(59) Park, H.; Jeong, D.; Shin, M.; Kwak, S.; Oh, E. J.; Ko, J. K.; Kim, S. R. J. A. m.; biotechnology. Xylose utilization in Saccharomyces cerevisiae during conversion of hydrothermally pretreated lignocellulosic biomass to ethanol. **2020,** *104* (8), 3245.

(60) Beisl, S.; Quehenberger, J.; Kamravamanesh, D.; Spadiut, O.; Friedl, A. J. P. Exploitation of Wheat Straw Biorefinery Side Streams as Sustainable Substrates for Microorganisms: A Feasibility Study. **2019,** *7* (12), 956.

(61) Lodi, G.; Pellegrini, L. A.; Aliverti, A.; Torres, B. R.; Bernardi, M.; Morbidelli, M.; Storti, G. J. J. o. C. A. Recovery of monosaccharides from lignocellulosic hydrolysates by ion exclusion chromatography. **2017,** *1496*, 25.

(62) Zhang, T.-Y.; Wang, X.-X.; Wu, Y.-H.; Wang, J.-H.; Deantes-Espinosa, V. M.; Zhuang, L.-L.; Hu, H.-Y.; Wu, G.-X. J. B. t. Using straw hydrolysate to cultivate Chlorella pyrenoidosa for high-value biomass production and the nitrogen regulation for biomass composition. **2017,** *244*, 1254.

(63) Zheng, Y.; Yu, X.; Li, T.; Xiong, X.; Chen, S. Induction of D-xylose uptake and expression of NAD(P)H-linked xylose reductase and NADP + -linked xylitol dehydrogenase in the oleaginous microalga Chlorella sorokiniana. *Biotechnology for Biofuels* **2014,** *7* (1).

(64) Bajguz, A.; Czerpak, R.; Piotrowska, A.; Polecka, M. J. A. s. b. P. Effect of isomers of hydroxybenzoic acid on the growth and metabolism of Chlorella vulgaris Beijerinck (Chlorophyceae). **2001,** *70* (4), 253.

(65) Larson, L. J. J. H. Effect of phenolic acids on growth of Chlorella pyrenoidosa. **1989,** *183* (3), 217.

(66) Yu, S.; Forsberg, Å.; Kral, K.; Pedersén, M. J. B. P. J. Furfural and Hydroxymethylfurfural inhibition of growth and photosynthesis in Spirulina. **1990,** *25* (2), 141.

(67) Slocombe, S. P.; Ross, M.; Thomas, N.; McNeill, S.; Stanley, M. S. J. B. t. A rapid and general method for measurement of protein in micro-algal biomass. **2013,** *129*, 51.

(68) Smith, P. e.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. J. A. b. Measurement of protein using bicinchoninic acid. **1985,** *150* (1), 76.

(69) Porra, R.; Thompson, W.; Kriedemann, P. J. B. e. B. A.-B. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. **1989,** *975* (3), 384.

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The approved original version of this thesis is available in print at TU Wien Bibliothek.
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(70) Liang, Y.; Sarkany, N.; Cui, Y. J. B. l. Biomass and lipid productivities of Chlorella vulgaris under autotrophic, heterotrophic and mixotrophic growth conditions. **2009,** *31* (7), 1043.

(71) Cai, Y.; Liu, Y.; Liu, T.; Gao, K.; Zhang, Q.; Cao, L.; Wang, Y.; Wu, X.; Zheng, H.; Peng, H.et al. Heterotrophic cultivation of Chlorella vulgaris using broken rice hydrolysate as carbon source for biomass and pigment production. *Bioresource Technology* **2021,** *323*, 124607.

(72) Sakarika, M.; Kornaros, M. J. B. t. Effect of pH on growth and lipid accumulation kinetics of the microalga Chlorella vulgaris grown heterotrophically under sulfur limitation. **2016,** *219*, 694.

(73) Komor, E.; Schobert, C.; Cho, B.-H. J. F. L. The hexose uptake system of Chlorella: Is it a proton symport or a hydroxyl ion antiport system? **1983,** *156* (1), 6.

(74) Tanner, W. The Chlorella hexose/H+-symporters. **2000**.

(75) Hawkins, R. L. J. C. m. Utilization of xylose for growth by the eukaryotic alga, Chlorella. **1999,** *38* (6), 360.

(76) Zhang, T.-Y.; Wu, Y.-H.; Wang, J.-H.; Wang, X.-X.; Deantes-Espinosa, V. M.; Dao, G.-H.; Tong, X.; Hu, H.-Y. Heterotrophic cultivation of microalgae in straw lignocellulose hydrolysate for production of high-value biomass rich in polyunsaturated fatty acids (PUFA). *Chemical Engineering Journal* **2019,** *367*, 37.

(77) Sui, Z.; Gizaw, Y.; Bemiller, J. N. Extraction of polysaccharides from a species of Chlorella. *Carbohydrate Polymers* **2012,** *90* (1), 1.

(78) Ho, S.-H.; Huang, S.-W.; Chen, C.-Y.; Hasunuma, T.; Kondo, A.; Chang, J.-S. Characterization and optimization of carbohydrate production from an indigenous microalga Chlorella vulgaris FSP-E. *Bioresource Technology* **2013,** *135*, 157.

(79) de Freitas, B. C. B.; Brächer, E. H.; de Morais, E. G.; Atala, D. I. P.; de Morais, M. G.; Costa, J. A. V. J. E. t. Cultivation of different microalgae with pentose as carbon source and the effects on the carbohydrate content. **2019,** *40* (8), 1062.

(80) Tam, N.; Wong, Y. J. B. t. Effect of ammonia concentrations on growth of Chlorella vulgaris and nitrogen removal from media. **1996,** *57* (1), 45.

(81) Xie, T.; Xia, Y.; Zeng, Y.; Li, X.; Zhang, Y. J. B. t. Nitrate concentration-shift cultivation to enhance protein content of heterotrophic microalga Chlorella vulgaris: Over-compensation strategy. **2017,** *233*, 247.

(82) Mohammad Mirzaie, M.; Kalbasi, M.; Mousavi, S.; Ghobadian, B. J. P. b.; biotechnology. Investigation of mixotrophic, heterotrophic, and autotrophic growth of Chlorella vulgaris under agricultural waste medium. **2016,** *46* (2), 150.

(83) Shen, X.-F.; Qin, Q.-W.; Yan, S.-K.; Huang, J.-L.; Liu, K.; Zhou, S.-B. J. J. o. A. P. Biodiesel production from Chlorella vulgaris under nitrogen starvation in autotrophic, heterotrophic, and mixotrophic cultures. **2019,** *31* (3), 1589.

(84) Tokuşoglu, O.; Uunal, M. K. Biomass Nutrient Profiles of Three Microalgae: Spirulina platensis, Chlorella vulgaris, and Isochrisis galbana. *Journal of Food Science* **2003,** *68* (4), 1144.

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9 Appendix

9.1 Additional fermentation data

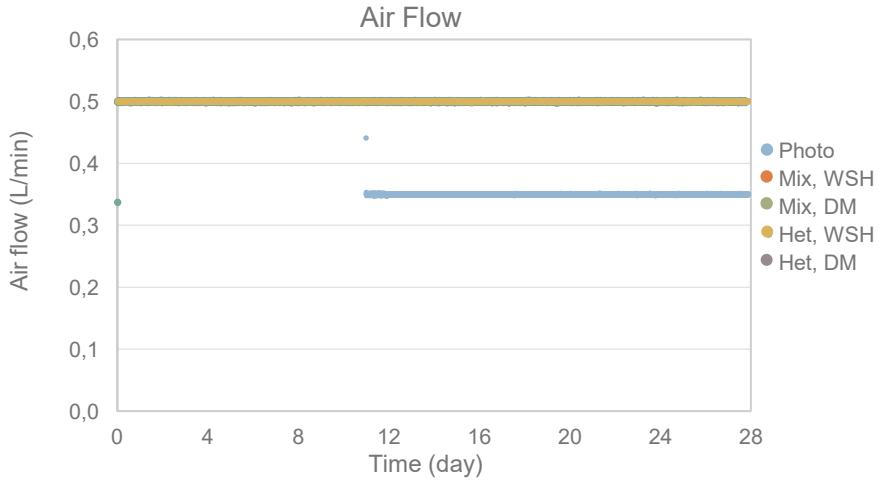

Figure 66: Airflow during fermentation in different media and cultivation modes

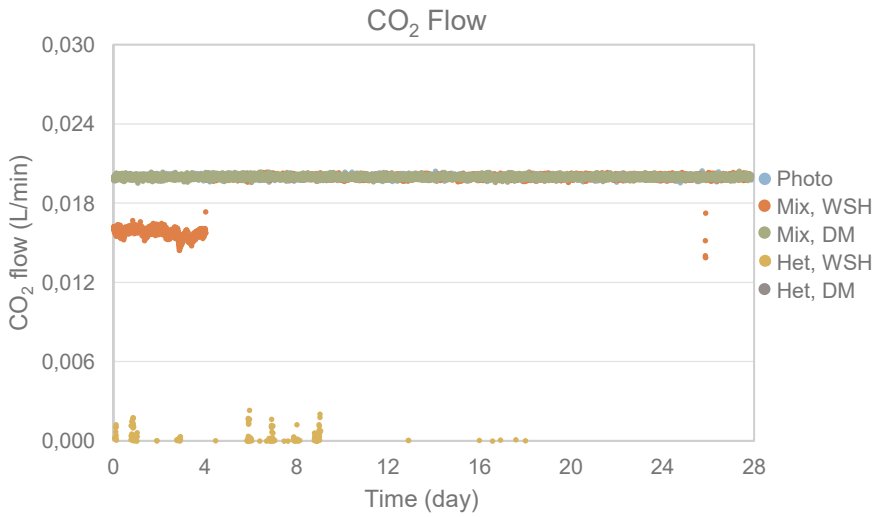

Figure 67: CO_2 flow during fermentation in different media and cultivation modes

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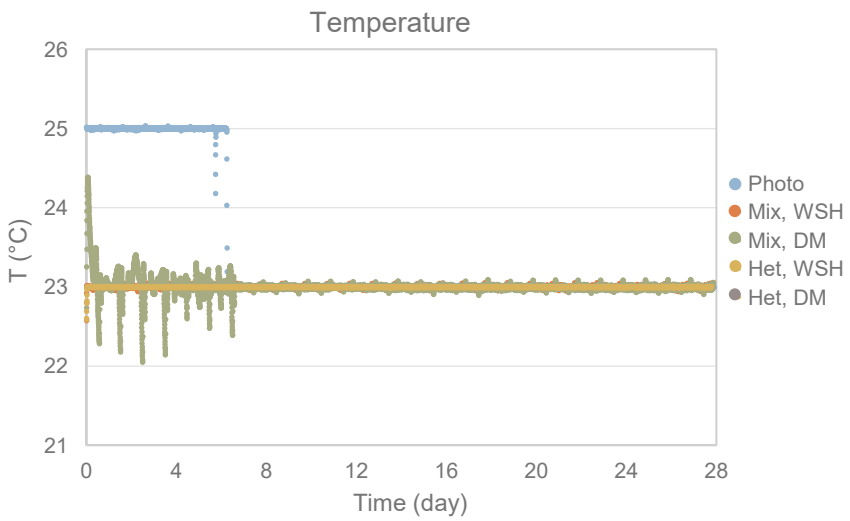

Figure 68: Temperature during fermentation in different media and cultivation modes

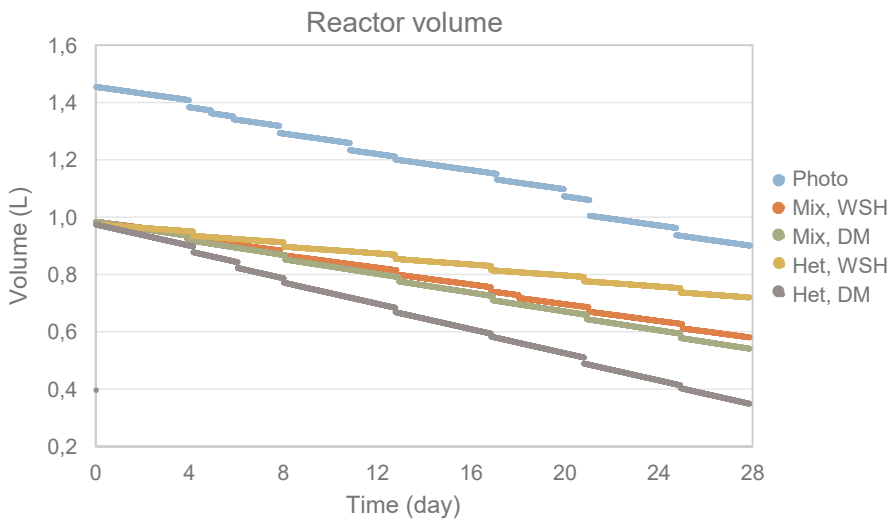

Figure 69: Reactor volume during fermentation in different media and cultivation modes

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