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EXPLORING CO_2 AS FEEDSTOCK FOR PRODUCTION OF BIOPOLYMERS BY THE BACTERIA *CHLOROFLEXUS AURANTIACUS*

MASTERS IN BIOTECHNOLOGY

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Abstract

With CO_2 and plastic waste rising globally, developing a method that allows CO_2 capture and its transformation into biodegradable polymers is an appealing prospect.

One organism capable of such feature is *Chloroflexus aurantiacus*, a filamentous anoxygenic bacteria that presents a very versatile metabolism. This extremophile is capable of using solar energy for CO_2 fixation and adverse components as electron donors (e.g., hydrogen sulphide), to produce polyhydroxyalkanoates (PHA) and accumulate them as internal reserves. PHAs are microbially synthesized polyesters that offer a wide range of properties, similar to petrochemical plastics, yet are fully biobased, biodegradable, and more biocompatible than conventional polymers.

In this work we plan to outlay a possible alternative to the conventional methods of PHA production, using CO_2 capture and hydrogen sulphide removal by *C. aurantiacus* Y-400-fl (DSM 637) to provide a path for a sustainable and inexpensive PHA production process. Hopefully, this work will contribute to lower PHA production costs, and thus make it viable for new markets. In this work the growth of *C. aurantiacus* Y-400-fl (DSM 637) was optimized in lab conditions, analysing its response to variations in the culture medium composition, which impacted the overall behaviour of the organism and its polymer production, both in quantity and monomeric composition of the PHA produced.

In the course of this thesis *C. aurantiacus* Y-400-fl (DSM 637) was grown under several different media, with different carbon sources (glycylglycine, glycine and sodium carbonate), sulphide concentrations from 0.078 mM to 0.625 mM and with light intensities from 0.58 to 4.4 W/l.

The results showed that *C. aurantiacus* Y-400-fl (DSM 637) has an ideal sulphide concentration of around 0.313 mM when waking up and maintaining growth using organic carbon sources (glycylglycine), that it is a fast adapting organism, that shows similar growth profiles regardless of whether its inoculum was grown under a low or high light intensity, that the bacteria is able to assimilate inorganic carbon (Na_2CO_3) and grow normally without the presence of glycylglycine, and that under organic carbon conditions it's possible to replace the expensive glycylglycine with glycine, without affecting the organism's growth profile.

Grown under anaerobic conditions with continuous illumination, *C. aurantiacus* Y-400-fl (DSM 637), was able to achieve 15% PHA content per cell dry weight when fed with an organic carbon substrate (glycylglycine). Preliminary tests to evaluate *C. aurantiacus* growth on inorganic carbon-based medium resulted in low PHA content, indicating the importance of future tests to target operation under ideal accumulation conditions (e.g., nutrient limitation, adjusted sulphide concentrations.

Resumo

Com os níveis de CO_2 e os resíduos de plástico a aumentar globalmente, desenvolver um método que permita a captura de CO_2 e a sua transformação em polímeros biodegradáveis é uma perspetiva apelativa.

Um organismo capaz de tal é *Chloroflexus Aurantiacus*, uma bactéria filamentosa anoxigénica que apresenta um metabolismo muito versátil. Este extremófilo é capaz de utilizar energia solar para a fixação de CO_2 e compostos adversos como dadores de eletrões (p. ex.: sulfureto de hidrogénio) para produzir polihidroxialcanoatos (PHA) e acumulá-los como reservas internas. Os PHAs são poliésteres sintetizados microbialmente que oferecem uma larga gama de propriedades, semelhante aos plásticos petroquímicos, no entanto são completamente biobaseados, biodegradáveis e mais biocompatíveis do que os polímeros convencionais.

Neste trabalho planeamos elaborar uma possível alternativa aos métodos convencionais de produção de PHAs, utilizando captura de CO_2 e remoção de sulfureto de hidrogénio por *C. aurantiacus* Y-400-fl (DSM 637) para providenciar um caminho para um processo sustentável e acessível para a produção de PHA. Com esperança, este trabalho contribuirá para baixar os custos de produção de PHA, e assim torná-los viáveis para novos mercados. Neste trabalho o crescimento de *C. aurantiacus* foi otimizado em condições de laboratório, analisando a sua resposta a variações na composição do meio de cultura, cujo impacto se fez sentir tanto no comportamento do organismo em si, como na produção de polímeros, tanto a quantidade deste, como a sua composição monomérica.

Os resultados demonstraram que 0.625 mM de sulfuretos é a concentração ideal para acordar e manter o crescimento com fontes orgânicas de carbono(glicilglicina) em *C. aurantiacus* Y-400-fl (DSM 637). Demonstramos também que *C. aurantiacus* Y-400-fl (DSM 637) é um organismo de adaptação rápida, que demonstra um perfil de crescimento semelhante independentemente de ser inoculado de uma cultura crescida em baixa ou alta intensidade de luz. Mostramos ainda que *C. aurantiacus* Y-400-fl (DSM 637) é capaz de assimilar carbono inorgânico (Na_2CO_3) sem a presença de glicilglicina, e que em condições de crescimento orgânico é possível substituir a dispendiosa glicilglicina com glicina sem afetar o perfil de crescimento substancialmente.

Crescida em condições anaeróbicas com iluminação continua *C. aurantiacus* Y-400-fi (DSM 637) consegui atingir 15% de teor de PHA por peso seco total quando alimentada com um substrato de carbono orgânico (glicilglicina). Testes preliminares para avaliar o crescimento de *C. aurantiacus* Y-400-fl (DSM 637) em meio com fonte de carbono inorgânica (Na_2CO_3) resultaram em baixas quantidades de PHA acumulados, indicando a importância de futuros testes para alcançar as ideais condições de acumulação (p. ex.: limitação de nutrientes, ajustes de concentração de sulfureto).

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List of Abbreviations

APB Anoxygenic Phototrophic Bacteria
Bchl Bacteriochlorophyll
CDW Cell Dry Weight
GG Glycylglycine
Gly Glycine
IC Inorganic Carbon
MMC Mixed Microbial Culture
OD Optical Density
PHA Polyhydroxyalkanoates
PHB Polyhydroxybutyrate
PHBV Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PHO Polyhydroxyoctanoate
PHV Polyhydroxyvalerate
PNSB Purple Non-sulphur Bacteria
SBR Sequential Batch Reactors
SS Soluble Sulphides
TOC Total Organic Carbon
TS Total Sulphides
TSS Total suspended solids
VSS Volatile suspended solids

1. Introduction

1.1. The growing levels of CO_2 in the atmosphere

Carbon dioxide (CO_2) is a gas that is naturally produced in many ways, from organisms' respiration to volcanic eruptions and a multitude of chemical reactions that exist between minerals and the elements. However, human activity through the extraction, processing and consumption of fossil fuels, combined with the high rates of deforestation all over the world, has caused global CO_2 levels to grow exponentially in the last half century, from around 315 ppm in 1960 to 420 ppm in 2022 (Figure 1.1).(GISS, 2022)

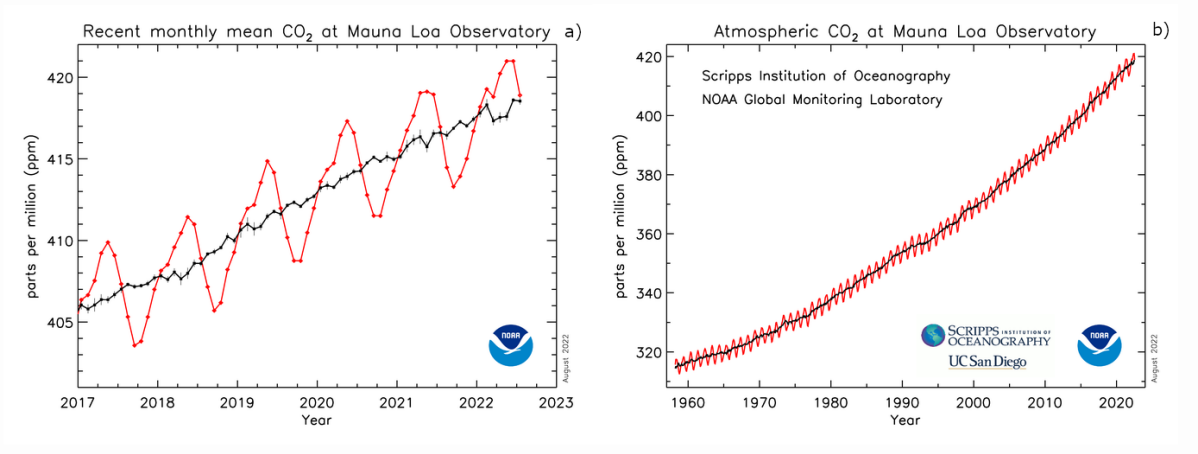

Figure 1.1 Mean CO_2 levels in the atmosphere a) last 5 years (2017-2022) b) last 65 years (1957-2022) (GISS, 2022)

While moderate concentrations of greenhouse gases help sustaining life on Earth by preventing frigid night temperatures, their increasing concentration is drastically increasing the atmosphere's ability to retain heat.

This heat retention has manifested itself in a mean average increase of 0.85°C (Figure 1.2) in global temperatures(GISS, 2022). Although this may seem a relatively minor value, its effects are significant since peak temperatures have risen by several degrees in multiple regions, weather records are being broken and the occurrence of extreme weather events is becoming more and more common.

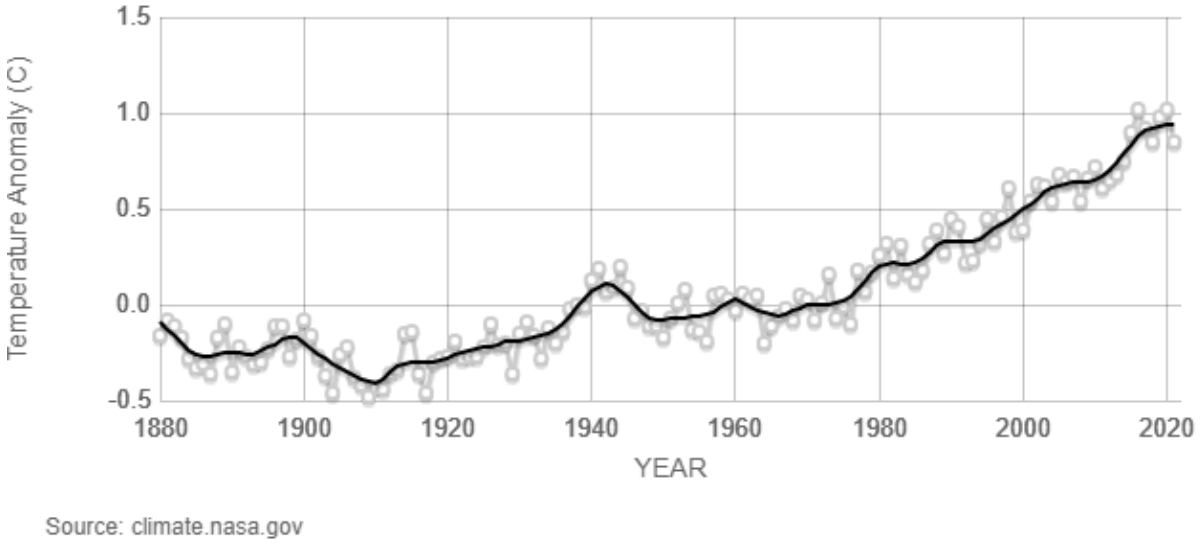

Figure 1.2: Global temperature anomaly (°C)(NASA, 2021)

1.2. Conventional carbon fixation techniques

In an attempt to minimize the emissions of CO_2 to the atmosphere, several carbon capture techniques have been developed. These techniques are primarily applied to fossil fuel (coal, natural gas, and petroleum derivatives) powered plants but can be expanded to work in other sectors like the cement industry(Benhelal et al., 2013).

In the case of fossil fuels, the carbon capture can occur either pre- or post-combustion. The former consists in converting the fuel to syngas (mixture of hydrogen and carbon monoxide), which is then reformed with steam (a process of partial oxidation), capturing the carbon, and forming pure hydrogen that can be burned without release of carbon. Post-combustion capture involves passing the combustion products through a separation step that can trap large amounts of CO_2 (Wilberforce et al., 2021).

The separation step takes place in 2 steps, adsorption, and stripping. In the adsorption step the gas stream is fixed with a solvent stream and in the stripping step, the solvent is heated up to release the CO_2, regenerating the solvent.

One proposed solution for the captured CO_2 is for it to be pumped underground into saline formations, coal beds or depleted gas and oil reserves, in these underground hydrocarbon pockets it isn't able to rapidly escape into the atmosphere, preventing build-up of greenhouse gases, and the pressure causes it to react with the surroundings to form added value hydrocarbons, partially regenerating some of the consumed hydrocarbons.(Wilberforce et al., 2021)

1.3. Use of microorganisms to mitigate harmful gas pollution.

Since several microorganisms can capture CO_2 and convert it to organic matter, many studies have targeted their utilization in filtration systems of high CO_2 producing processes (such as the burning of fossil fuels, cement industry), in wastewater treatment facilities and in a series of biogenic CO2 producing industries (e.g., dairy, brewery)(George et al., 2020; Razzak et al., 2017).

In this field there are 2 major types of organisms that can be used. Cyanobacteria and microalgae that perform oxygenic photosynthesis, and anoxygenic phototrophic bacteria (APB) that do not use water as an electron donor (thus do not evolve oxygen) but can use other compounds (such as sulphide and hydrogen in lithotrophy, or acetate and glucose in organotrophy).(Kondratieva et al., 1992; van der Meer et al., 2005; Zarzycki & Fuchs, 2011)

Due to their specific metabolism APB present unique possibilities for carbon fixation in environments not suitable for oxygenic photosynthesis such as sulphur rich water streams and other media containing compounds that react adversely to the presence of oxygen.(George et al., 2020)

1.4. The propagation of plastic

Together with the increase in CO_2 emissions, the plastics production and usage also increased greatly in the last decades. Plastic is a material with very appealing properties, from its light weight to the ease in which it is shaped, to the unique strength and elasticity provided by its polymeric crystalline structure, to its relatively low cost and high availability(Martín et al., 2021). All these properties make plastic one of the most important building blocks of our day-to-day life, with around 360 Mt being produced each year. But, alongside with the increase in its production, there is also an increase in the waste it generates (Figure 1.3). Plastic pollution is quickly becoming one of the most pressing environmental issues we face, and the production of single-use plastics and other disposables sees no end in sight.

As of 2020, worldwide, only around 12% of plastic is recycled and around 1% is transformed into other value-added products (Figure 1.3), with the vast majority of the rest ending up in landfills, bodies of water or straight up lost long the process. There's also around 27% that is incinerated to recover energy, which releases yet more CO_2 into the atmosphere (Martín et al., 2021).

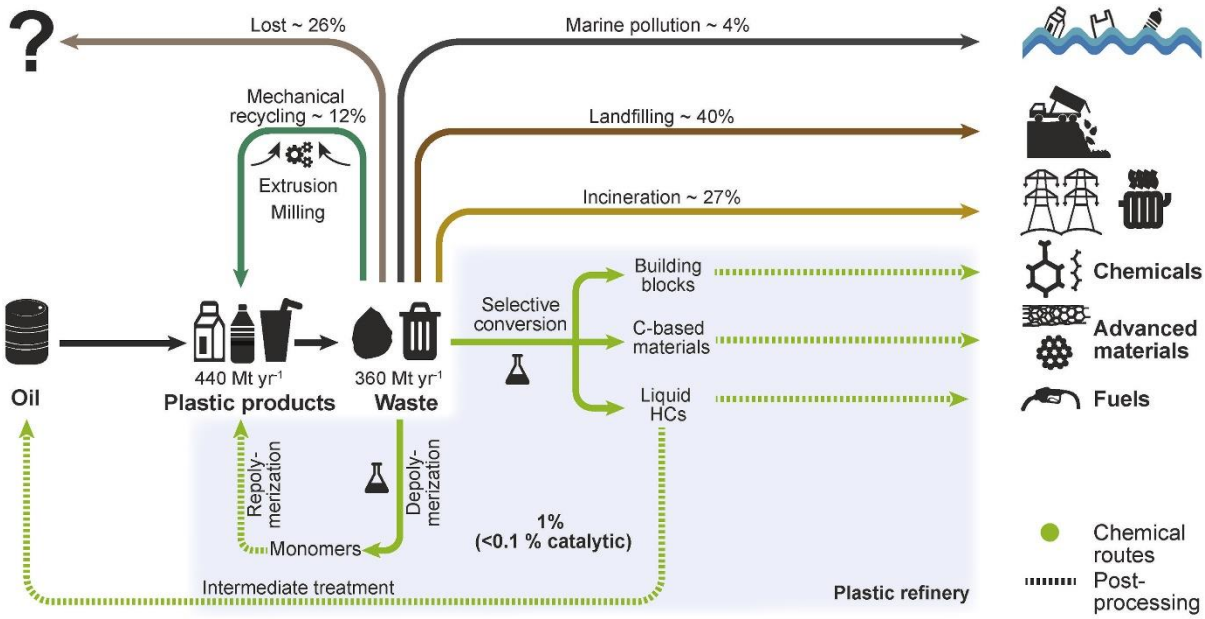

Figure 1.3: Main plastic waste management strategies at work(Martín et al., 2021)

1.5. The search for a biobased and biodegradable plastic

Due to the many aforementioned shortcomings of petroleum-based plastics, there is a growing demand for a material that offers properties similar to petroleum-based plastics while being fully biodegradable and biobased.

The goal is to replace traditional plastics in many areas where other renewable materials aren't viable, with a biodegradable and biobased polymer.

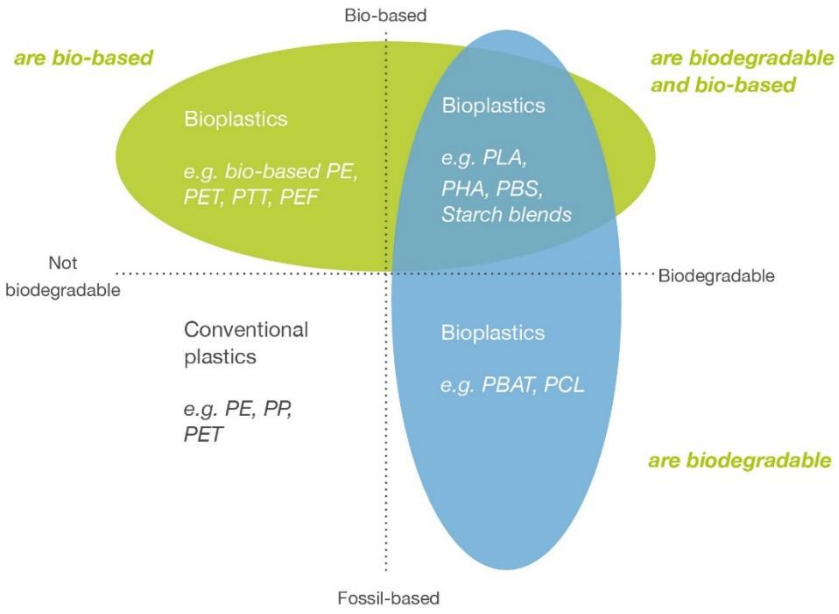

Figure 1.4:Bioplastics refers to different types of materials with different properties(European Bioplastics, 2022)

1.6. Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs)are a group of bioplastics produced by several microbial organisms (bacteria and certain extremophilic archaea) that are both biobased and biodegradable. PHAs are accumulated inside the cytoplasm as water insoluble granules and serve as reserve substances for the organism, helping it to survive famine conditions and other challenging environmental conditions (Figure 1.6)(Koller, 2018).

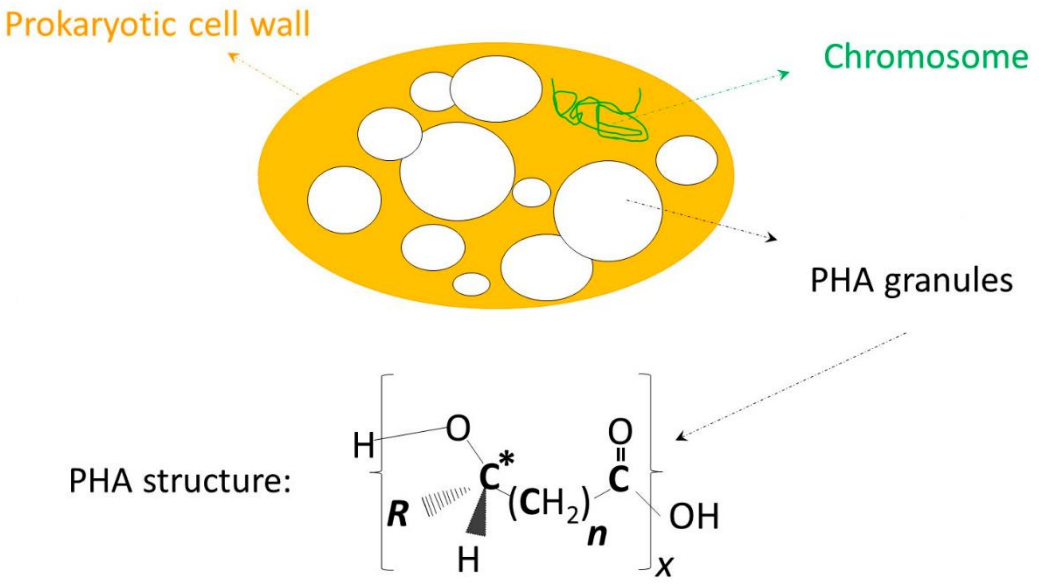

Figure 1.5:Diagram of cell accumulating PHA in carbonossomes and general chemical structure of PHA(Koller, 2018)(modified). R: side chain of PHA monomers; n: number of methylene groups in monomers; x: degree of polymerization.

PHAs are microbial polyesters whose properties (stiffness, elasticity, crystallinity, and degradability) are highly adjustable by tuning the monomeric composition, microbial strain selection, carbon source, production parameters and post-synthesis processing.

PHAs are considered capable biological alternatives for plastics derived from the petrochemical industry and highly sought after for medical applications, due to their large biocompatibility derived from their biobased nature. Currently, PHAs are starting to replace polymers like polyurethane, polycaprolactone and polyethylene glycol, that had been on the forefront of medical polymers for decades(Koller, 2018).

PHAs can be primarily classified into 3 categories(Sharma et al., 2021):

- **Short chain length PHA (scl-PHA):** They consist of monomers 3-5 carbons long. E.g., polyhydroxyvalerate (PHV) and polyhydroxybutyrate (PHB). In their monomeric forms, these polymers are very brittle and rigid and are not suitable for the kinds of uses needed by the medical and packing industries(Li et al., 2016), however as heteropolymers, their properties change, and they can fulfil some of these usecases.
- **Medium chain length PHA (mcl-PHA):** They consist of monomers 6-14 carbons long. E.g., polyhydroxyoctanoate (PHO). These polymers are generally considered elastomers and as such they have lower mechanical strength which can also limit their use(Nomura et al., 2004). However, these shortcomings can once again be overcome by the use of carefully tuned co-polymers.
- **Long chain length PHA:** They consist of monomers of 15 carbons or more. E.g., polyhydroxypentadecanoate.

PHAs are very versatile in the sense that different monomers can be mixed together to achieve the desired properties. If no single homopolymer suits the desired usecase, tuning of the ratios of different polymers in the final mix allows for adjustment of several of their properties, such as the tensile strength, toughness, elasticity modulus and strain at break.

These combined polymers are called heteropolymers, or in the specific case of PHA, heteropolyesters, which in turn are subdivided into different categories:

- Copolyesters, which feature monomers of either different backbones or side chains.
- Terpolyesters, which consist of monomers differing in both the backbone and the sidechain.

Different species of bacteria have the ability to produce both homopolyesters and heteropolyesters, depending on what substrate and other characteristics of the growth medium(Koller, 2018).

One of PHA's strengths is how easily it can be bio-degraded by multiple organisms. This property results from the fact that PHA originates as energy reserves for microorganisms. Bacteria that accumulate PHA have specific enzymes that catabolize PHA back into more basic forms of energy that the cell can use in its multiple systems. These enzymes are named PHA depolymerases, carboxyesterases that catalyse the hydrolysis of the hydrophobic polymers into water soluble monomers and then further hydrolyse these into water, CO_2, and methane(Amara & Moawad, 2011).

1.7. PHA microbial production

Currently, chemotrophic PHA production primarily uses one of 2 systems: pure bacterial cultures and mixed microbial consortia (MMC). Both have their advantages and drawbacks, as follows:

1.7.1. State of the art PHA production with pure microbial cultures

Pure cultures have been used in the industry for quite some time, but PHA production via this method has proved economically challenging due to the cost of the high purity substrates they use, accounting for about 45% of the total production cost(Kourmentza et al., 2017). Therefore, the use of agro-industrial residues/by-products as feedstocks enables the decrease of production costs and contributes to circular economy and a better resource usage(Kourmentza et al., 2017; Monroy & Buitrón, 2020).

Several different carbon sources can be used as their feedstocks, including glucose, several types of starch, cellobiose, xylan, propionate, valerate, heptanoate, octanoate, benzene, toluene and several by-products/ wastes from food and other industries who process organic raw materials (e.g. pulp fruits, glycerol by-product) (Khatami et al., 2022; Kourmentza et al., 2017)

The ideal organism depends on the type of feedstock in use. Some bacteria have the ability to accumulate PHA while growing like *Alcaligenes latus* or *Paracoccus denitrificans*. Moreover, there are bacteria that need nutrient limitation, such as nitrogen or phosphorus for PHA accumulation (PHA accumulation disassociated from growth) like *Cupriavidus necator*, which is considered the ideal organism to achieve the best accumulation yields.(Kourmentza et al., 2017)

The highest yields obtained on a pure culture test, according to Kourmentza et al, 2017(Kourmentza et al., 2017), were with *Haloferax mediterranei*, a halophilic Archaea, using as substrate 25% v/v pre-treated vinasse(Bhattacharyya et al., 2012), obtaining 70% of its cell dry weight (CDW) as PHA. More recently other groups such as Cruz et. Al, have been able to achieve productivities of 84% of CDW in PHA, totally 21.3 g/l, using *Cupriavidus necator* and used cooking oil as a substrate(Cruz et al., 2019).

These systems traditionally had the primary advantage of allowing for high yields, although with the cost of the aforementioned expensive, non-waste, feedstocks, something that is changing as in recent years as more waste feedstocks prove viable with pure cultures (Khatami et al., 2022; Kourmentza et al., 2017).

1.7.2. PHA production with Mixed Microbial Consortia (MMC)

The use of MMCs for PHA production is primarily based on decreasing the process costs, as these cultures employ selected mixtures of organisms which allow for a greater harnessing of the feedstock at hand. The cultures used in MMCs are selected using ecological principles imposed on the system by the reactor's conditions, these conditions select the organisms that best harness the feedstock and accumulate it internally as PHA (Nguyenhuynh et al., 2021)

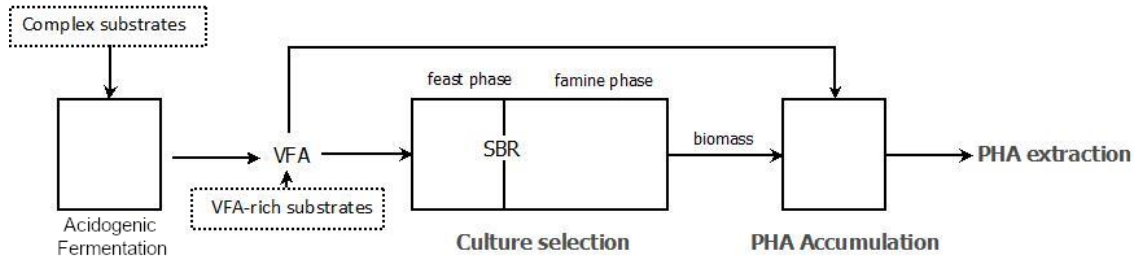

Figure 1.6: OHA production by mixed microbial cultures(Kourmentza et al., 2017), modified.

PHA production through MMC is usually performed in 2 stages (Figure 1.7). In the first stage sequential batch reactors (SBR) are used to enrich and select a microbial population with high capability of PHA production through the application of transient conditions. In the second stage the SBR's culture is subjected to conditions that favour PHA accumulation(Kourmentza et al., 2017).

The selection of PHA accumulating organisms is primarily done through feast and famine cycles.

In the feast phase, ecological selection principles give organisms who accumulated reserves in the form of PHA a survival advantage, enriching the culture in high PHA accumulating bacteria(Kourmentza et al., 2017; Nguyenhuynh et al., 2021).

While glucose is the most commonly used substrate in pure cultures, MMC make use of volatile fatty acids (VFA) as precursors for PHA production because, in these conditions, carbohydrates tend to form glycogen instead of PHA(Reis et al., 2011). As such, an intermediate step of acidogenic fermentation of complex substrates is usually employed to generate the VFAs used in PHA accumulation stages. It's noteworthy to mention that the composition of the VFA mixture affects the polymers composition produced by the bacteria and therefore, can be used to modulate the final polymer's properties. In particular, VFAs with even number of carbon atoms tend to produce Hydroxybutyrate (HB) monomers while the ones with off carbon atoms tend to produce Hydroxyvalerate (HV) monomers(Kourmentza et al., 2017).

The downside of this process is the higher inconsistency in the final produced polymer and the lower yields per total biomass, although both have been substantially improved by recent innovations in synthetic substrates, with some reporting 90% of the cell dry weight (CDW) in PHA(Jiang et al., 2011).

As mentioned previously MMCs typically feature the advantage of lower cost, in both operating costs, equipment setup and feedstock(Kaur et al., 2017), while pure cultures offer the benefits of very high yields at the cost of substantially higher costs of production, these however have potential for improvement, as multiple breakthroughs in waste management and culture selection can make the use of pure cultures with mixed media and high yield MMC's possible (Monroy & Buitrón, 2020).

Currently most PHA production systems use chemoheterotrophic bacteria that rely on their substrate's components as their energy sources. But several bacteria exist that can accumulate PHA while obtaining most of their energy though photosynthesis. These photoautotrophic organisms present an interesting prospect for the development of new PHA production processes with a lower substrate cost and possible integration with carbon capture systems.

1.8. Phototrophic systems

Photosynthesis has existed on this planet for billions of years, being one of the earliest forms of producing energy developed by microorganisms. Today it is employed by a wide range of organisms, from microscopic bacteria in the harshest environments to trees spawning dozens of meters in the air. It is at the source of most food chains in the planet, being of up-most importance to life on earth, and in the case of oxygenic photosynthesis, provides the oxygen used by all aerobic organisms(Shih et al., 2017).

Two major types of photosynthesis exist: oxygenic photosynthesis, used by cyanobacteria, algae, and plants, and anoxygenic photosynthesis, used by several groups of bacteria, some of them from extremophile conditions where most oxygenic photosynthesis organisms cannot thrive.

The primary difference in the two types of photosynthesis is their electron donor source, with oxygenic photosynthesis using water as electron source, and generating molecular oxygen as a by-product, while anoxygenic photosynthesis uses more reduced compounds as electron donors, such as hydrogen gas or hydrogen sulphide(Stephens et al., 2021).

Anoxygenic photosynthesis is a simpler system than oxygenic photosynthesis as it only possesses a single photosystem, as opposed to oxygenic photosynthesis that possesses two photosystems. (Shih et al., 2017; Stephens et al., 2021).

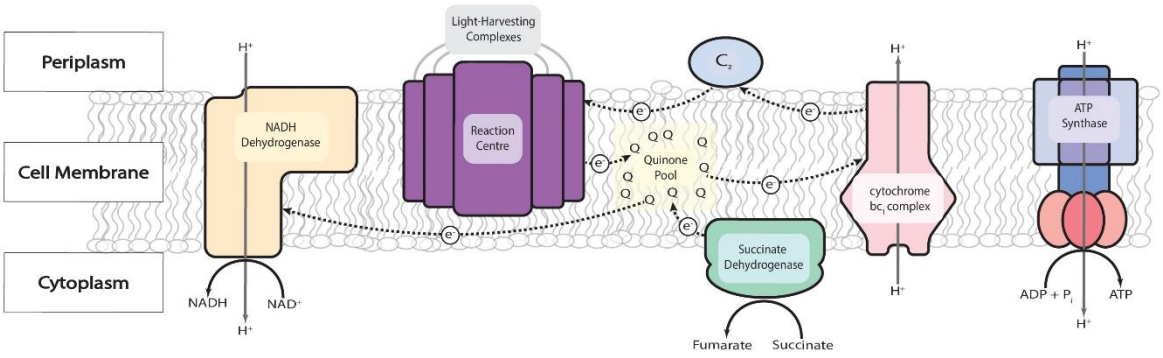

Figure 1.7: Electron transport chain of anoxygenic photosynthesis(Stephens et al., 2021)

In anoxygenic photosynthesis (figure 1.8) the light harvesting complexes accept photons and relay energy to the reaction centres where the excitation of electrons occurs, these then travel along the electron transport chain to the quinone, the cytochrome bc_1 and cytochrome c_2. This causes a proton motive force through which ATP can be synthesized via ATP synthase. Also, of note is how the electron flow in this process is cyclical, unlike what happens in oxygenic photosynthesis (Stephens et al., 2021).

Several anoxygenic phototrophic bacteria (APB) present the ability to produce PHAs. There are many different families of APB that are known to accumulate PHA, such as the *Chromatiaceae* and the *Ectothiorhodospiraceae* families of purple sulphur bacteria, a large range of purple non-sulphur bacteria, green sulphur bacteria like the *Chlorobiaceae* family, and the *Chloroflexaceae*, *Oscillochloridaceae* and *Roseiflexaceae* families of filamentous oxygenic bacteria(George et al., 2020).

From these the most commonly tested for PHA production are purple non-sulphur bacteria (PNSB), such as *Allochromatium vinosum*, *Rhodovulum sulfidophilum* and *Rhodospirillium rubrum* (George et al., 2020).

Cyanobacteria are the only know organisms also capable of producing PHA through oxygenic photosynthesis, however PHA production through cyanobacteria has its disadvantages, such as their more expensive growth medium, although recently the use of genetically engineered strains, such as *Aulosira fertilisima*, that can metabolize waste streams shows a possible solution for this problem.(Kamravamanesh et al., 2018).

Finally, there's also promising extremophile filamentous anoxygenic bacteria, *Chloroflexus aurantiacus*, that has been studied for multiple decades and is known to be able to accumulate PHA while growing photoautotrophically at high temperatures and sulphide concentrations, presenting an interesting path to lower PHA production costs(Kondratieva et al., 1992; Pierson & Castenholz, 1974; Sirevåg & Castenholz, 1979).

1.9. Chloroflexus aurantiacus

Chloroflexus aurantiacus is a filamentous anoxygenic bacterium from the *Chloroflexota* phylum, a member of the *Chloroflexia* class, that can be found in thermal springs. Due to its natural habitat, it has developed several adaptations that provide it the capability of withstanding conditions that would prove lethal to most organisms, such as high temperatures and sulphide concentrations.(Frigaard & Dahl, 2008; Hanada, 2003; van der Meer et al., 2005)

When grown in anaerobic conditions and light availability, this bacterium can use sulphide compounds, as well as hydrogen, as electron donors for its photosynthetic reaction. *C. aurantiacus* also has the ability to use inorganic carbon as their anabolic carbon source, despite being mostly found metabolizing organic carbon sources, as these have higher availability and are more easily metabolized.

C. aurantiacus naturally thrives in environments usually considered extremophilic, like the sulphide-rich alkaline hot springs of Yellowstone national park, Wyoming, USA. These environments' rich sulphide content explains *C. aurantiacus'* ability to utilize hydrogen sulphide as an electron donor. *C. aurantiacus* is often found in microbial mats of a gel-like consistency, often in combination with cyanobacteria or microalgae (Pierson & Castenholz, 1974; van der Meer et al., 2005; Ward et al., 1998).

C. aurantiacus can also tolerate oxygenic environments, which gives it an evolutionary advantage over many other anoxygenic bacteria, it enables it to form the aforementioned mats with oxygenic photosynthesis organisms. In these mats, *C. aurantiacus* utilizes the metabolites of the oxygenic organisms as carbon sources to realize photoheterotrophic growth, which other studies show are the bacterium's most productive growth conditions (Pierson & Castenholz, 1974; van der Meer et al., 2005). When *C. aurantiacus* is found in anaerobic springs with high sulphide content it tends to be the dominant organism and performs photoautotrophic growth, utilizing inorganic carbon as a carbon source, sulphide compounds as electron donors and solar radiation as energy source(Pierson & Castenholz, 1974; van der Meer et al., 2005).

C. aurantiacus has the ability to use longer wavelength light than that most oxygenic photosynthetic organisms can, with Bacteriochlorophyll *a*, that absorbs light at 795 nm (near-infrared), supplemented by bacteriochlorophyll *c*, that absorbs light at 740 nm (visible red light)(Savikhin et al., 1998).

The optimal conditions for C. aurantiacus growth, as proposed by Pierson and Castenholz, 1974, are at 55 ℃ in 1 g/l yeast extract medium they dubbed "Roux", with light between 20000 (~158 W/m^2) and 50000 lux (~395 W/m^2) with these conditions providing a duplication rate of 0.3 h^{-1}, 6x higher than the ones obtained at lower light intensity (300 lux/~2.37W/m^2)(Pierson & Castenholz, 1974).

C. aurantiacus presents a filamentous morphology, with very long filaments stretching up to 300 µm under ideal growth conditions. Cell division happens by the formation of septum with invaginations of the cytoplasmic membrane and centripetal ingrowth of the inner cell wall layer(s)(Pierson & Castenholz, 1974).

It also possesses a gliding mobility that varies from 0.01 to 0.04 µm at 55-60 ℃ on 1.5% agar medium(Pierson & Castenholz, 1974).

1.9.1. C. aurantiacus' metabolism

C. aurantiacus presents a very versatile metabolism, being able to use different energy, carbon, and electron sources, under both aerobic and anaerobic conditions.(Kondratieva et al., 1992)

Under illuminated anaerobic conditions, it can grow as either a photoheterotroph or photoautotroph organism, depending on the carbon composition of the surrounding medium. Under light anaerobic conditions it can perform photoautotrophic growth, and under dark aerobic and anaerobic conditions it performs chemoheterotrophic growth(Kondratieva et al., 1992).

C. aurantiacus can utilize a wide range of organic carbon sources under aerobic conditions (light or dark phase), including amino acids, hexoses, short chain fatty acids, several different organic acids, and alcohols(Krasilnikova et al., 1986).

However, under anaerobic conditions the same behaviour was not observed, with most bacteria presenting no growth in the dark phase, confirming that photoheterotrophy is this species' preferred growth condition (Madigan et al., 1974). Madigan et al. (1974) also shows that photoautotrophic growth only occurred in one of the tested strains (OK-70-fl) and at anaerobic conditions, explained by the fact the electron donors used under photoautotrophy are highly reactive with oxygen and therefore not stable in these redox potentials and overall conditions(Madigan et al., 1974; Pierson & Castenholz, 1974; Sirevåg & Castenholz, 1979).

C. aurantiacus presents a special metabolic pathway called the 3-hydroxypropionate (3HP) bicycle, a pathway that it uses to assimilate both organic and inorganic carbon sources.

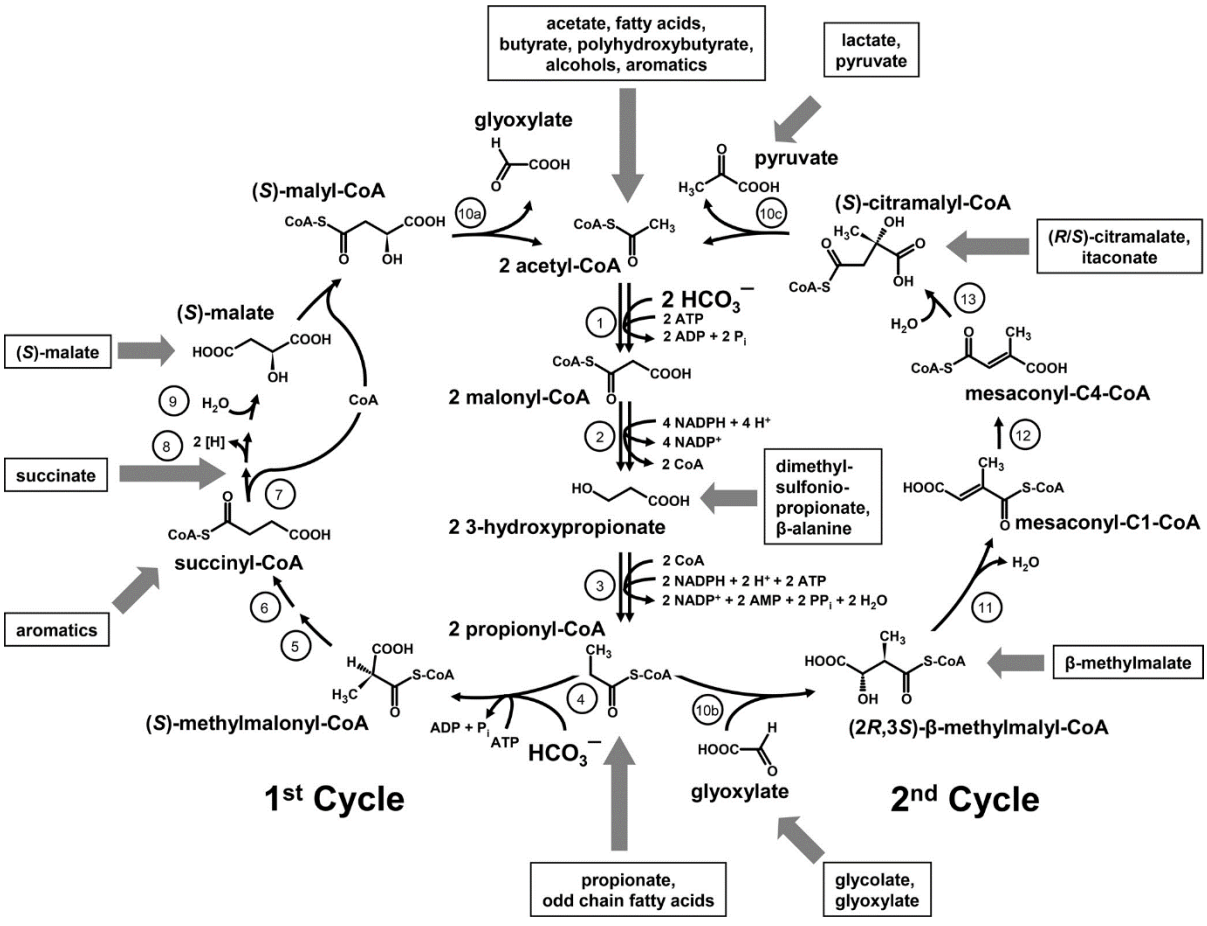

Figure 1.8: The autotrophic 3-hydroxypropionate bi-cycle in Chloroflexus aurantiacus and potential entry sites for various organic cosubstrates. 1,acetyl-CoA carboxylase; 2, malonyl-CoA reductase; 3, propionyl-CoA synthase; 4, propionyl-CoA carboxylase; 5, methylmalonyl-CoA epimerase;6, methylmalonyl-CoA mutase; 7, succinyl-CoA:(S)-malate-CoA transferase; 8, succinate dehydrogenase; 9, fumarate hydratase; 10a/b/c, (S)-malyl-CoA/-methylmalyl-CoA/(S)-citramalyl-CoA lyase; 11, mesaconyl-C1-CoA hydratase (-methylmalyl-CoA dehydratase); 12, mesaconyl-CoA C1:C4 CoA transferase; 13, mesaconyl-C4-CoA hydratase(Zarzycki & Fuchs, 2011)

This cycle (Figure 1.9) consists of 13 enzymes involved in 19 steps, and results in the formation of one molecule of pyruvate and fixation of 3 bicarbonate molecules (2 in step 1 and 1 in step 4). It is called a bi cycle since after the 3rd step, the generated propionate molecule can either absorb an extra bicarbonate molecule and be converted to (S)-methylmalonyl-CoA, which will eventually lead to glyoxylate, or absorb a glyoxylate molecule and be converted into (2R,3S)-β-methylmalyl-CoA, which will eventually lead to the desired final product, pyruvate.

C. aurantiacus synthesizes 4 major pigments, bacteriochlorophyll *a*, bacteriochlorophyll *c*, β-carotene, and γ-carotene.

Growth conditions have a direct impact on the ratio of bacteriochlorophyll *a* to bacteriochlorophyll *c*. Bacteriochlorophyll *c* is primarily found in structures named chlorosomes, whose amount is independent of cell overall growth. Low light anaerobic conditions stimulate the production of chlorosomes, and the subsequent enrichment in bacteriochlorophyll *c* of the cells, whereas bacteriochlorophyll *a* content is closely related to overall cell growth.(Oelze, 1992a; A. G. Yakovlev et al., 2021)

The principle behind this modulation is the acclimatization of *C. aurantiacus* to low light environments, with the intent of capturing more of the little available light. Low light stimulates the production of more chlorosomes, that in turn capture more light. Under conditions with abundant light, there isn't a need to channel resources for extra chlorosomes, as a base ammount is capable of capturing enough light for the cell's phototrophic metabolism to function, so the cell channels resources to overall cell growth, leading to a lower ratio of chlorosomes in the total biomass(Oelze, 1992a). Oxygen also downregulates the synthesis of pigments as it mediates the synthesis of ALA (5-aminolevulinic acid), a precursor amino acid to both bacteriochlorophyll *a* and *c* forms. As such, high oxygen environments *cause C. aurantiacus* to diminish pigment production and grow in almost exclusive chemoheterotrophic conditions(Oelze, 1992a).

1.10. Thesis framework

In this thesis the primary goal is to study the behaviour of *C. aurantiacus* Y-400-fl (DSM 637) and develop novel, practical and sustainable ways to produce PHAs at scale using the organism.

With the increase in demand for more sustainable energy, biogas has surged as a compelling way to harness the methane that is naturally produced in the agricultural and livestock industries. However, these streams can often contain large quantities of hydrogen sulphide and carbon dioxide. Sulphide levels, in particular, can be concerning since they tend to range from 500 to 3000 ppm, while the recommended levels for the biogas-fuelled generator engines range the 200 ppm and biogas boilers have maxima around 1000 ppm(Skerman et al., 2017). Most commonly sulphide is removed from these streams using activated forms of iron or zinc, which react with sulphide to produce precipitates. This obviously carries a cost, with the replacement of the filter materials being around 6% of the raw energy gain obtained by harnessing biogas. (Skerman et al., 2017) On a positive note, hydrogen sulphide is a reductive species that several phototrophic microorganisms are able to use as an electron source by oxidizing it to less toxic/corrosive compounds, like sulphates.

C. aurantiacus is one such organism, that can grow phototrophically using CO_2 as a carbon source and hydrogen sulphide as an electron donor, this metabolic pathway makes integrating *C. aurantiacus* in a biogas harnessing process an alluring prospect, allowing it to capture the CO_2 and sulphide in the streams.

The knowledge that this bacterium regularly accumulates carbon and energy reserves in the form of PHA(George et al., 2020; van der Meer et al., 2005), makes it an even more interesting subject of study that might tackle some of the cost issues associated with the feedstocks used for PHA production in pure cultures. C. aurantiacus has been known to accumulate PHA under certain conditions, such as under anaerobic phototrophic conditions (Pierson & Castenholz, 1974; Zarzycki & Fuchs, 2011) for a long time. But to date no major commercial processes have employed *C. aurantiacus*, so in this thesis we plan to test *C. aurantiacus*' polymer accumulation potential with the aim of prototyping a possible system for a large-scale production of polymer using *C. aurantiacus* pure cultures.

The ideal outcome of this thesis would be to be able to integrate a *C. aurantiacus* culture into an industrial stream of hydrogen sulphide and carbon dioxide, supplemented with minimal cost in micronutrient supplements, that can produce PHA in a cost effective and sustainable way. *C. aurantiacus'* extremophile nature also presents an opportunity for minimizing sterility costs as its growth habitat is considered inhospitable for most microorganisms and virtually all multi-cell organisms. Its ideal growth temperature of 50°C, with tolerance of even higher ones, is also useful as it allows for hot effluents of industrial processes to transfer their heat into the medium without worries of accidentally killing the culture.

1.10.1. Thesis objective

The objective of this thesis is to use a pure culture of *C. aurantiacus* Y-400-fl (DSM 637) and characterize its growth, due to the little amount of literature covering its ideal growth conditions, see how it reacts to different medium conditions, and analyse the impact they have on the growth rate, pigment content and internal accumulation of polymers.

2. Materials and Methods

2.1. Preparation of microorganism

2.1.1. Cryopreservation of *C. aurantiacus* Y-400-fl (DSM 637)

Glycerol solution and new cryovials inside a recipient were autoclaved at 121 ℃ for 20 minutes. Each cryovial was filled with 200µl of glycerol inside a sterile laminar flow chamber and 800µl of cultivation broth was transferred to the cryovials and homogenized. The vial was closed, labelled, placed in a box and stored at -80℃.

2.1.2. Waking up cryopreserved cultures of *C. aurantiacus* Y-400-fl (DSM 637)

The cryovials were left to defrost at room temperature and then inoculated into 100 ml serum flasks containing M1 medium supplemented with sodium sulphide solution (0.313 mM S end concentration) inside a sterile flow chamber (Heraeus SB48). These flasks were left to grow on an incubating shaker (IKA KS 3000 I control) at 175 rpm, at 50 ℃, with constant illumination of around 1.5 W/l, provided by a halogen bulb. These flasks were left to grow, and their optical density followed (VWR V1200 spectrophotometer) until they entered exponential phase.

2.2. Solutions and media preparation

2.2.1. Preparation of M1 medium

M1 medium is based on the DSMZ reference medium for *Chloroflexus*, with the only change being the use of disodium phosphate dibasic heptahydrate ($Na_2HPO_4.7H_2O$), instead of the recipe's dihydrate form ($Na_2HPO_4.2H_2O$), and the subsequent adjustment of its mass.

In certain tests some components were removed or replaced to test the reaction of the organism to the changes, but all the omitted compounds remain the same.

Below is the reference recipe used for all preparations of unmodified M1, proportionally adjusted to the necessary volume:

Table 2.1: Components of M1 medium for reference volume (~1060ml)

Yeast extract	1.000 g
Glycyl-glycine	1.000 g
$Na_2HPO_4.7H_2O$	0.144 g
$MgSO_4.7H_2O$	0.100 g
KNO_3	0.100 g
$NaNO_3$	0.500 g
NaCl	0.100 g
$CaCl_2.2H_2O$	0.050 g
Fe (III) citrate solution (0.1g/100ml)	5.000 ml
Trace element solution SL-6 (Appendix 1)	1.000 ml
Distilled water	1050.000 ml

2.2.2. Preparation of neutralized sulphide solution

100ml of distilled water in a 100ml serum flask was bubbled with nitrogen for 5 minutes to remove any oxygen dissolved inside it. To this serum flask 3g of nonahydrate sodium sulphide ($Na_2S.9H_20$) was added, and without mixing the two, the headspace was flushed with nitrogen, after which the flask was closed with a butyl rubber septum and an aluminium capsule. This solution was then hand-shook until the sodium sulphide was fully solubilized, after which the solution was autoclaved for 20 minutes at 121 ℃. After the autoclave, the solution was left to cool down and once at room temperature, the bottle was injected with small doses of 2M sulfuric acid until its colour turned

yellow, after which the pH was measured with pH strips and further lowered until the strips indicated around pH 7. Final concentration of the sulphide neutralized solution was 30 g/l of $Na_2S.9H_20$ (125 mM S).

2.2.3. Preparation of resazurin solution

Resazurin is a non-toxic compound that indicates the reduction potential of the medium based on the changes in colour and as such was chosen to follow the anaerobic conditions of *C. aurantiacus* without the use of more invasive methods.

To prepare the stock solution 13mg of resazurin powder reagent was weighed and placed in a 50ml volumetric flask and filled with distilled water, leading to a final concentration of 260mg/l. After mixing the solution was transferred to a 50ml serum flask, capped with a butyl rubber septum and aluminium, followed by a 5-minute flush with nitrogen gas and then autoclaved at 121℃ for 20 minutes.

2.2.4. Preparation and supplementation of sodium carbonate solution

18g of anhydrous sodium carbonate (Na_2CO_3) were weighed into a 100 ml serum flask containing 100ml of distilled water. The flask was quickly capped with a rubber septum and aluminium to prevent losses and agitated by hand to dissolve the carbonate.

Supplementation of the carbonate solution was done by drawing the desired volume plus extra 2 ml from the stock solution bottle, placing a sterile 0.2µm pore filter in between the syringe and a sterile needle and pressing the plunger until a droplet left the needle, point at which the needle was inserted in the destination flask and the desired volume injected.

2.2.5. Preparation of sulphide colouring reagent

In 100 ml of 6M hydrochloric acid (HCl), 0.6 g of iron (iii) chloride hexahydrate and 0.4 g of N,N-dimethyl-p-phenylenediamine sulphate were dissolved and stirred until fully mixed.

2.3. Experimental methods

2.3.1. Growth with M1 medium in 100 ml serum flasks

M1 medium (Table 1.1) was prepared in a 1L *Schott* flask, of which 90ml doses were transferred to 100ml serum flasks. These flasks were subsequently closed with a rubber septum and encapsulated with an aluminium capsule. Once encapsulated the flasks' liquid was bubbled with nitrogen gas for 5 minutes to remove the oxygen inside it and the flasks were autoclaved at 121 ℃ for 20 minutes.

After cooling down, 0.25ml of neutralized sulphide solution was added to the flasks (end concentration of 0.313 mM S), followed by a variable volume of inoculum (3.75 to 7.5 ml) depending on the intended initial optical density (OD).

These flasks were then placed in a shaker incubator at 50℃ with 150 rpm agitation and constant illumination provided by a halogen light bulb. The light intensity was adjusted according to the desired test. Optical density was followed daily at 610nm, from the point that turbidity of the medium was detected onwards.

2.3.2. Study of the effect of sulphide concentration in waking up culture cryovials

After several unsuccessful attempts at waking up cryovials with the recommended protocol (using a 1.25 mM S end concentration of sodium sulphide nonahydrate solution in the waking up flask), a test study was conducted to determine the best sulphide concentration to wake up cryovials.

Seven 100ml serum flasks containing sterile M1 medium were inoculated with 1 cryovial each and 4 different concentrations of sulphide were tested: 1.25 mM S (1 flask), 0.625 mM S (2 flasks), 0.313 mM S (2 flasks) and 1 drop of the 125 mM S neutralized sulphide solution (as prepared in the previous section) (2 flasks). Total and soluble sulphide concentration was followed, alongside the optical density at 610nm until the cultures reached the decay stage (around 9 days of test). The test culture was grown at 7 ± 2 W/L light intensity, 50℃ and 175 rpm.

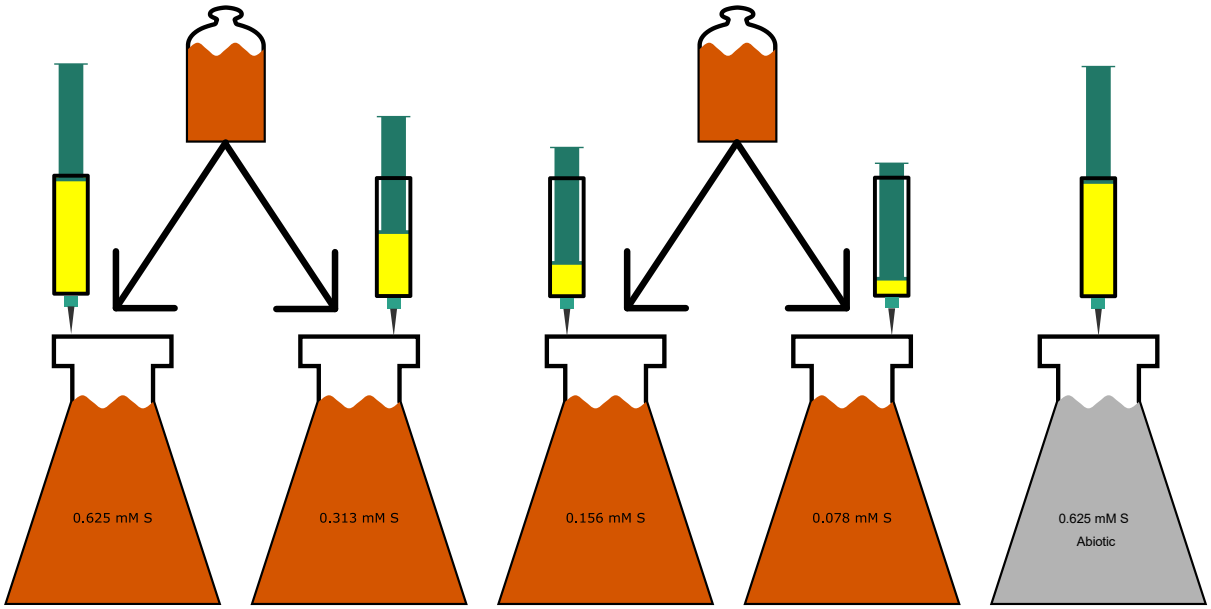

Figure 2.1: Experiment: study of the effect of different sulphide concentrations in exponentially growing cells

2.3.3. Study of the effect of sulphide concentrations in exponentially growing cells

500 ml Erlenmeyer flasks containing M1 medium were capped with standard *Schott* lids and autoclaved. In parallel, matching butyl rubber equipped *Schott* lids were also autoclaved. Once autoclaved and cooled, the shake flasks' lids were swapped for the butyl rubber Schott lids inside a sterile laminar flow chamber. The flaks were subsequently flushed for 10 minutes with sterile nitrogen and stored until inoculation day.

Five 500 ml Erlenmeyer flasks containing M1 medium (Figure 2.1) were supplemented with 1ml of resazurin colouring solution and a variable volume of neutralized sulphide solution to test *C. aurantiacus* growth at four different sulphide concentrations: 0.625 mM S (2 flasks), 0.313 mM S (1 flask), 0.156 mM S (1 flask) and 0.078 mM S (1 flask). One of the high sulphide concentration flasks was used as abiotic control, and the remaining flasks were inoculated (starting OD ≈ 0.05) with an exponentially growing *C. aurantiacus* culture (grown in M1, same conditions as section 2.3.1). The test was conducted for 8 days, and samples were taken every day to determine optical density, soluble and total sulphide. The culture was grown at 2.26 ± 0.12 W/L light intensity, 50℃ and 175 rpm.

Samples to determine the Total organic carbon (TOC), PHA content, total suspended solids (TSS) were taken on the exponential stage and at the end of the test. Samples to determinate phosphate and ammonia concentration, and glycogen content were taken only at the end of the test.

2.3.4. Test of the effect of different light conditions on cell pigment and growth

2.3.4.1. Inoculum preparation

Identical 100ml serum flasks containing 90ml of M1 medium were supplemented with of sulphide (0.313 mM S end concentration) and inoculated (starting OD ≈ 0.05) with an exponentially growing

C. aurantiacus culture inoculated from a cryovial. One flask was constantly illuminated with 5.08 W/l and another with 1.92 W/l, in both cases provided by halogen bulbs, with 175 rpm agitation, at 50 °C, until exponential phase was achieved in either flask (7 days for high light intensity and 11 days for low light intensity). Optical density was followed, and the final pigment content was determined through acetone-methanol extraction (section 2.4.5), before being used as inoculum for the next stage of the test.

2.3.4.2. Full test

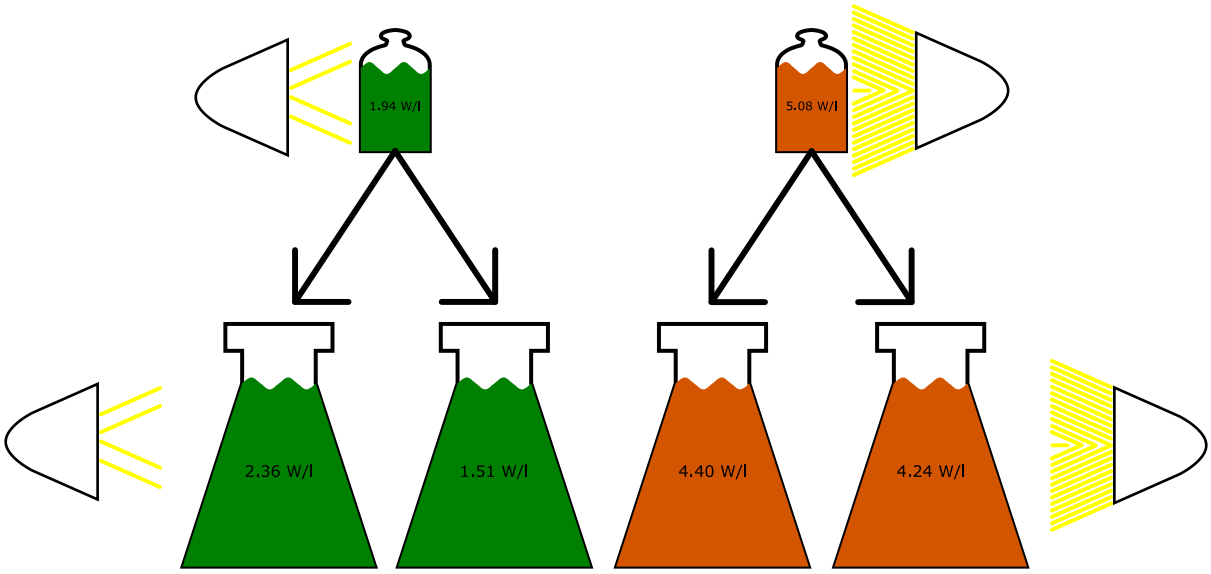

Figure 2.2: Experiment: Impact of inoculum light intensity on growth

To allow for larger samples, 4 shake flasks of 500 mL (Figure 2.2) containing M1 medium were inoculated with 27-29 ml of inoculum culture from the previous test (section 2.4.3.1) (end optical density of 0.05) and supplemented with resazurin (0.52 mg/L end concentration) and sulphide (0.313 mM S end concentration).

Growth was followed daily with optical density, total and soluble sulphide analysis. Samples for pigment analysis, TOC, PHA, glycylglycine, glycogen, phosphate and ammonia were taken at the start of the exponential phase and the end of the test.

2.3.5. Growth with inorganic carbon source (Na_2CO_3)

2.3.5.1. Preparation of inoculum flask

Modified M1 medium without glycylglycine (M1 GG free) was prepared into both 500 ml shake flaks and 100 ml serum flasks. The medium was flushed with nitrogen and autoclaved at 121 °C for 20 minutes. Sodium carbonate solution (1698 mM C) was filtered using sterile 0.2 µm syringe filters and added into the flasks, for an end inorganic carbon concentration of 29 mM C, pH was then adjusted to around 8 using 6M HCl and neutralized sulphide solution was added (0.625 mM S end concentration). Flasks were inoculated with the cultivation broth coming from a woken up cryovial containing M1 medium (prepared as section 2.1.2). The flasks were grown in the same conditions, 175 rpm, 50 °C and 24h light presence at 4.11 $\pm$ 0.25 W/l. When the cultures reached exponential phase, they were used as inoculum for the 500 ml shake flasks of the next section, containing modifier M1 medium with inorganic carbon source in the place of glycylglycine.

2.3.5.2. Growth in 500 ml shake flasks

500 mL shake flasks containing medium M1 GG free (prepared as in section 2.2.1 but without glycylglycine) were supplemented with sodium carbonate solution (1698 mM C) for an end inorganic

carbon concentration of 29 mM C. Afterwards, sulphide solution was added to attain a concentration in the flasks of either 0.313 mM S end concentration (3 flasks) or 0.625 mM S end concentration (1 flask), followed by 1 ml of the resazurin solution (final concentration in the medium of 0.52 mg/l) (Figure 2.3).

Two inocula were used one grown in M1 (section 2.3.1) and the other grown in M1 GG free + IC (section 2.3.5.1).

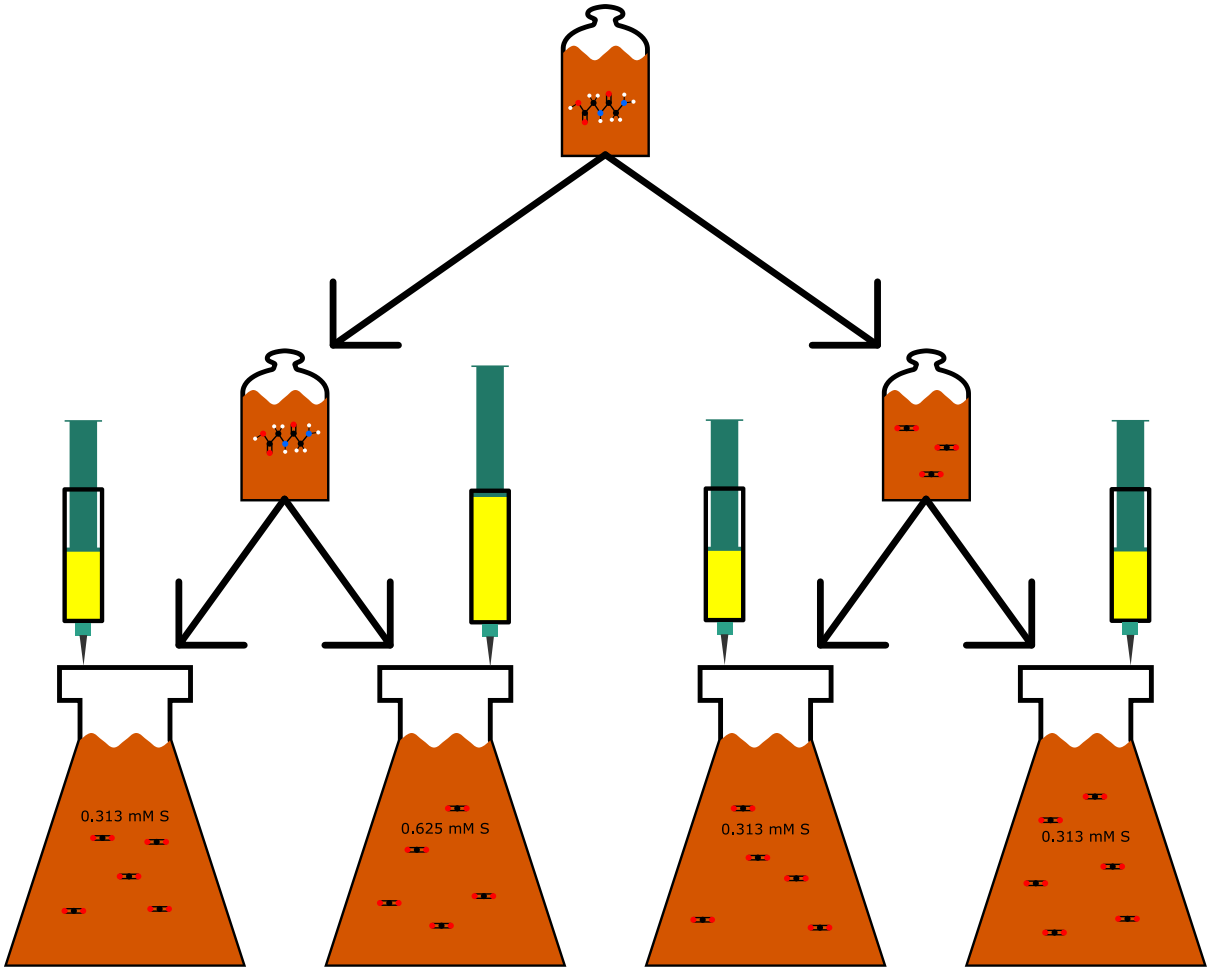

Figure 2.3: Experiment: Growth with inorganic carbon feedstock. One serum flask with M1 medium was grown until exponential phase and used to inoculate an identical flask and another with M1 GG free + IC. These 2 flasks were then grown to exponential phase and used to inoculate two 500ml shake flasks containing M1 GG free + IC medium. One of the 500ml flask inoculated with inoculum grown in M1 medium was supplemented with double the sulphide concentration.

2.3.6. Growth with inorganic carbon source (Na_2CO_3) in 500 ml shake flasks with yeast extract limitation

A medium based on the M1 GG Free + IC medium from section 2.3.5 was prepared, but with 1/10 the total yeast extract, this new medium, M1 GG Free + IC, LYE (low yeast extract) was inoculated in a similar fashion to the flasks in figure 2.3, using 0.625 mM of sulphide and an inoculum from regular M1 GG Free + IC.

2.3.7. Growth in M1 and M1 Gly medium with 250ml shake flasks.

Due to the high cost of glycylglycine (GG), one of the primary carbon sources of M1 (alongside yeast extract), an experiment was designed to determine whether its monomeric form, glycine, could be used as a less expensive alternative (so forth called M1 Gly medium).

M1 GG free was prepared in a 2L *Schott* flask, and 250 ml of the medium was transferred to 250 ml shake flasks, 3 of which were added 236g of Glycylglycine (flasks GG1 up to GG3), and 3 of which were added 269 mg of Glycine (flaks Gly1 up to Gly3), a value calculated to have the same amount of Glycine monomers as the 236g of Glycylglycine. All flasks were purged of oxygen using pressurized nitrogen and autoclaved at 121 ℃ for 20 minutes.

On the inoculation day it was added to the flasks 22.5 ml of 5.5 mg/l resazurin solution (resulting in an end concentration of 0.5 mg/l), 2.5 ml of sulphide solution (totalling a theoretical 1.25 mM S), 0.25 ml of vitamin B12 solution. The flasks were inoculated with 5ml of cultivation broth from the 100ml serum flask M1 inoculum (2.29 W/L, 150 rpm, 50℃).

These 250 ml flasks were placed on the incubating shaker at 175 rpm 50 ℃ with 2 incandescent lightbulbs illuminating them from the side and one from the top (1.219 W/L $\pm$0.17). Samples were taken daily for sulphide analysis and optical density. Samples for total organic and inorganic carbon and volatile fatty acids were taken at 3 points, 24h, 72h and 168h, with samples for glycogen content and total and volatile solids taken at 72h and 168h. At 168h the test was ended as the culture density was starting to decrease.

2.4. Analytical methods

2.4.1. Measurement of light intensity

With all lamps in place and the shaking platform stopped a light sensor was placed in each of the spots of the incubator, aiming the light source. An average of the light intensity in W/m^2 of that spot was taken. The volumetric light intensity (W/l) in each flask was calculated by multiplying the light intensity of that spot (in W/m^2) by the illuminated surface area of the flask (m^2) and divided by the working volume of the flask (l).

2.4.2. Determination of cellular growth by optical density

To determine the optical density of the cultivation broth a cuvette of distilled water was used to blank the spectrophotometer at 610nm, followed by a measurement of the raw culture at the same wavelength. Then, the culture was centrifuged at 10500 g for 3 minutes and the absorbance of the supernatant at the same wavelength was measured. The resulting value was subtracted from the raw absorbance to assure only the optical density of the suspended cells was being measured.

2.4.3. PHA staining method with Nile Blue

To a 1.5ml Eppendorf containing fresh cultivation broth, a drop of Nile blue staining reagent was added and mixed thoroughly, after which the tube with the culture was left to hybridize for 15 minutes in a warm bath at 50 ℃. This sample was then observed in an epifluorescent microscope and the presence of lipids or PHA could be detected by the observation of fluorescent granules.

2.4.4. Analysis of volatile and total suspended solids

5 to 7 ml of the cultivation broth were filtered using 0.7 µm pore (General Electric GF/F) fiberglass filters (previously muffled at 550℃) and placed for 24h at 100℃ to remove all moisture and thus determine the total suspended solids (TSS). d Afterwards, the filters were muffled at 550℃ for 20 minutes to remove volatiles and thus determine the ashes portion of the biomass. Volatile suspended solids (VSS) were determined by subtracting the ashes from the TSS.

2.4.5. Analysis of bacteriochlorophylls and carotenoids

To quantify of bacteriochlorophylls and carotenoids concentration, 1.5ml of a 7:2 acetone-methanol solution was added to 1.5ml *Eppendorf* tubes containing defrosted biomass pellets, obtained by centrifuging 1.5 ml of the cultivation broth, and thoroughly mixed with a micropipette through up and down movements. The *Eppendorfs* were let to rest overnight at room temperature protected from ambient light with aluminium foil. In the following day, these were centrifuged at 10500 g for 3 minutes to settle all biomass and the supernatant was placed in a quartz cuvette and its absorbance measured in a scan from 190 to 900 nm.

Absolute pigment concentrations were calculated using the following formulas, derived from papers published by Kuo et al.(Kuo et al., 2012) and Yakovlev(A. Yakovlev et al., 2017).

$$[Carotenoids](\mu g\ g(VSS)^{-1}) = \frac{Abs_{450} * 10000}{250 * L(cm) * W}$$

$$W = \frac{biomass(g)}{solvent\ volume\ (ml)} = \frac{VSS\ (g\ l^{-1}) * sample\ volume(l)}{solvent\ volume\ (ml)}$$

$$[Bchl\ a](mg\ g(VSS)^{-1}) = \frac{\frac{Abs_{769}}{L(cm) * 68.6} * 911.594}{VSS(g\ L^{-1})} * 1000$$

$$[Bchl\ c](mg\ g(VSS)^{-1}) = \frac{\frac{Abs_{663}}{L(cm) * 74} * 841.5}{VSS(g\ L^{-1})} * 1000$$

2.4.6. Carbohydrates analysis through high-performance liquid chromatography

2-4 mg of freeze-dried biomass pellets were weighed to a digestion tube and 2ml of 0.6M HCl was added. The tubes were subsequently placed in a dry bath at 100 °C for 2 hours. The resulting digested mixture was cooled down in ice and collected with a syringe, being subsequently filtered with 0.2 µm syringe filters to HPLC vials. Analysis in HPLC (High Performance Liquid Chromatography) was conducted using an IR detector in a Hitachi Chromaster (RI detector 5450, UV-Visible detector 5420, column oven 5310, Auto-sampler 5260, Pump 5160) at a temperature of 30°C with an eluent of 0.01N H_2SO_4 and a flow rate of 0.5 ml/min. For the standards glucose concentrations from 1.42 to 22.8 mg/l were used.

2.4.7. Collection and analysis of total and soluble sulphide concentration

2.4.7.1. Collection

For soluble sulphides, a 2ml sample of cultivation broth was collected with a syringe and filtered with a 0.45µm filter to a 1.5ml Eppendorf, of which 1ml was transferred to another 1.5ml Eppendorf containing 400µl of NaOH 6M and 100µl of 0.5M zinc acetate solution.

For total sulphides a syringe was first prepared with 2ml of HCl 6M, then it was used to collect 2ml of cultivation broth and after agitating the syringe, the mixture was filtered to a 1.5ml Eppendorf pre-prepared with a drop of 2M zinc acetate solution. From this tube 1ml was transferred to another 1.5ml Eppendorf containing 400µl of NaOH 6M and 100µl of 0.5M zinc acetate solution.

Both final tubes were then frozen until the analysis day and the intermediate tube was discarded.

2.4.7.2. Analysis

200 µl of sulphide colouring reagent (section 2.25) and 200 µl of sample from the *Eppendorfs* prepared in the previous step were pipetted to a 5ml capped glass tube, while avoiding the contact with oxygen the as much as possible during the transfer, since the sulphide oxidizes quickly, and left to react for 15 minutes, after which 5ml of HCl 0.1M was added to the tubes to dilute the samples to the desired range.

Their subsequent absorbance was measured at 666nm, and its results checked against standards prepared with a similar technique from solutions with known concentrations of sulphide (0.58 to 0.009 mM S). Blanking of the spectrophotometer was done with a solution containing 200µl of Iron (III) chloride solution (6g/l) and 5ml of 0.1M HCl.

2.4.8. Analysis of phosphate and ammonia content in the medium

0.5 ml of filtrated supernatant sample was added to 2ml of mil-Q water and stored in the provided vials with a parafilm cover in the fridge until analysis in the Skalar San++ segmented flow analyser. Standards from 2mg/l to 20 mg/l of ammonia and phosphate were used for the internal calibration curve.

2.4.9. Analysis of total organic carbon and inorganic carbon

Total organic carbon (TOC) and inorganic carbon (IC) concentration was determined by diluting 2ml of filtered supernatant sample with a drop of 6M NaOH in 8ml of mil-Q water and the values read in TOC-V_{CSH} Analyser (SHIMADZU). Value of the inorganic carbon stock solution was also determined in the same equipment using a 1/20 dilution.

2.4.10. Analysis of PHA content through Gas chromatography

Freeze dried pellets totalling around 2-4 mg were weighed to digestion tubes. 1ml of acidic methanol (5% H_2SO_4 v/v) and 1ml of chloroform with 1g/l of heptadecane (internal standard) was added. The tubes were closed with screw caps and placed in a dry heat thermoblock for 2h at 105 ºC, with the tubes being shaken after 1h to help dissolving the pellets.

The tubes were left to cool overnight and in the following day 1ml of water was added, the tubes were homogenized in a vortex for 1 minute, and after settling the methanol phase removed with a glass pipette.

1ml of water was added, the tubes were re-closed and homogenized in a vortex for 1 minute. The phases were left to separate for 1h, the organic phase was removed with a glass pipette and transferred to a GC vial with molecular sieves for a few seconds and afterwards the liquid was filtered to an empty GC vial and encapsulated.

Samples were run through the GC (Thermo scientific Trace 1300 with a GOLD GC column) with 7 standards ranging from 1.189 mg/l to 0.119 mg/l of hydroxybutyrate, 0.193 mg/l to 0.019 mg/l of hydroxyvalerate, 0.456 mg/l to 0.046 mg/l of hexanoic acid, 0.212 mg/l to 0.021 mg/l of hidroximetilvalerate and 0.5 mg/l of heptadecane. Helium was used as eluent and the heptadecane added to the chloroform solution serves as the internal standard during the analysis.

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3. Results and discussion

3.1. Determination of relation between VSS and TSS and optical density of culture

To establish a relation between optical density (OD) and actual cell density, 2 plots (Figures 3.1 and 3.2) of optical density over, respectively, volatile, and total suspended solids, were created. Only days with total solids above 0.4 g/l were used for the plots as the lower ones were deemed too error prone. Volatile suspended solids (VSS) obtained a regression line with a slope of 0.5393 and an R^2 of 0.9521. Total suspended solids (TSS) obtained a marginally higher regression slope at 0.5454, these values also had a slightly greater variance than the VSS, with an R^2 of 0.949. The TSS to OD ratio is substantially lower than to the 0.702 ratio obtained by Farnocchia (2021) with the same organisms, this can be partly due to differences in how the values were obtained, while in this experiment we used filters, Farnocchia (2021) used the raw weight of freeze-dried biomass pellets, a technique that does expend less sample volume but by using less total mass, also reduces accuracy. The VSS/TSS ratio is of 0.988, which results in an average ashes content of 1.12%, this means that our cells had a low mineral content, meaning that no substantial accumulation of inorganic material was being performed by our cultures, both in inorganic and organic carbon medium.

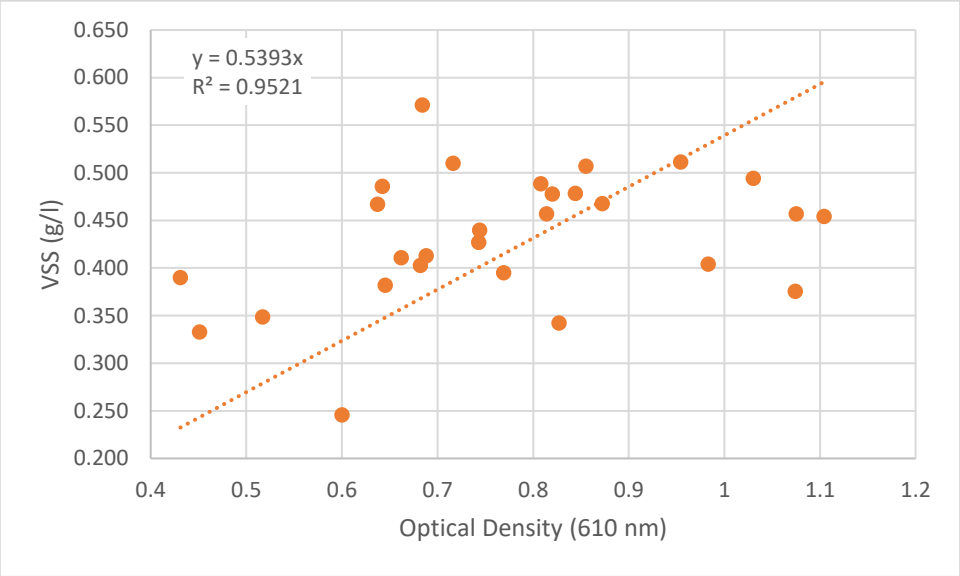


Figure 3.1: Volatile suspended solids (g/l) concentration over optical density (610 nm)

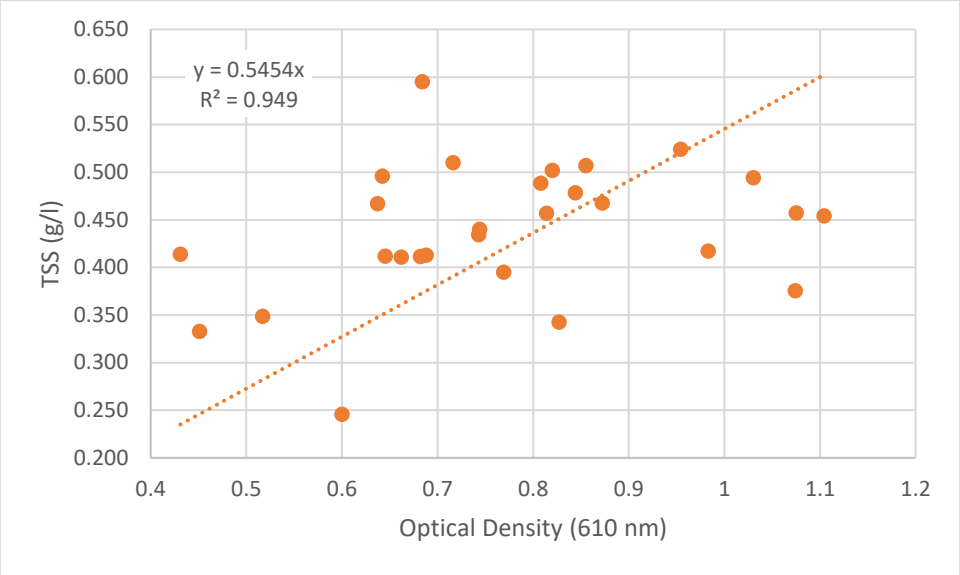


Figure 3.2: Total suspended solids (g/l) concentration over optical density (610nm)

3.2. Impact of sulphide concentration on *C. aurantiacus* growth

Initially tests were done to determine which sulphide concentrations (1.25, 0.625, 0.313 mM and 1 drop of S) cryovials could be woken up with. Tests were done in triplicate with 3 different cryovials split evenly into 3 to rule out potentially inviable cryovials. From the first cryovial, only the test with 0.313 mM of sulphide had meaningful growth, with OD peaking at 1.403. The test flasks with 0.625 and 0.156 mM of sulphide peaked at OD's of 0.170 and 0.184 respectively.

The second cryovial had no meaningful growth at any of the 3 concentrations, suggesting a potentially defectively prepared vial.

The final cryovial had growth in both the 0.313 and 0.156 mM test flasks, peaking at OD's of 1.235 and 1.641 respectively but looking in the microscope, the 0.156 mM flask didn't resemble the expected appearance of *C. aurantiacus*, hinting at a contamination caused by the lower selective pressure caused by the lower sulphide concentration, the 0.625 mM test flask developed some turbidity and faint yellow colour but upon microscope inspection, no signs of *C. aurantiacus* were found.

From this test we concluded that 0.313 mM of sulphide was the most reliable way to wake up cryovials and was the concentration used for all subsequent tests' cryovial wakeup stages.

3.2.1. Test of impact of sulphide concentration in 500 ml flasks inoculated with exponentially growing inocula.

With the aim of getting larger biomass amounts for more analysis, a larger test, in 500 ml baffled shake flasks, was conducted to evaluate the effect of different sulphide concentrations on the cellular growth of *C. aurantiacus* after it had already been woken up. In this test the highest cellular growth (OD = 0.821) was observed after 72h of assay in the medium with 0.156 mM of sulphide (Figure 3.3). The flasks containing a sulphide concentration of 0.313 and 0.078 mM obtained peaks at OD = 0.736 and OD = 0.722, respectively, but maintained density relatively constant until the end of the test while in the experiment with 0.156mM of sulphide showed a sharp decline after 96h. The flask with 0.625 mM of sulphide was the worst performing flask obtaining a peak of only OD = 0.457, suggesting an inhibition of growth caused by the high sulphide concentration. At 168h a contamination was detected in the abiotic culture flask, prompting the use of better asepsis methods (sterilizing the lids, use of different sterile needles, changing sterile gas filters between samples, changing the order of sampling to start with the abiotic) in the future. pH was checked daily, and it showed a trend to gradually decrease from the 8.2 of the original media to around 6.5 at the end of the experiment, this trend isn't particularly concerning as it didn't appear to change much past that value and 6.5 is still relatively close to a neutral pH, but it is a trend worth keeping an eye for and would likely necessitate a pH control system if it was being grown under CSTR.

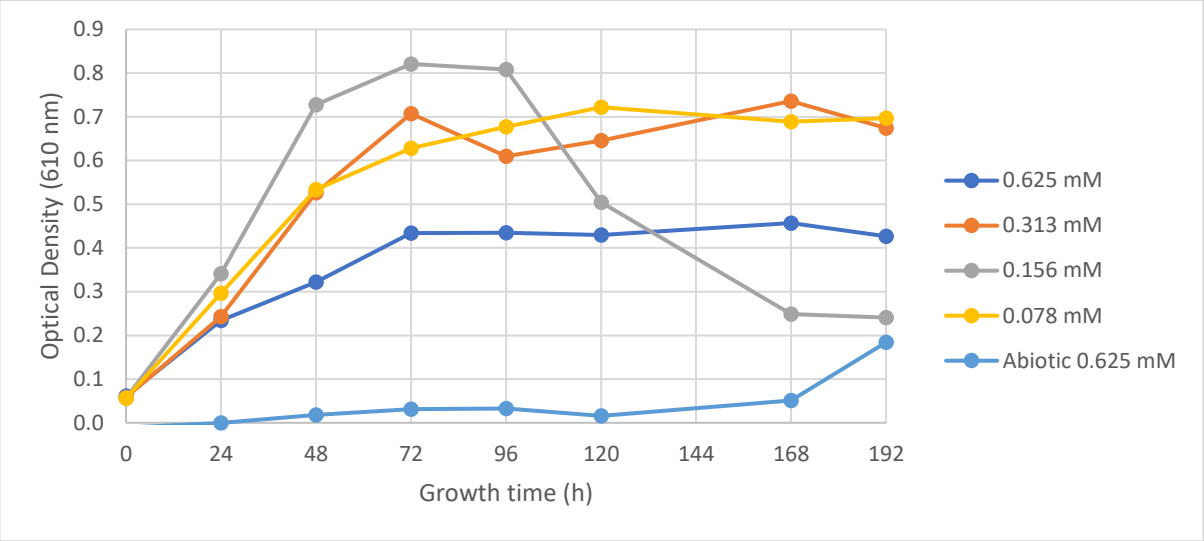


Figure 3.3: Optical density (610 nm) over growth time (hours) and respective light intensities of cultures with different starting sulphide concentrations. Light intensities: dark blue 0.625 mM: 2.30 W/l; orange 0.313 mM 2.05 W/l; grey 0.156 mM: 2.33 W/l; yellow 0.078 mM: 2.36 W/l; light blue abiotic: 0.58 W/l. Inoculum was grown in 2 identical 100ml serum flasks at 0.313 mM of sulphide and 3.92 W/l.

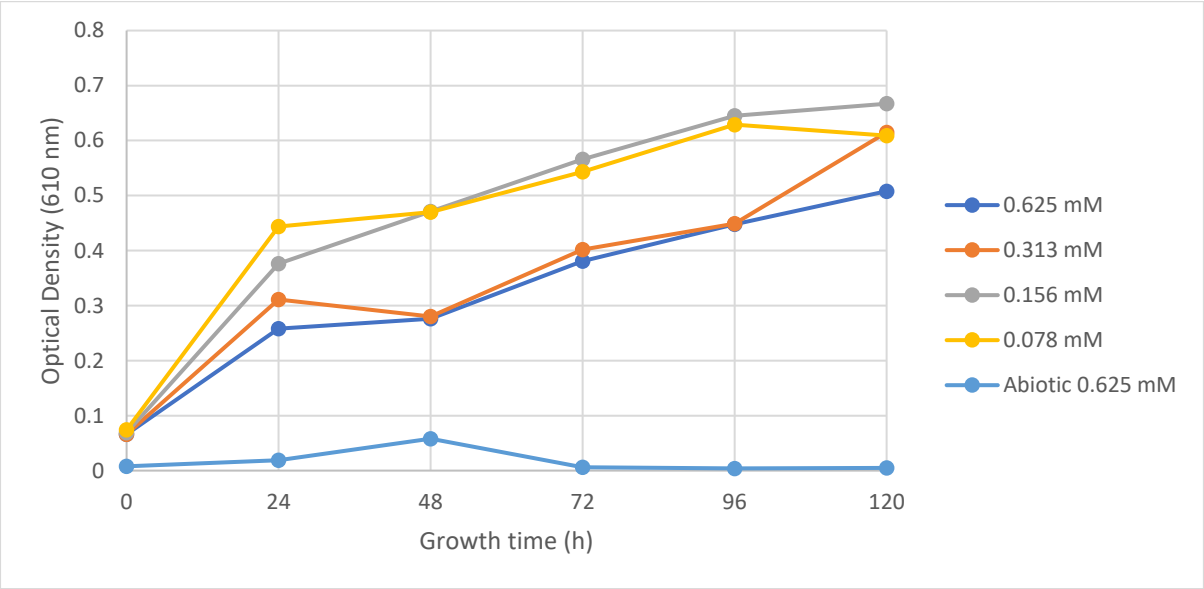


Figure 3.4: Optical density (610 nm) over growth time (h) and respective light intensities of the replica test of cultures with different starting sulphide concentrations. Light intensities: dark blue 0.625 mM: 2.30 W/l; orange 0.313 mM 2.05 W/l; grey 0.156 mM: 2.33 W/l; yellow 0.078 mM: 2.36 W/l; light blue abiotic: 0.58 W/l. Inoculum was grown at 0.313 mM of sulphide in a single 500 ml shake flask with 0.58 W/l.

For result validation, the experiment was repeated in order to have duplicates, but due to constraints of space in the shaker incubator the inoculum was grown with a lower light intensity than the first test, 0.58 W/l instead of 3.92 W/l. This resulted in an inoculum with a visible green colour, as opposed to orange of the previous experiment's inoculum. Perhaps due to the different inoculum conditions or due to another factor outside of the ones analysed, this duplicate (Figure 3.4) showed a different growth profile (Figure 3.4), with the 2 lowest sulphide concentration flasks (0.156 mM and 0.078 mM S) following a very similar trend of higher density, and the 2 highest concentration flasks (0.625 and 0.313 mM S) also following a similar trend until the last day when

the 2nd highest (0.313 mM S)had a growth surge not seen in the highest concentration flask (0.625 mM S).

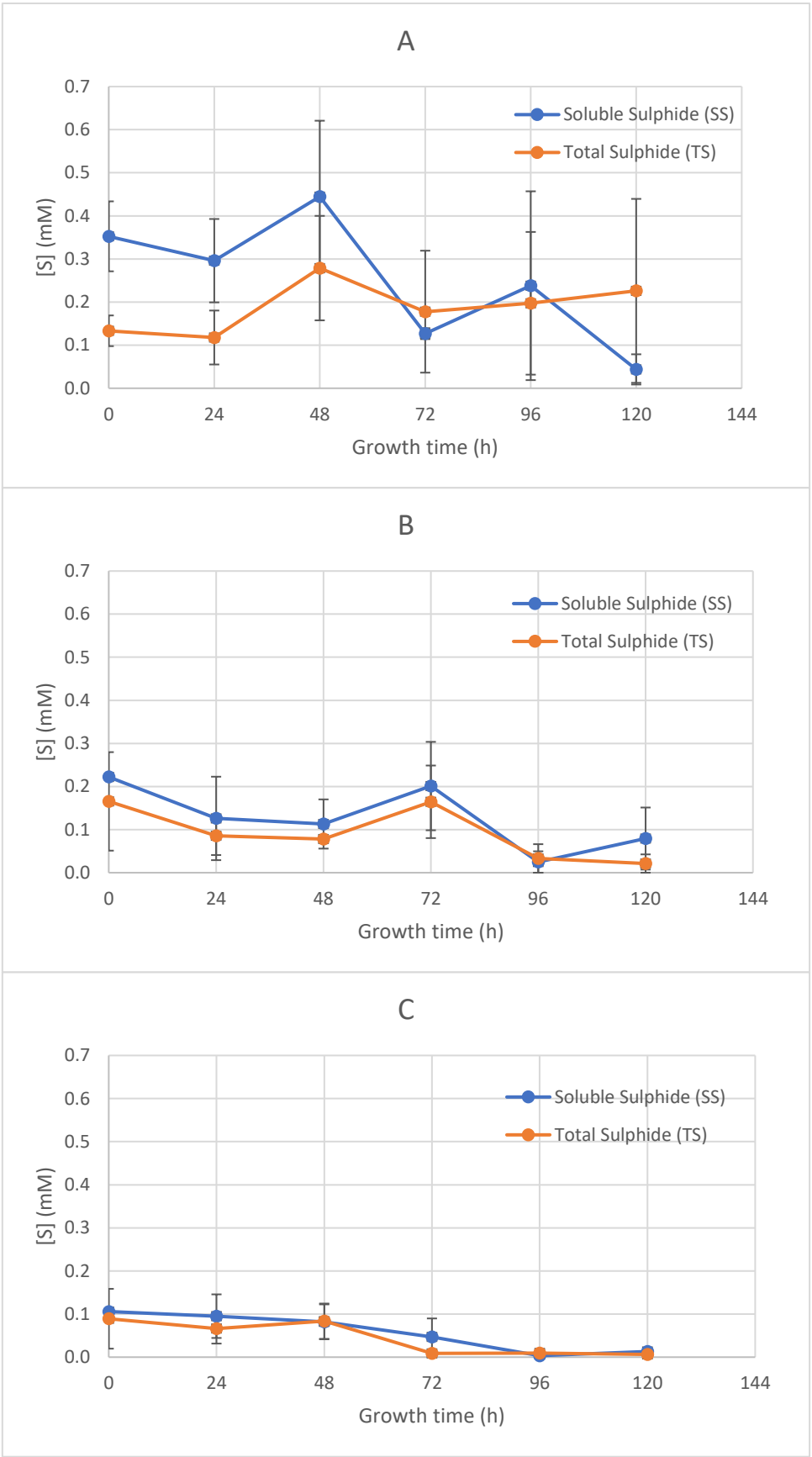
[S]	Test 1 μ(d^{-1})	Test 1 Replica μ(d^{-1})	Average μ(d^{-1})	Std deviation
0.625 mM	2.84	2.91	2.87	0.04
0.313 mM	3.19	3.71	3.45	0.26
0.156 mM	4.98	4.45	4.72	0.27
0.078 mM	4.29	5.00	4.64	0.36

Table 3.1: Specific growth rates of the cultures from test 1 (d^{-1}).

Specific growth rates (Table 3.1) confirm the pattern observed in the OD graph, with both tests showing the 2 lowest sulphide concentrations (0.156 mM and 0.078 mM) having faster growth, possibly due to the higher sulphide concentration in the other flasks causing some inhibition. The relatively low standard deviation shows that it might be possible to infer the 2 tests as being replicas, despite the difference in inoculum conditions.

The cultures entered steady state at around 72-96h in most experiments conducted, there are many factors that can drive an exponentially growing culture to enter steady state. Several analyses were conducted to the medium and the cells to try to find which was the main factor in slowing down growth. Under both tests ammonia levels remained in the range of 45 to 108 mg/l and phosphate levels on the range of 13 to 26 mg/l, on the original test both were consumed/lost, and, in the replica, both had their concentrations increase, making very unlikely these were the cause of the culture entering steady state.

Unexpectedly sulphide analysis (Figure 3.5) revealed total sulphide (TS) values lower than the soluble sulphides (SS), this indicates some sort of problem with how the samples were collected, prepared, or analysed that we were unable to track down. Nonetheless the results allow us to draw the conclusion that the sulphide concentration decreased over time, taking longer to reach trace levels on the higher concentration flasks. Sulphide concentrations reaching trace levels is likely to not the cause for the culture reaching steady state, as the cultures with lower starting sulphide concentrations didn't have meaningful inhibition compared to the other cultures, in fact the opposite was true on average, with the lower concentration flasks reaching trace levels relatively early on and maintaining growth.


A
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
[S] (mM)
Soluble Sulphide (SS)
Total Sulphide (TS)
0
24
48
72
96
120
144
Growth time (h)
B
0.7
0.6
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0.2
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[S] (mM)
Soluble Sulphide (SS)
Total Sulphide (TS)
0
24
48
72
96
120
144
Growth time (h)
C
0.7
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0.2
0.1
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Growth time (h)

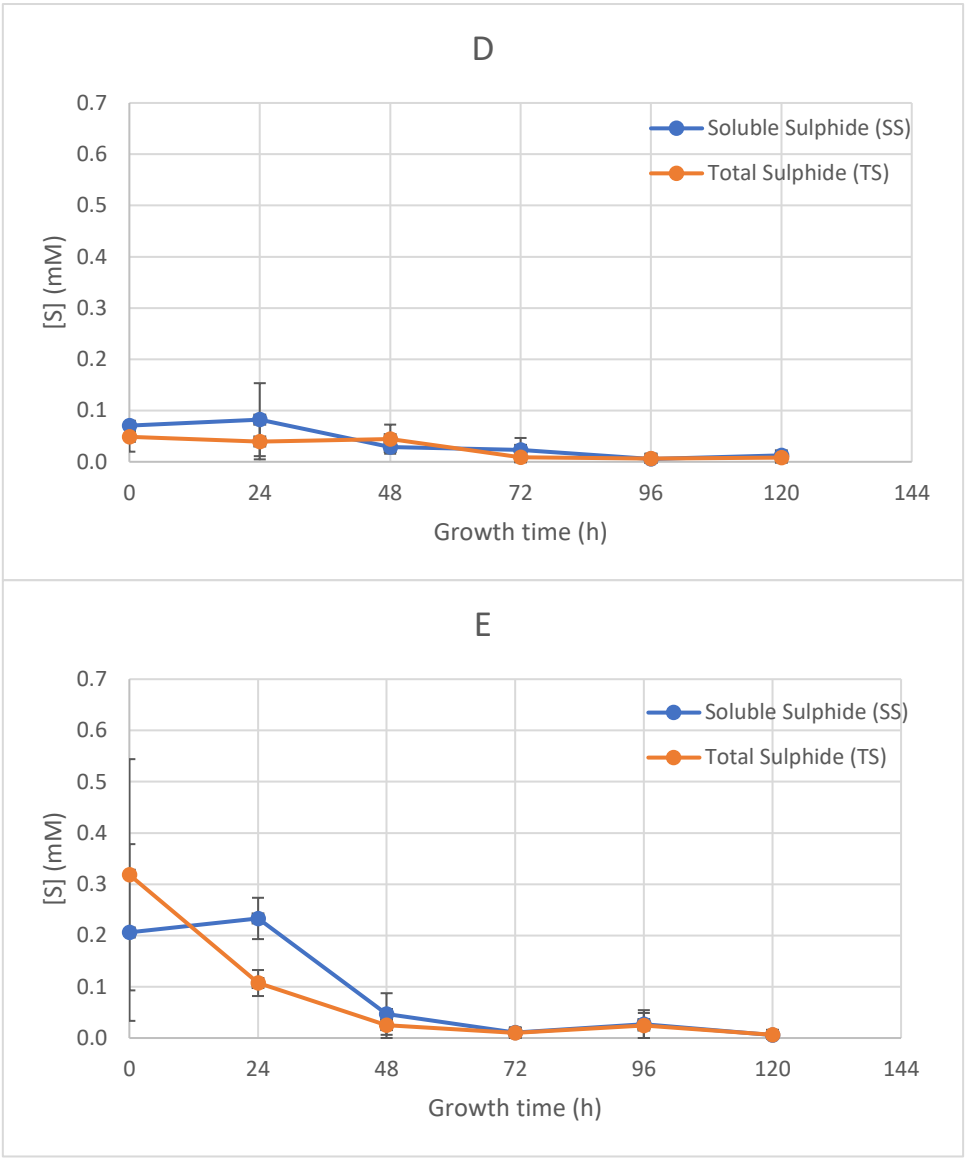

Figure 3.5: Total (orange) and soluble (blue) sulphide concentrations (mM) over growth time (h). A: 0.625 mM initial sulphide concentration; B: 0.313 mM initial sulphide concentration; C: 0.156 mM initial sulphide concentration; D: 0.078 mM initial sulphide concentration; E: Abiotic with 0.156 mM initial sulphide concentration.

Despite not feeding the culture with volatile fatty acids, we conducted an analysis of their content as due to the composition of the medium containing yeast extract, it's very common for volatile fatty acids to be produced by the cells while metabolizing the complex organic compounds in the yeast extract. The analysis showed butyric acid as being the prevalent in all flasks and its concentration decreasing over time but never fully running out (Appendix 2), due to the large timespan between the analysis it's not possible to conclude they were not responsible for causing the culture to enter stationary phase but seems unlikely given the butyric acid always remained above 3 mM [C] in the original test, even if it decreased in the end of replica, possibly due to sampling losses.

Total organic carbon and inorganic carbon analysis (Figure 3.6) shows a trend of decreasing total organic carbon and a less significant increase of inorganic carbon, suggesting that *C. aurantiacus* was consuming the organic carbon compounds in the medium and using it for the production of biomass, with only a small number of inorganic by-products being produced. Average uptake rate of carbon on most cases was between 0.42 and 0.57 µM h^{-1} (mg VSS)$^{-1}$, with the exception for the assay with 0.078mM sulphide assay in the replica test, which, strangely, had increase in organic carbon content. Due to the organic carbon levels largely staying above 30-40 mM it's safe to assume this was not the cause of the culture entering stationary stage, however it's noteworthy that the contaminated abiotic had some consumption of organic carbon and release of inorganic carbon, indicating that whatever organism grew there was metabolizing organic carbon into inorganic carbon.

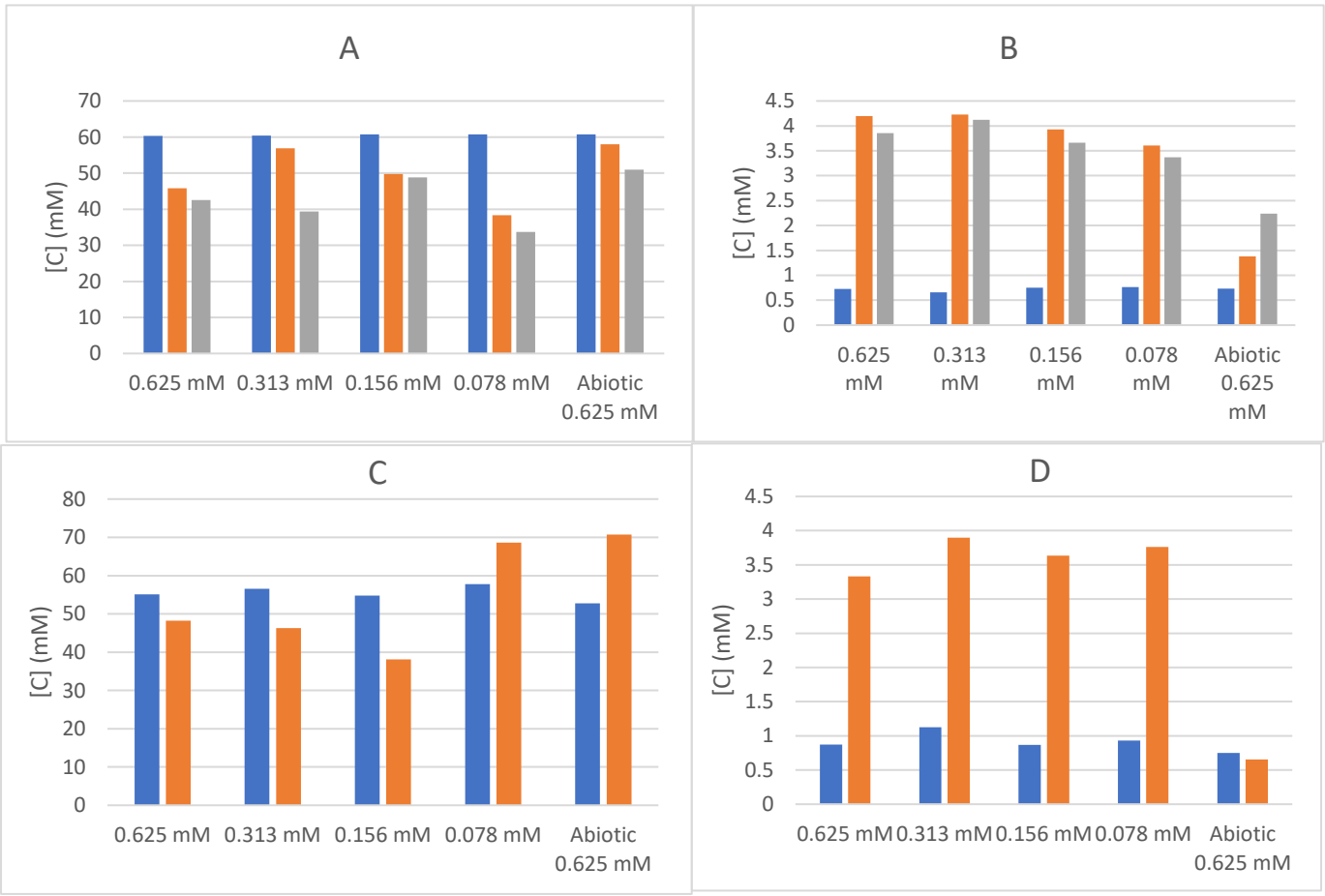

Figure 3.6: Total organic and inorganic carbon content in the culture medium. A: Total organic carbon concentration (mM C) of first test: blue 0h, orange 168h, grey 192h; B: Inorganic carbon concentration (mM C): blue 0h, orange 168h, grey 192h; C: Total organic carbon concentration (mM C) of replica test: blue 0h, orange 120h; D: Inorganic carbon concentration (mM C) of replica test: blue 0h, orange 120h.

3.2.2. Polymer content

PHA content (above 1% gPHA/gVSS) (Figure 3.7) was found in 2 of the experiments of the first test and in all the replica tests. Notably the highest sulphide concentration flask of the replica showed exceptional polymer accumulation with 6% (36nM) of polyhydroxybutyrate and 8% (50 nM) of polyhydroxyvalerate, which when combined with 1% of polyhexanoic acid (also by far the most significant accumulation of this polymer in any flask), amounts to a total of 15% of the cell's dry weight in PHA.

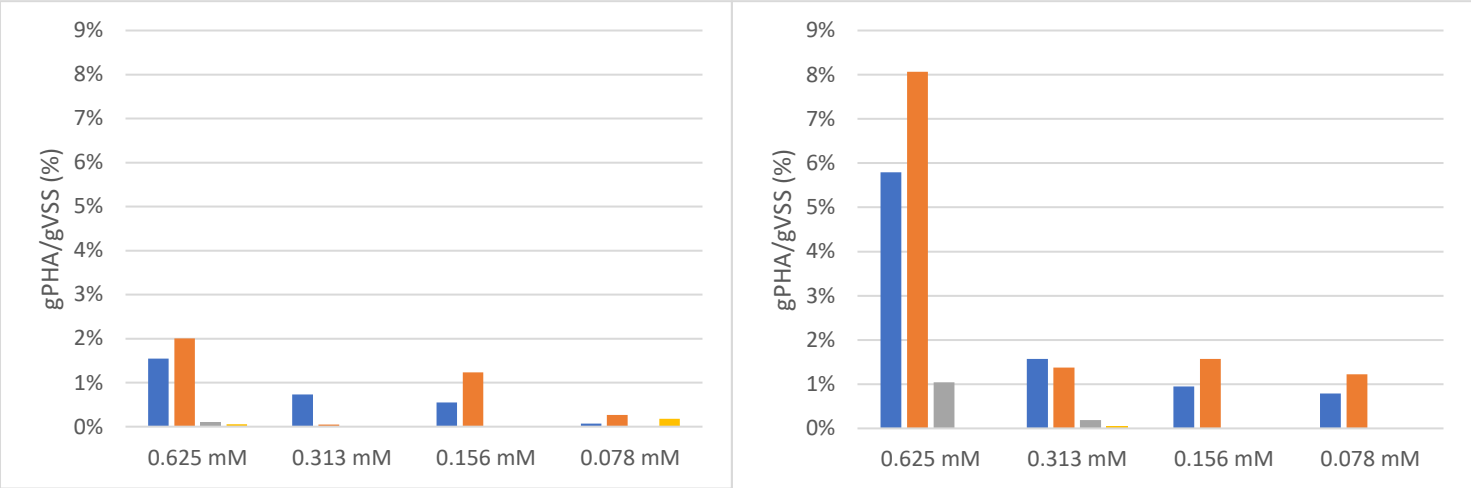

Figure 3.7: PHA content at end of the tests for both original (left) and replica (right), % of VSS. Blue: HB, Orange: HV; Gray HHX; Yellow HMV.

The higher PHA accumulation in the highest sulphide concentration tests points to this condition being one that favours PHA accumulation, a finding that matches up with what's known about secondary PHA accumulation, since it usually takes place in conditions where cell growth is inhibited but macronutrients are still available, as is the case here. The higher accumulation in the tests grown from low light intensity inoculum again fall in line with the expected as the lower amount of available energy promotes accumulation of PHA. From the preliminary results obtained here it suggests that a good strategy for PHA accumulation in *C. aurantiacus* will be a high sulphide concentration and a low light availability during the inoculum stage.

Glycogen content was analysed but only minimal amounts were detected (< 0.2% gGly/gVSS), indicating that the carbon was being used preferentially for cell growth and PHA accumulation instead of glycogen.

3.2.3. Pigment content

Pigment analysis (Appendix 3) of the first test with orange inoculum showed a tendency for bacteriochlorophyll *c* and carotenoid content to increase over time with the exception of the experiment with 0.156 mM sulphide concentration test. On the replica the trend of growing carotenoids continued, together with an increase in bacteriochlorophyll *c* as before, with the exception of the bacteriochlorophyll *c* in the experiment with 0.625mM of sulphide, this experiment showed a steady decline in both bacteriochlorophylls over time while the experiment with 0.313mM had a marginal increase followed by a decrease in both bacteriochlorophylls. On average the flasks in the replica had lower concentration of carotenoids at the start although they tended to increase at a higher rate than the ones from the original test, as expected from their adaptation from a low to a high light environment.

3.3. Study of the impact of light intensity on cell growth, pigment and PHA production

This test's main goal was to determine how different light intensities affect the pigment production in *C. aurantiacus* and how these changes might affect the cell growth profile. This question arises from the previous section results, where the inoculum was grown at different light conditions and presented an orange pigmentation when grown at high light and green pigmentation when grown at low light intensity. Also, it will give indications if cultures grown with differently pigmented inocula can be considered replicas. This confirmation is necessary as other authors working with *C. aurantiacus* have reported the organism expends energy trying to best adapt to its operating conditions(Oelze, 1992b), meaning changes in the medium can potentially have a negative impact on the measurable growth.

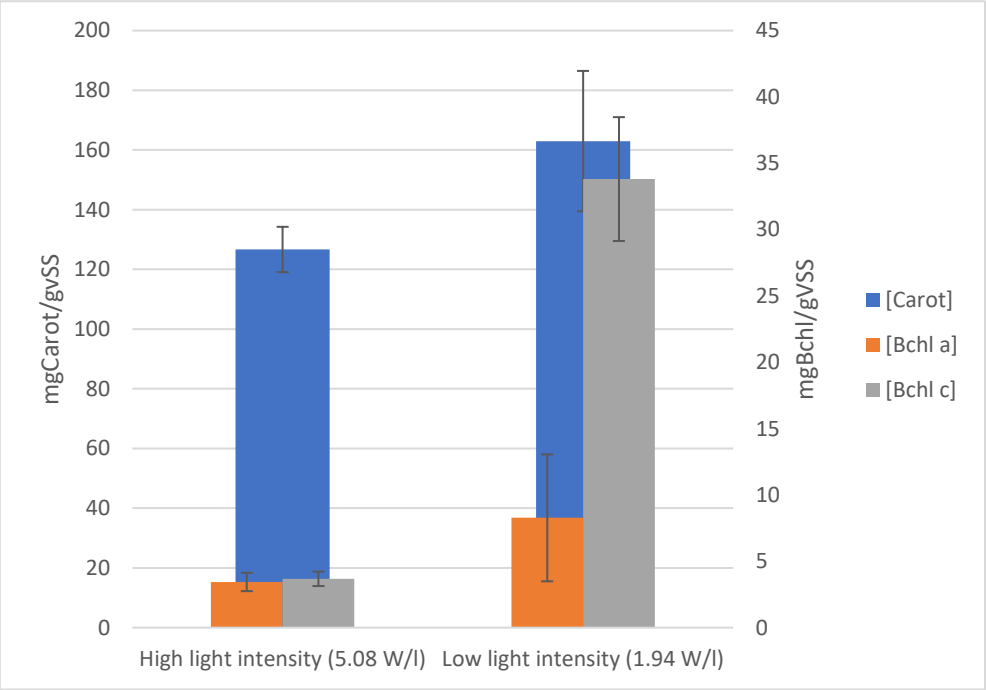

Figure 3.8: Pigment concentration in the high and low light intensity inoculum (mgPigment/g VSS). High light intensity (5.08 W/l); Light intensity (1.94 W/l) Left axis: blue: carotenoids; Right axis: orange: Bchl a; Right axis: orange: Bchl c.

In this new test all factors, except the inoculum light, were constant (M1 medium with sulphide concentration of 0.313 mM S), 2 inocula were grown at 5.08 w/l and 1.94 w/l respectively. Once they reached exponential stage each inoculum was inoculated to two 500 ml shake flaks with identical medium The low light inoculum took longer to achieve exponential stage, so its experiments were delayed by 96h.

The experiment with high light intensity saw lower concentrations in all pigments (Figure 3.8), in particular bacteriochlorophyll *c*, who registered a concentration of 37 mgBchl c/gVSS while the high light intensity experiment registered 3.7 mgBchl c/gVSS, these results match up with the literature as what is known about *C. aurantiacus*' response to low light is that it increases concentrations of chlorosomes, structures rich in bacteriochlorophyll *c* (Oelze, 1992a).

Figure 3.9: High (front) and low (rear) light inoculums 48h after inoculation

In order to measure the impact of the inocula conditions on the culture growth, from each of these flasks two 500 ml flasks (Figure 3.9) were inoculated and grown at similar conditions to their respective inoculum (M1 medium and 0.313mM of sulphide). See section 2.3.4 for further experimental details.

High and low light intensity cultures followed fairly similar growth pattern (Figure 3.10 C). The results from this test show that *C. aurantiacus* is a fast-adapting microorganism that does not incur a large impact on growth rates, with the bottles always in high light intensity having growth rates of $7.598 \pm 2.402\ d^{-1}$ and the ones from low light intensity at $5.798 \pm 0.948\ d^{-1}$, there was greater difference between the 2 experiments in each environment than between the averages of the 2 (Figure 3.9 C), with this data we conclude that the inoculum light condition does not have a measurable impact on the growth of the cultures, meaning we can take the second test of Section 3.2 as a valid replica of the first.

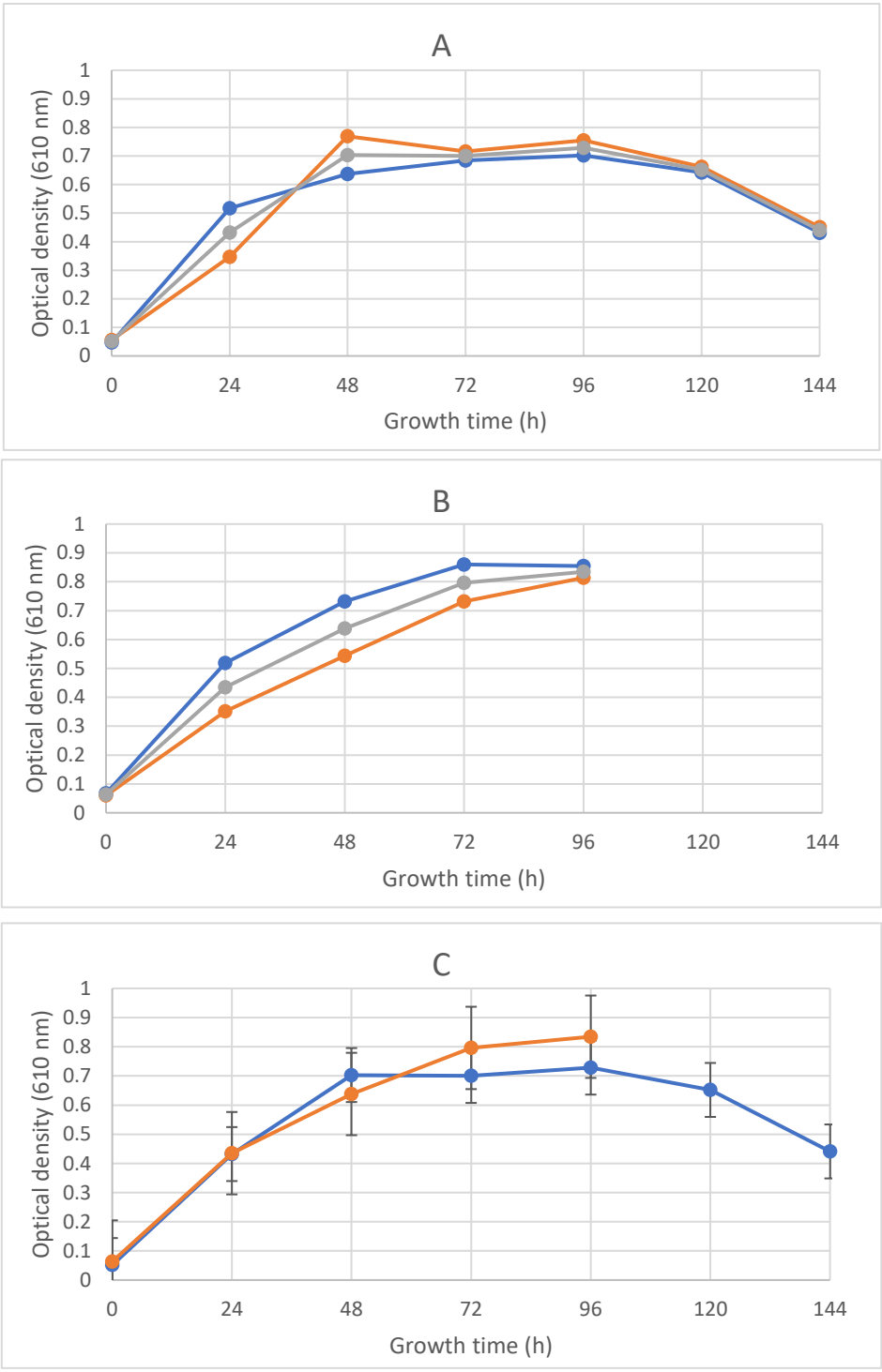

Figure 3.10: Optical density (610 nm) over growth time (h).A: orange and blue: bottles 1 and 2 at high light intensity(4.40 and 4.24 w/l), grey: average; B: orange and blue: bottles 1 and 2 at low light intensity(2.36 and 1.51 w/l), grey: average; C: averages of both tests, blue: high light intensity, orange: low light intensity.

3.3.1. Pigment contents

As expected from what is known about *C. aurantiacus'* reaction to changes in light intensity, in the test, the lower light intensity experiments had substantially more bacteriochlorophyll *c* than the ones grown in high light intensity, this happens because bacteriochlorophyll c is mostly located in discrete structures, called chlorosomes, whose number can rapidly vary without change in the total cell biomass, bacteriochlorophyll a, on the other hand is a pigment that is part of the membrane-bound light harvesting systems, as such it isn't able to change its concentration as dramatically as bacteriochlorophyll c in response to changing light intensities(Oelze, 1992a), and that fact reflects itself in our results, with the concentration of bacteriochlorophyll a being very similar in the low and high light intensity experiments, at around 10-15mgBchl a/gVSS.

The two high light intensity flaks (Figure 3.11 A, B) followed a similar pattern after the first day, carotenoids stayed in the range of 70 to 95 mg/g and there was a marginal decrease in bacteriochlorophyll. The low light intensity experiments (Figure 3.12 A, B) showed different behaviours however, as a consequence of the 1st low light intensity experiment's higher than expected light intensity, the 1st low light intensity experiment saw a roughly constant concentration of and carotenoids, while the 2nd saw increase in all pigments, most significantly bacteriochlorophyll *c* that reached over 25 mg per gram of biomass at 96h, these increases in pigments on the lower light intensity experiments fall in line with the usual *C. aurantiacus* response to lower light intensities, of increasing pigment production.

The high adaptation time of the inoculum flasks makes growing the cultures in lower light intensities not as practical as keeping the cultures always under high light intensities, even if the end results have little variance in terms of growth profile, and as such can still be useful datapoints. Due to the high carotenoid content, it might be worth investigating which types of carotenoids *C. aurantiacus* expresses in each condition in future research.

The Bchl *a* and *c* values are lower but follow the same trend as the values obtained by Oelze, 1992a at 6.1 mg/g (VSS) of Bchl *a* and 58.9 mg/g (VSS) of Bchl *c* obtained at (5 klux, ~39.5 W/m^2, 0.7 W/l) and 5.3 mg/g (VSS) of Bchl *a*, 29.4 mg/g (VSS) of Bchl *c* obtained at (25 klux, ~197.5 W/m^2, 3.7 W/l)(Oelze, 1992a)

Carotenoid values in all tests are substantially higher than those obtained by Pagels et al., 2021 in cyanobacteria, with values ranging from 3.3 to 45 mg/g (VSS), depending on the species and test conditions, although it's worth mentioning these tests were conducted with substantially lower light intensities (0.07 -0.4 W/l) (Pagels et al., 2021), meaning there isn't as much need to generate carotenoids to protect from light oxidation.

On average, the carotenoid content in the flask was 40.0 ± 91 mgCarot/l, the Bchl *a* content was 14.94 ± 3.0 mgBchl a/l and the Bchl *c* content was 0.35 ± 0.53 mgBchl c/l, these values are substantial given the volumes we're working and if they scale linearly would make *C. aurantiacus* a very promising carotenoid source.

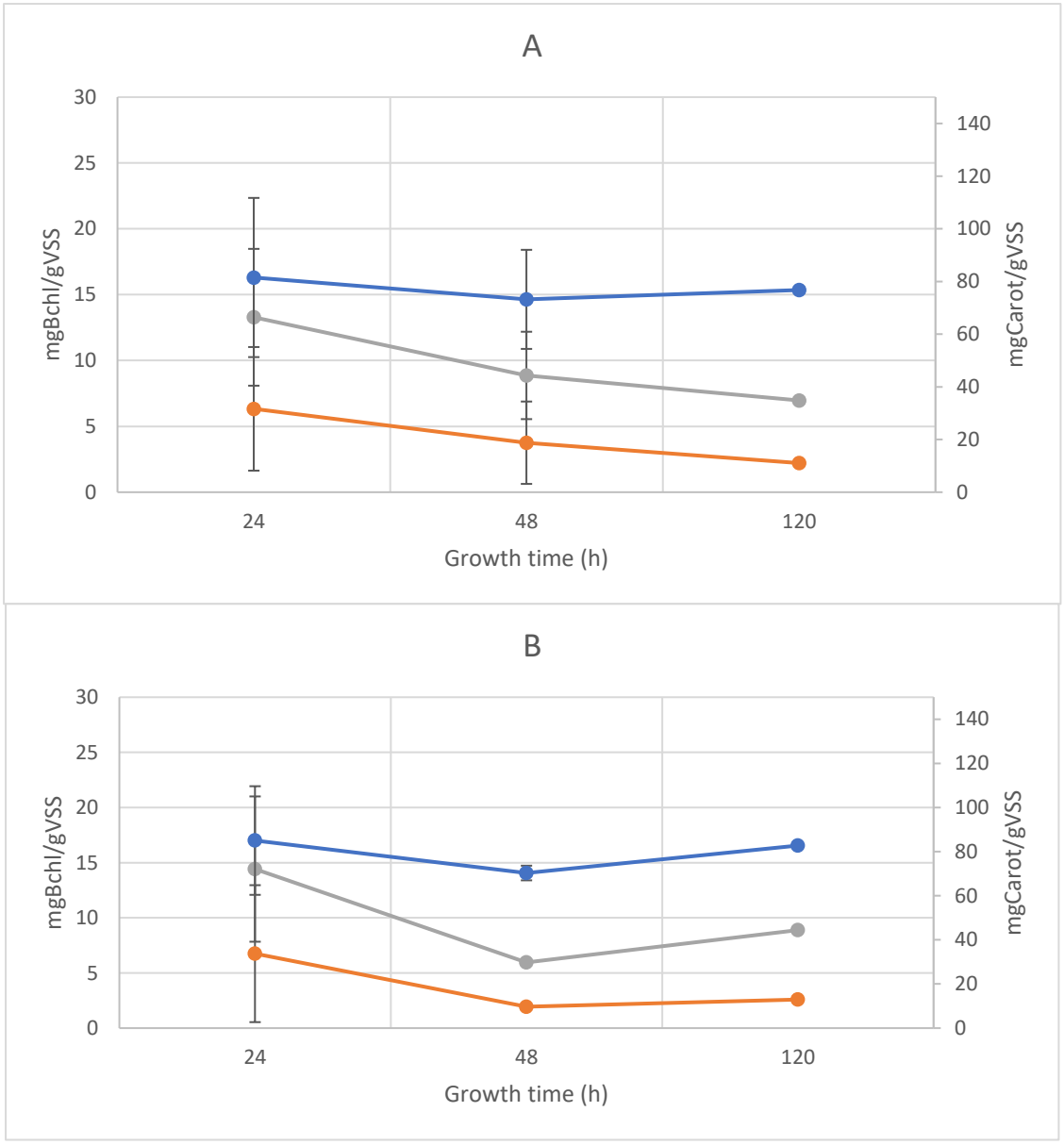

Figure 3.11: Pigment concentration (mg/g VSS) over growth time (h) in the growth flasks derived from high light intensity inocula. Right scale: blue: carotenoids; Left scale: orange: Bchl-a; Left scale: grey: Bchl-c. A: light intensity (4.40 W/l); B: light intensity (4.24 W/l) Error bars from multiple samples of the same culture.

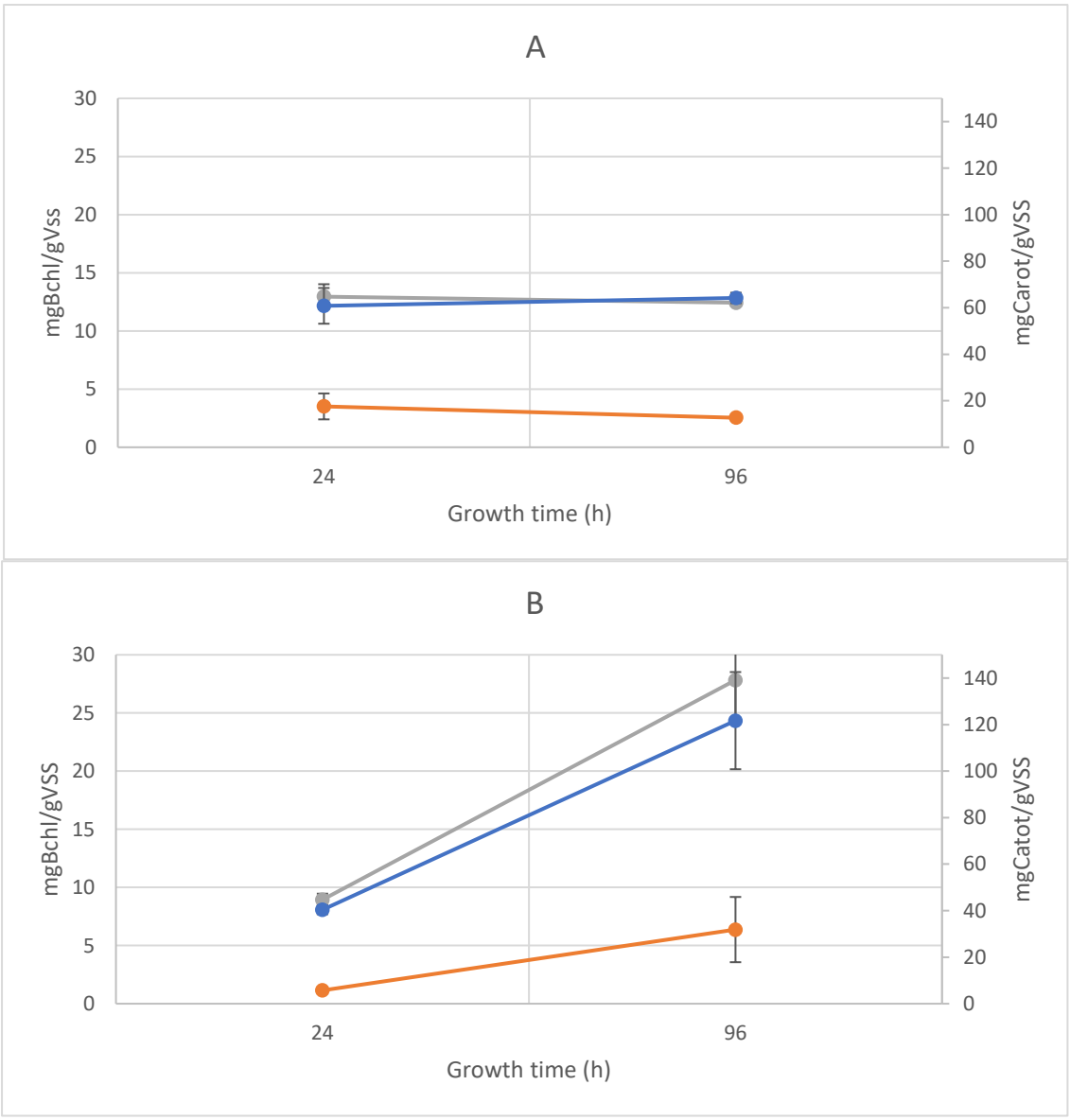

Figure 3.12: Pigment concentration (mg/g VSS) over growth time (h) in the growth flasks derived from low light intensity inocula. Right scale: blue: carotenoids; Left scale: orange: Bchl-a; Left scale: grey: Bchl-c. A: Light intensity (2.36 W/l); B: light intensity (1.51 W/l). Error bars from multiple samples of the same culture.

Since there was no conclusive answer as to why the culture achieved steady state in the previous test, further medium and biomass analysis were conducted to this samples from this culture, with the aim of finding if the limiting factor is one of the carbon sources, the nitrogen source, the phosphate source, the sulphide, or some other component of the medium.

Medium analysis showed marginal changes in ammonia and phosphate concentrations. Ammonia concentrations remained in the 41.9-80.0 mg/l range throughout the entire test and phosphate in the 14.7-22.2 mg/l range, discarding their concentrations as possible growth limiting nutrients.

pH of the medium stayed in the range of 8 to 7 in all bottles during the course of the entire test.

TOC and IC analysis shows organic carbon remained in the 33.2-58.5 mM range and inorganic carbon concentrations never reached higher than 4.2 mM.

Volatile fatty acid analysis showed all tested acids were below the 1 mM [C] threshold in all tested days.

PHA analysis, done on all experiments at the end of the test, showed only minor accumulation of polymers in the second low light intensity experiment, where 2.13% PHB/g VSS was accumulated, these results fall in line with the ones from the previous test, where the highest sulphide concentration (not used in this test) was the one with the most PHA content, and lower light inocula produced marginally higher PHA content on average.

In glycogen analysis no accumulation was detected.

Sulphide analysis (Appendix 4) showed that all flasks consumed sulphide at similar rates, with all flasks ending the experiment with under 0.035 mM S, down from the theoretical 0.313 mM S at the start. No flask ended with a concentration below 0.022 mM S, discarding the idea of this being a limiting factor.

Microscope observation during exponential stage (Figure 3.13) with Nile blue staining showed presence of fluorescent granules, which indicate accumulation of PHA or lipids, despite the low amount detected with the samples taken from steady stage.

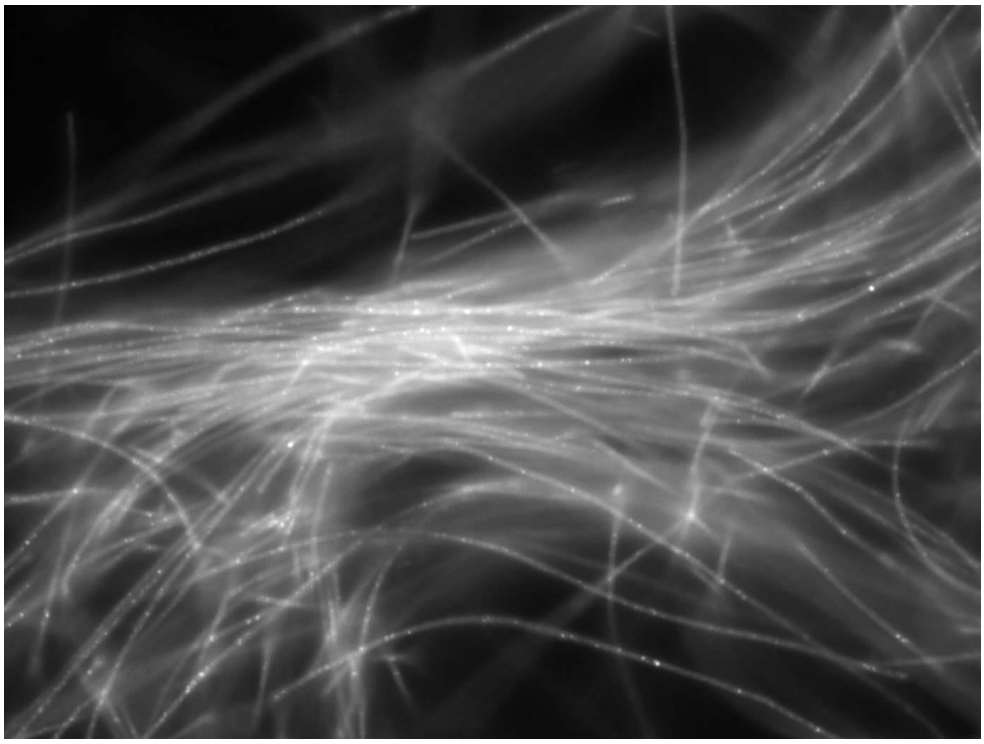

Figure 3.13: Nile blue fluorescence microscope photograph of bottle B2 (1.51 W/l) at 96h of growth (end of exponential phase/start of stationary phase).

3.4. Test of the gradual replacement of organic carbon source with inorganic carbon

With the aim of testing how *C. aurantiacus* would grow with an inorganic carbon source the following test was devised: in four shake flasks of modified inorganic M1 medium (IC medium), 2 inoculated directly from an inoculum grown in standard M1 and 2 inoculated from an inoculum prepared with the same modified inorganic M1 medium. The goal of this test was to check which inoculum type yielded better results on the actual test and to assess the overall growth profile under inorganic carbon feedstock.

The inoculum flasks prepared for the test were 100 ml serum flasks prepared according to Section 2.3.1, the inoculum flasks grown in M1 achieved steady state 5 days before the M1 IC flasks, so they were inoculated to the 500ml shake flasks earlier. Due to an error in the preparation of the replica 2 from M1 inoculum, in which double the sulphide concentration was added (0.625 mM instead of 0.313 mM), and the lack of growth in replica 1 from IC inoculum, the test was repeated to validate the results, this replica confirmed that inorganic carbon culture inoculated from cultures originating from M1 medium and IC medium had similar growth characteristics. pH once again remained steady at 8 and other factors of the test remained constant (yeast extract, nutrients, light).

The flasks inoculated with the inoculum grown in regular M1 medium achieved stationary state at roughly the same times as ones inoculated in IC medium (Figure 3.14), this indicates that *C. aurantiacus* adapted quickly to change in medium in these conditions, despite the same not happening in the inoculum.

In all tests the pH was followed and remained steady at 8 throughout.

Specific growth rates had some variance, with the highest one achieved being in one of the experiments inoculated from M1 medium and 0.313 mM of sulphide at 7.636 d^{-1}, however its replica obtained only 2.694 d^{-1}, closer to the average of the tests with M1 medium and 0.625 mM of sulphide and the test with IC inoculum, at 3.22 ± 0.96 d^{-1} and 2.48 ± 0.16 d^{-1} respectively.

This run-to-run variance is clearly visible in the average optical density graph (Figure 3.14) where the standard deviation bars commonly surpass the other lines, the initial test's M1-inoculated flasks had an advantage, but this disappears once averaged out with the replica, suggesting that neither sulphide concentration nor the inoculum composition has a consistent impact on the growth of *C. aurantiacus*.

From these results we conclude that *C. aurantiacus* can sucessfully grow with this modified medium, it's not enough to say that it can grow on inorganic carbon sources alone as the medium still contains yeast extract, but it shows it does not have a strict dependency on glycylglycine. It's hard to draw any conclusions regarding the impact of sulphide concentrations in growth in IC medium as the run to run variance puts any differences inside the standard deviation of the tests.

The lack of an impact from 0.625 mM of sulphide in the culture like what happened under M1 medium does suggest that *C. aurantiacus* might be taking more advantage of this compound in the medium than it did under M1 growth, something that would line up with the literature as it's known that *C. aurantiacus* can reduce sulphide compounds when it's assimilating inorganic carbon sources.

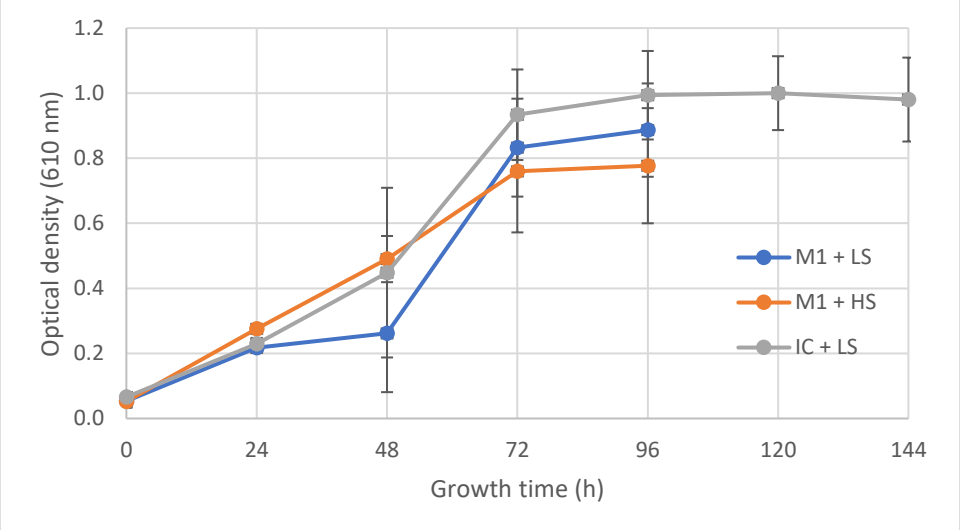

Figure 3.14: Average optical density (610 nm) over time (h). Light intensity: 1.46 W/l ± 0.087. Blue: 0.313 mM [S] + M1 inoculum; Orange: 0.625 mM [S] + M1 inoculum; Gray: 0.313 mM [S] + Modified inorganic carbon M1 inoculum. Average values from original test and replica, error bars: standard deviation.

3.4.1. Growth in inorganic carbon feedstock with higher sulphide concentrations and yeast extract limitation

On the previous test (section 3.4), the experiments with the higher concentration of sulphide (0.625 mM) appeared to maintain exponential phase for longer on average and achieve slightly higher peak densities, as such the following test was made to check if the same occurs on cultures inoculated from inorganic carbon medium.

This however had a severe impact on the growth of the culture(Figure 3.15), with cellular growth roughly halved on the best performing culture and not even achieving an OD of 0.300 in the worst

performing one, specific growth rate was of 3.75 ± 0.69 d^{-1}, higher than the 2.48 ± 0.17 d^{-1} obtained with the same conditions and half the sulphide in the previous test, this was however not sustained and the culture showed no meaningful growth path 72h (section 3.4).

Wondering if these stress conditions would be ideal for PHA accumulation, the same conditions were repeated with a nutrient limitation, in the form of cutting the yeast extract contents of the medium to 1/10 of the amount recommended for optimal growth, this resulted in an even lower cell growth, with cell density varying without a clear trend past the 3rd day and a specific growth rate of 1.31 ± 0.10 d^{-1}, substantially below the ones obtained in the other tests. The pH was followed for all experiments and remained steady at 8.

The deficient growth in the low yeast extract experiments is not fully surprising, as it only had 0.1 g/l of yeast extract while 0.3 g/l was reported as the minimum viable by Pierson & Castenholz, 1974 (Pierson & Castenholz, 1974).

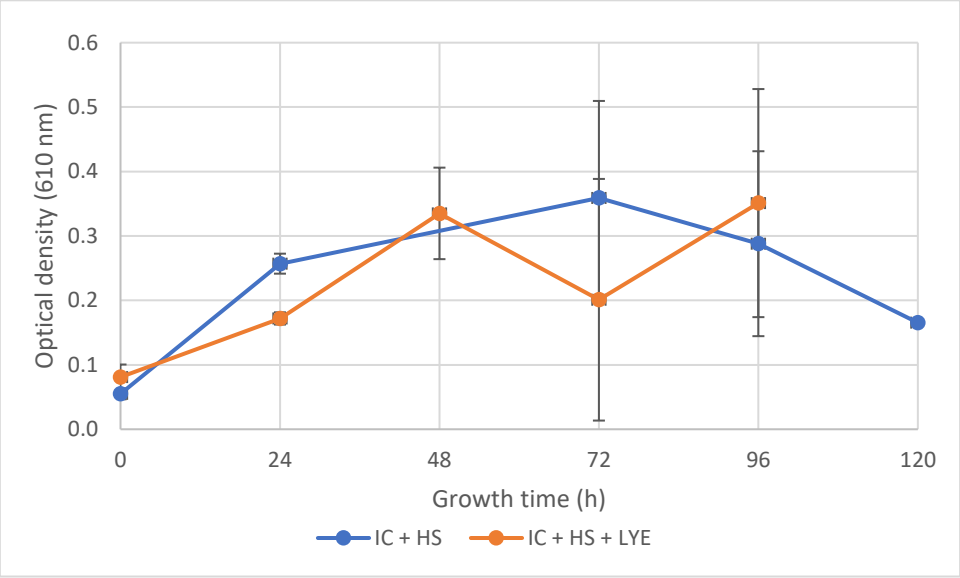

Figure 3.15: Optical density of cultures grown in inorganic carbon medium with high sulphide concentration. Light intensity: 1.46 W/l ± 0.087. Blue: Inorganic carbon modified M1 inoculum and 0.625 mM of Sulphide; Orange: Inorganic carbon modified M1 inoculum, 0.625 mM of Sulphide and yeast extract concentration reduced to 1/10.

3.4.2. Analysis conducted to the culture in inorganic carbon.

PHA samples from the original test, its replica and the low yeast extract culture were analysed and all fell under 1% suggests that ideal accumulation conditions were far from being reached, despite the low yeast extract experiment having a slightly higher concentration suggesting a shortage of nitrogen can, as predicted, stimulate accumulation, something confirmed by ammonia analysis that found no measurable quantities in the low yeast extract experiment, while the others all the other experiments (IC medium with full yeast extract) ended with 16 to 53 mg/l of ammonia, producing on average 4.47 mg l^{-1} d^{-1}.

Phosphate analysis showed all flasks had at least 5 mg/l by the end of their growth cycles, with average consumption around 1 mg l^{-1} d^{-1}.

Pigment analysis (appendix 5) showed that partially replacing the organic fraction with IC (tests with full yeast extract) resulted in no general difference in pigment production between any of the tests performed, all produced roughly equivalent amounts of pigment for their respective biomass that tended to increase only with growth time, with the maximum pigment quantities being 12.2% (Carot/VSS) of carotenoid, 2.8% (Bchl a/VSS) of bacteriochlorophyll *a* and 2.6% (Bchl c/VSS) of bacteriochlorophyll *c*. However, in the test with yeast extract limitation there was substantially lower concentration of all pigments, but since the culture didn't achieve a substantial density it's hard to gauge whether the results are due to the N limitation or simply correlated to the lack of growth.

Volatile fatty acid analysis (appendix 6) indicates that in the first test (0.313 mM of sulphide, half M1 inoculum, no yeast extract limitation) there was largely synthesis of volatile fatty acids, while on the replica the pattern was overall of consumption, with many of the inorganic carbon inoculum cultures depleting the medium of VFA's. In the high sulphide concentration + inorganic carbon inoculum tests the acetic acid was consumed totally and butyric acid was consumed partially, while the other acids were produced in large amounts. On the low yeast extract culture butyric and valeric were produced while the low amounts of the remaining acids were fully consumed, the only conclusions that can be drawn from this analysis are that medium composition and growth conditions aren't the only factors affecting volatile fatty acid synthesis and consumption.

TOC analysis (Appendix 7) clarified that little to no additional organic carbon was coming from the inoculum with organic carbon levels at the start of the culture similar between the cultures inoculated from M1 inoculum and inorganic carbon inoculum. It also showed that the low yeast extract flasks still contained about 30 mM of carbon at hour 0, of which only around 5 mM were consumed at hour 96 of the culture (Appendix 8), suggesting that it was not the direct lack of organic carbon responsible for their lower growth, but a limitation of another nutrient, like nitrogen, a hypothesis corroborated by this test's complete lack of detectable ammonia in the medium.

3.5. Using monomeric glycine as a substitute for glycylglycine

Chloroflexus reference medium, M1, contains a very costly component, glycylglycine, this cost limits any kind of large scale tests under organic conditions and makes it completely inviable for any applications outside lab scale.

Glycylglycine is a dimeric form of the basic amino acid, glycine, and other researchers have been able to use monomeric glycine as an organic feedstock for *C. aurantiacus* (He et al., 2015).

In theory, monomeric glycine provides an adequate substitute for its far costlier dimeric form. It is speculated, albeit unproven, that glycylglycine, like many other polymers, breaks down into glycine upon autoclaving, a theory we intended to test but were unable to due to lack of working test equipment for the necessary analysis. It's also possible that that glycylglycine is broken down to glycine as it is being assimilated by the organism, as is the case with most other polymers used as carbon and energy source (e.g., PHA).

For this test, six 250 ml shake flasks (1.219 W/l ± 0.166, 1.25 mM [S]), 3 with regular M1 with glycylglycine and 3 with modified M1 using monomeric glycine in the place of glycylglycine, with the same carbon concentration, were inoculated from identical glycylglycine-based inoculum cultures. Glycylglycine cultures achieved a higher cellular density at 48h but were quickly matched the next day, with at 96h cultures showing similar cellular densities.

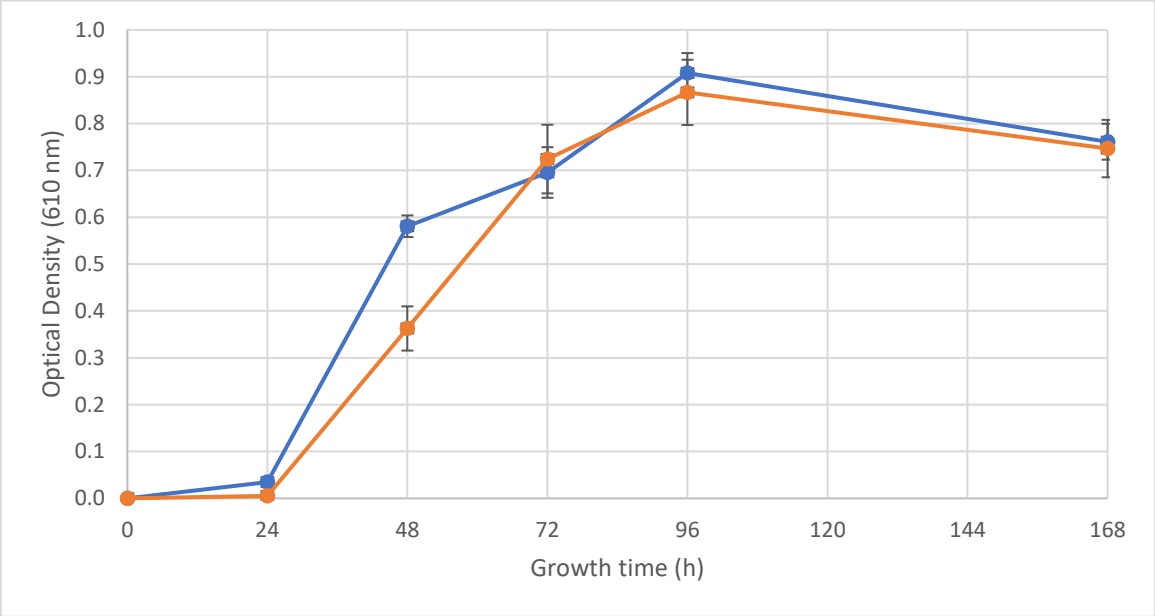

Figure 3.16: Optical density (610 nm) over time (h) of cultures grown with different organic carbon feedstocks: Blue: glycine; Orange: glycylglycine. Error bars derive from 3 replicas.

These cultures were grown with a sodium sulphide solution, that, upon later analysis, contained very little sulphide left, with inoculation day values regularly below 0.2 mM. As such this test is considered to have been conducted under trace sulphide levels. The growth profiles (Figure 3.16) don't follow a similar trend to the ones conducted under higher sulphide concentrations, such as in section 3.2. For comparison the specific growth rate for this test was 18.8 ± 3.8 d^{-1}, while the other tests with higher sulphide recorded values in the range of 3-10 d^{-1}, this, however, can also be down to multiple other factors, like the lower size of these bottles (lower total light absorbing surface), the lower starting concentration (0.009 mg/ml vs 0.04 mg/ml), and a different way to measure optical density (the supernatant value wasn't subtracted, instead the absorbance of a blank medium was).

The cultures of this test entered steady state sometime between 96 and 168h, the latter point being when the experiment was ended. At the end of the test the culture medium centrifuged for analysis and there was a noticeable difference in the coloration of the medium (Figure 3.17), with the glycine flasks showing an orange tint while the glycylglycine flasks were more yellow. This difference could be related to a difference in excreted metabolites produced during the metabolization of the carbon sources, or it could point to a different composition of pigments induced by a difference in carbon source, it is known that medium composition can affect the synthesis of pigments in *C. aurantiacus*(Oelze, 1992b), but no study has yet shown glycine to be one of the affecting compounds.

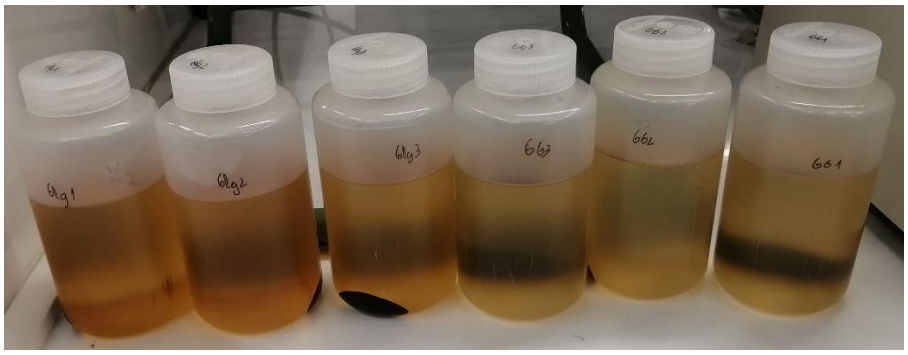

Figure 3.17: Centrifuged culture medium at 168h of growth showing the biomass pellet in the bottom, and the colouration of the medium caused by the release of pigments into the medium.

This experiment shows that glycine can be used to grow *C. aurantiacus* as an organic carbon source, just as effectively as glycylglycine, at least when under trace sulphide amounts.

Ammonia analysis content in all samples, with all finishing within the range of 12 to 15 mg/l. While phosphorous remained fairly stable with it between 2.3 and 3.4 mg/l in all flasks.

Volatile fatty analysis revealed that cultures grown in glycylglycine had levels of butyric acid throughout that were around 8 times higher (~40 ppm vs ~5 ppm) than those grown in glycine and all cultures showed a trend of consuming propionic acid at similar rates.

Glycogen analysis (Figure 3.18) showed that cultures fed with glycylglycine, accumulated substantially more glycogen at 72h (6.6%$\pm$2.1 vs 2.2%$\pm$0.6), although the results equalized at 168h, suggesting that even if glycylglycine might be a promoter of glycogen accumulation during the exponential stage, the results equalize once some growth limiting factor is in place, such as at stationary stage.

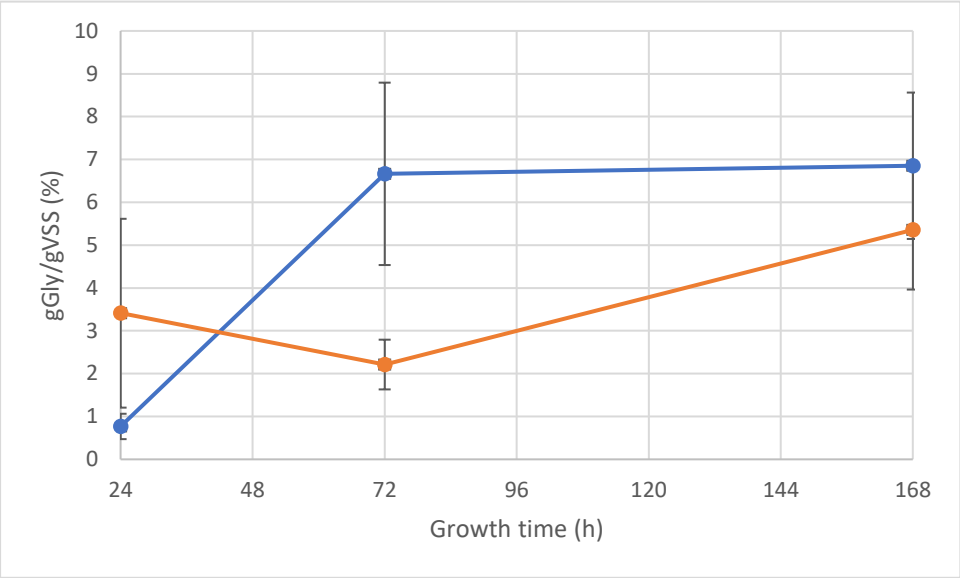

Figure 3.18: Glycogen content in % of volatile suspended solids over growth time (h). Blue: glycylglycine, orange: glycine. Error bars derived from 3 replicas each.

4. Conclusion and Future Work

C. aurantiacus Y-400-fl (DSM 637) proved a challenging species to work with. It demanded simultaneous sterility and anaerobic operating conditions which often required a redesign of operating methods that are usually used for aerobic species. It was also a new research line that was being initiated at the host research group and therefore the cultures' behaviour was novel and, at times, difficult to discern a pattern.

By testing the reaction of *C. aurantiacus* Y-400-fl (DSM 367) to different sulphide concentrations we determined that 0.313 mM of sulphide is the ideal concentration to wake cryovials up and also a good concentration to achieve a high growth rate under organic carbon medium (glycylglycine) (Table 3.1).

In the test of different light condition inocula (Table 3.2) we demonstrated the fast adaptation of *C. aurantiacus* Y-400-fl (DSM 367) to changing medium conditions and how it maintains much of its growth rate regardless of whether it originates from an inoculum grown under high or low light conditions.

By replacing one of the organic carbon sources (glycylglycine) with an inorganic carbon substitute (Table 3.3), we verified that *C. aurantiacus* Y-400-fl (DSM 367) is capable of incorporating inorganic carbon to sustain its growth.

We were not able to induce an accumulation scenario in *C. aurantiacus* Y-400-fl (DSM 637) by limiting the amount of yeast extract in the inorganic carbon based medium, as the cells struggled to grow in this nutrient deficient medium.

Replacing glycylglycine with glycine in the *Chloroflexus* reference medium showed that *C. aurantiacus* Y-400-fl (DSM 637) is capable of using relatively inexpensive substrates to grow organically, allowing for future studies to grow the organism in organic carbon conditions without the restrictions on scale posed by using an expensive reagent in the medium.

Some promising results were obtained for PHA production using M1 medium (0.58 W/l inoculum light, 2.30W/l culture light, 0.625 mM [S], 175rpm, 50°C), a 15% PHA content without any attempts at providing accumulation conditions. However, the same was not observed when using inorganic carbon as feedstock, with the best PHA accumulation being 0.6% of the cell dry weight in polymer.

It's also important to keep testing how *C. aurantiacus* will react under different conditions with inorganic feedstocks, in particular how far the yeast extract concentration can be brought down while still obtaining a normally growing culture, and also the possibility of replacing the yeast extract with the exact micronutrients *C. aurantiacus* needs to grow, as those are easier to handle. Establishing clear PHA accumulating conditions for *C. aurantiacus* and eventually building a lab scale reactor that will exert these conditions on the cultures and allow for repeated test cycles seems like a step to take after the growth conditions under inorganic feedstocks and glycine are better characterized.

It's also worth looking into the possibility of using *C. aurantiacus* Y-400-fl (DSM 637) for production of bacteriochlorophyll *c*, as it was shown that it is an excellent producer of the pigment when kept under low light conditions. Unlike chlorophylls used by oxygenic photosynthesis organisms, bacteriochlorophyll *c* is quite a rare pigment that isn't readily available, despite having tempting characteristics like absorption in near-infrared wavelengths, for applications such as nontoxic long wavelength inks for medical applications, light capturing devices or potentially even used in molecular semiconductors(Buscemi et al., 2022).

As a closing thought, this thesis' results demonstrated that *C. aurantiacus* Y-400-fl (DSM 637) is an organism with potential for many useful projects, and, even if the main objective of accumulating large quantities of PHA under inorganic feedstock conditions did not prove successful, the bacteria showed a lot of potential in other areas, such as a pigment-producing organism and in adapting to high sulphide environments.

Nevertheless, the possibility of achieving higher PHA production if more adequate medium conditions are met, such as the correct nutrient limitations that still allow the organism to grow and multiply but induce an accumulation state.

[S]	HB (gHB/gVSS)	HV (gHV/gVSS)	Carotenoids (mgCarot/gVSS)	Bchl A (mgBchl a/gVSS)	Bchl C (mgBchl c/gVSS)
0.625	1.69%	2.19%	180.00	2.76	12.50
	5.79%	8.07%	68.81	0.64	1.61
0.313	0.73%	0.05%	147.11	3.54	15.41
	1.72%	1.50%	93.21	1.77	5.39
0.156	0.61%	1.35%	165.16	4.32	7.20
	1.04%	1.72%	107.18	3.06	8.87
0.078	0.08%	0.29%	81.57	2.70	6.11
	0.79%	1.23%	106.17	2.00	4.48

Table 4.1: Summary of the pigment and PHA concentrations in tests with varying sulphide concentrations, samples taken at the end of exponential stage, polymers below 1% omitted.

Light	HB (gHB/gVSS)	Carotenoids (mgCarot/gVSS)	Bchl a (mgBchl a/gVSS)	Bchl c (mgBchl c/gVSS)
High light	0.04%	76.77	2.21	4.76
	0.14%	82.80	2.59	6.30
Low light	0.00%	64.20	2.54	9.89
	2.14%	121.69	6.37	21.45

Table 4.2: Summary of the pigment and PHA concentration in tests with varying light intensity inoculum, samples taken at the end of exponential stage, polymers below 1% omitted.

Condition	Carotenoids (mgCarot/gVSS)	Bchl a (mgBchl a/gVSS)	Bchl c (mgBchl c/gVSS)
0.313 mM [S] (M1 inoculum)	113.74	2.12	6.94
	80.56	1.89	10.44
0.625 mM [S] (M1 inoculum)	74.85	0.99	2.56
	70.88	1.99	8.37
0.313 mM [S] (IC inoculum)	96.93	4.43	11.06
	59.27	2.15	7.49
	50.86	1.37	5.80
0.625 mM [S] (IC inoculum)	122.78	20.77	20.27
	22.02	0.12	1.33
0.625 mM [S] + LYE (IC Inoculum)	3.54	0.06	0.30
	71.65	0.72	3.27

Table 4.3: Summary of the pigment concentration in tests grown in inorganic carbon medium, samples taken at the end of exponential stage.

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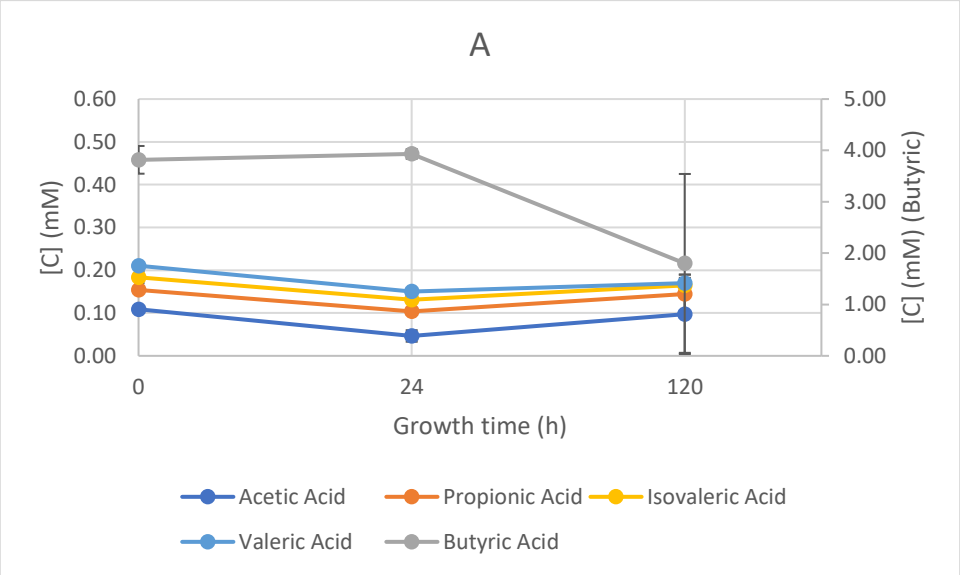
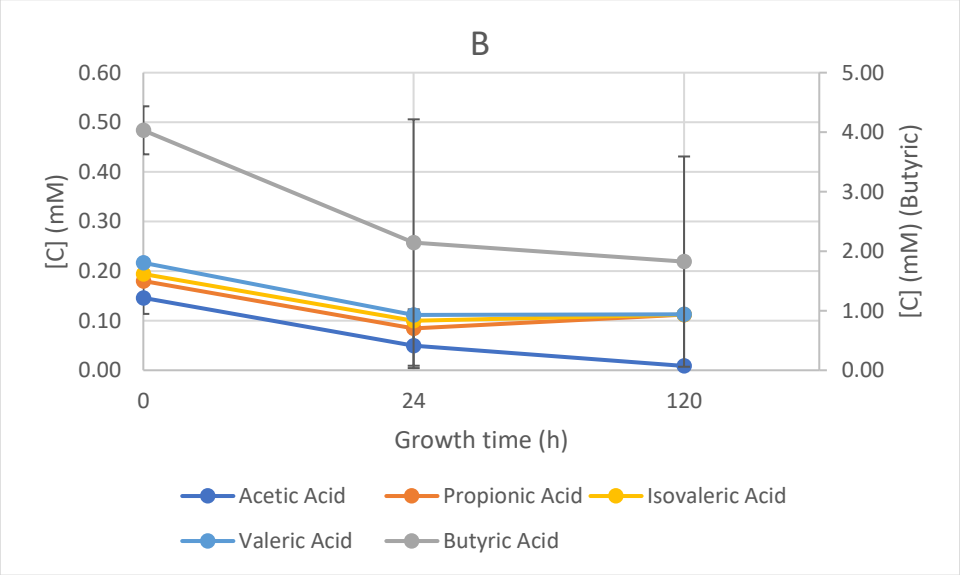
Appendices

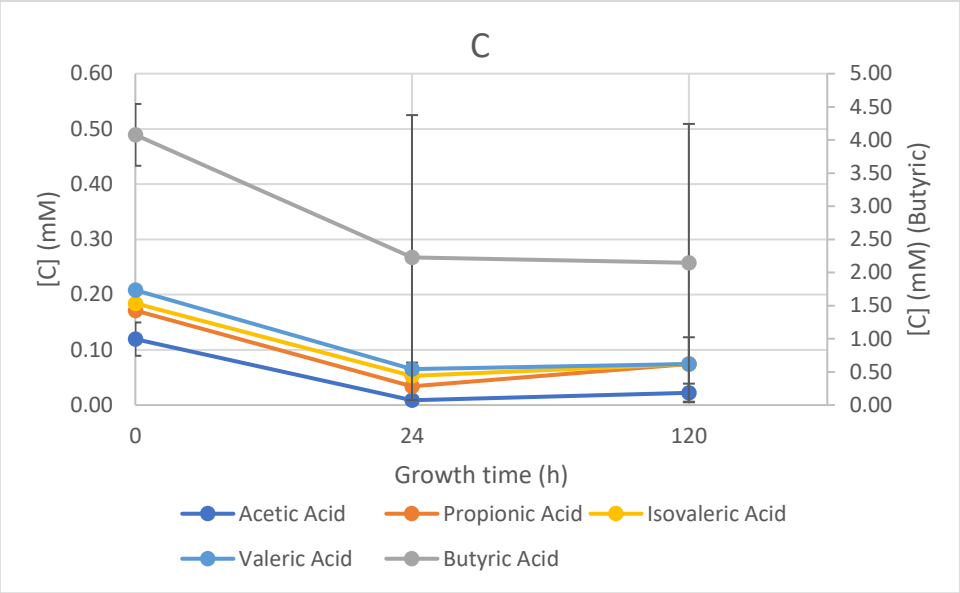
1. Composition of trace SL-6 medium

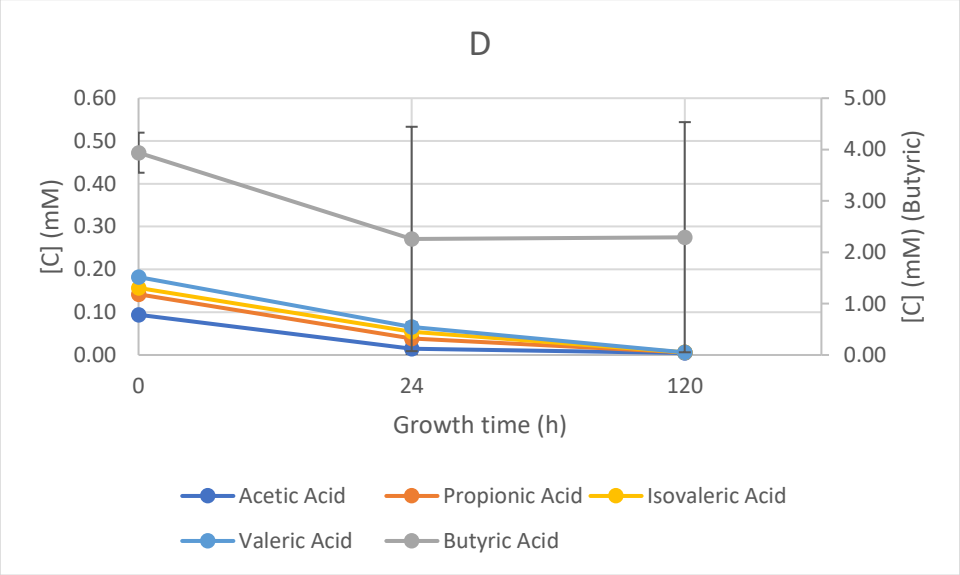
Compound	Mass/volume	unit
$ZnSO_4.7H_2O$	0.10	g
$MnCl_2.4H_2O$	0.03	g
H_3BO_3	0.30	g
$CoCl_2.6H_2O$	0.20	g
$CuCl_2.2H_2O$	0.01	g
$NiCl_2.6H_2O$	0.02	g
$Na_2MoO_4.2H_2O$	0.03	g
Distilled water	1000.00	ml

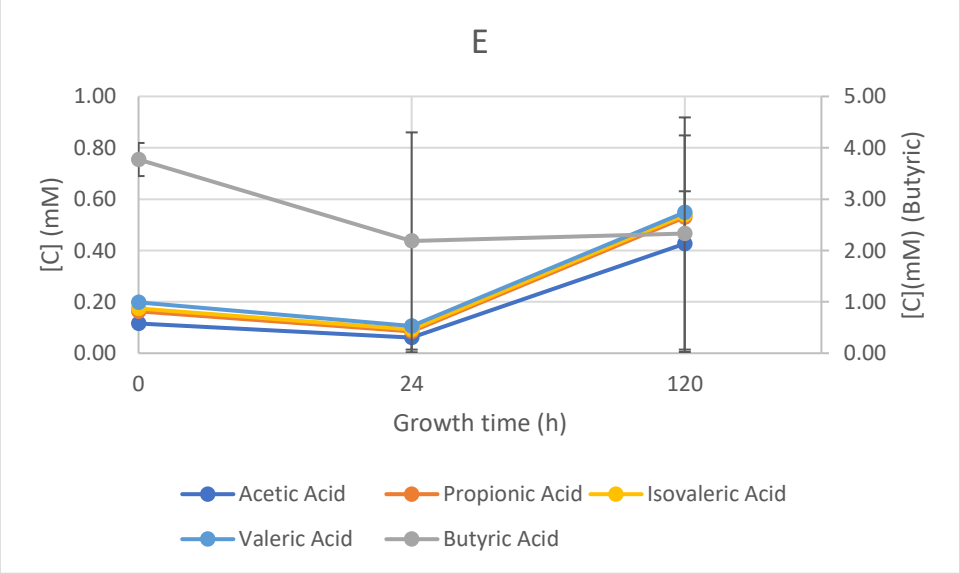
2. Volatile fatty acid content over time of tests with different sulphide. average mM [C] over time with std deviation error bars. A: 0.625 mM S; B: 0.313 mM S, C: 0.156 mM S; D: 0.078 mM S; E 0.625 mM S Abiotic. Right scale: butyric acid (grey); Left scale: acetic acid (dark blue), propionic acid (orange), Isovaleric acid (yellow) and valeric acid (light blue).



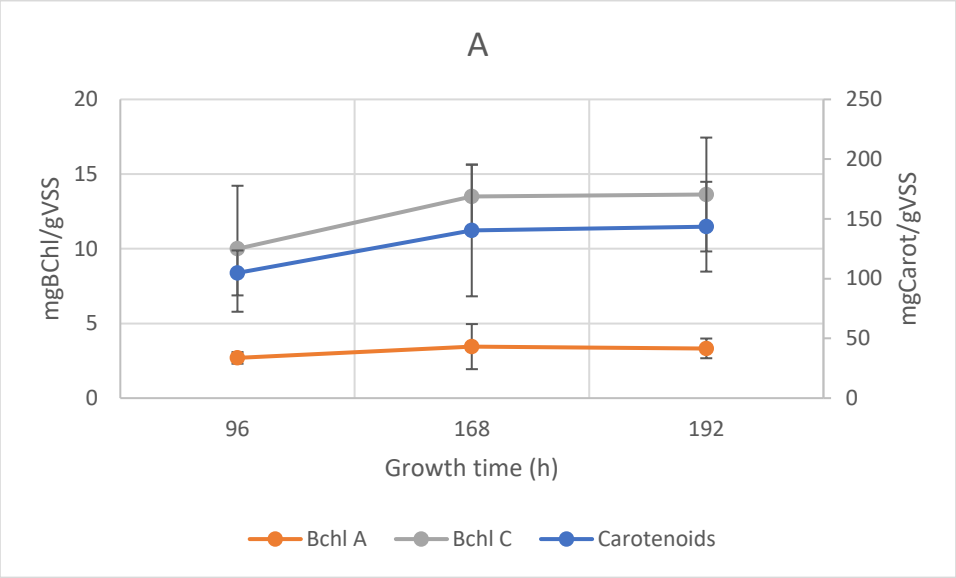


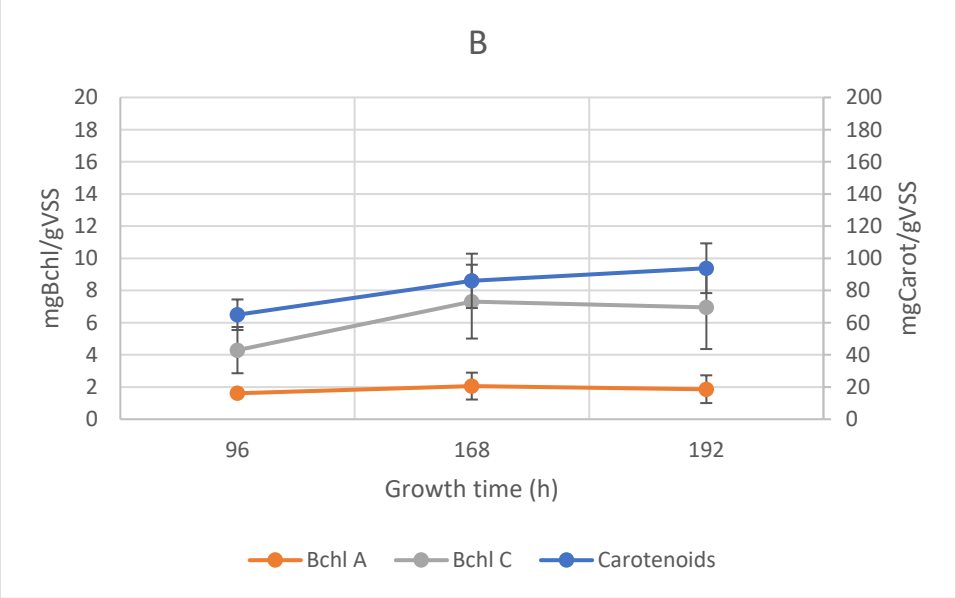

C
0.60
0.50
0.40
0.30
0.20
0.10
0.00
[C] (mM)
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
[C] (mM) (Butyric)
0
24
120
Growth time (h)
Acetic Acid
Propionic Acid
Isovaleric Acid
Valeric Acid
Butyric Acid


D
0.60
0.50
0.40
0.30
0.20
0.10
0.00
[C] (mM)
5.00
4.00
3.00
2.00
1.00
0.00
[C] (mM) (Butyric)
0
24
120
Growth time (h)
Acetic Acid
Propionic Acid
Isovaleric Acid
Valeric Acid
Butyric Acid

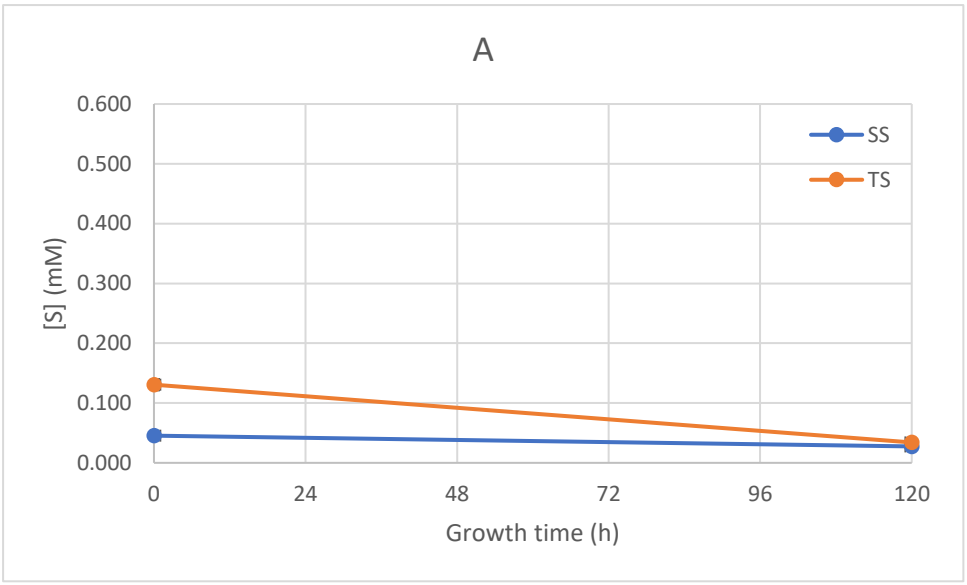

E
1.00
0.80
0.60
0.40
0.20
0.00
[C] (mM)
5.00
4.00
3.00
2.00
1.00
0.00
[C](mM) (Butyric)
0
24
120
Growth time (h)
Acetic Acid
Propionic Acid
Isovaleric Acid
Valeric Acid
Butyric Acid

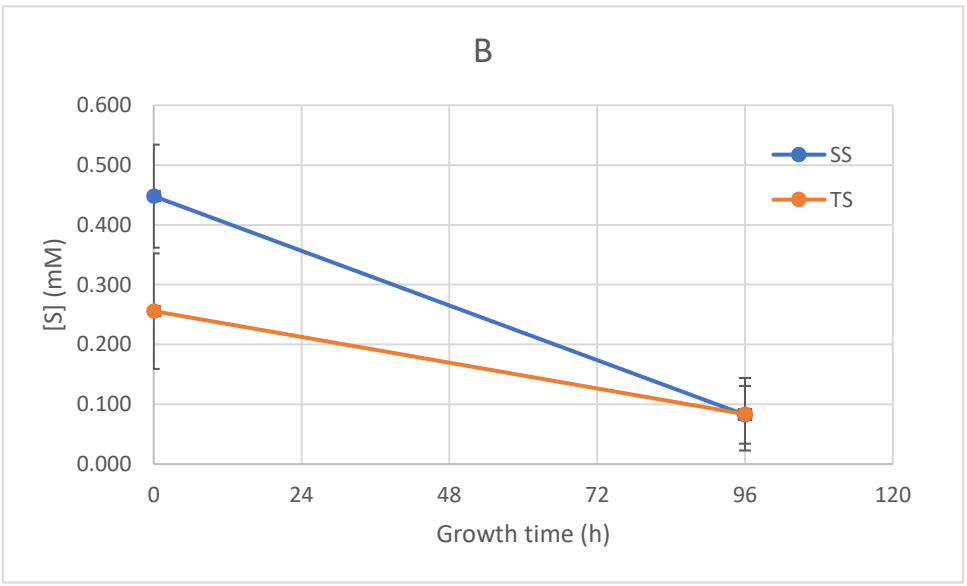
3. Pigment content (mg pigment/ g VSS) in growths with different concentrations of sulphide. A: High light intensity inoculum/first test of Figure 3.3; B: low light intensity inoculum/replica of Figure 3.4. Blue: carotenoids (right axis); Orange: Bchl *a* (left axis); Gray: Bchl *c* (left axis). Values are averages of all different sulphide concentration flasks as no meaningful trend was discovered between them and error bars represent the standard deviation.



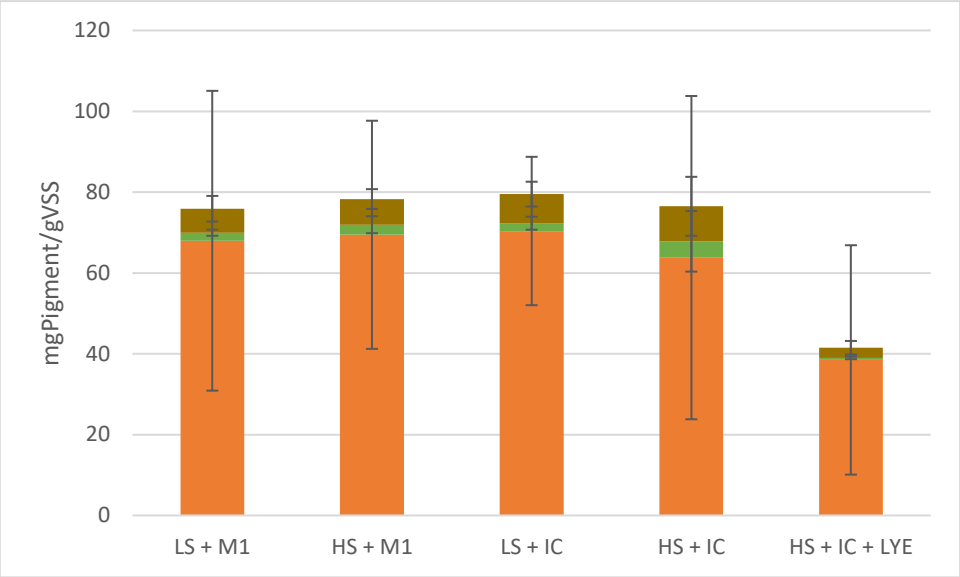


4. Concentration of sulphide in tests with varying light intensities. mM [S] over time. Blue: soluble sulphide, orange: total sulphide. A: high light intensity, B: low light intensity.





5. Pigment concentrations (mg pigment/ g VSS) in the tests with inorganic carbon feedstock. Orange: carotenoids; green: Bchl *a*; brown: Bchl *c*. LS + M1: 0.313 mM of sulphide + M1 inoculum; HS + M1: 0.625 mM of sulphide + M1 inoculum; LS + IC: 0.313 mM of sulphide + IC inoculum; HS + IC: 0.625 mM of sulphide + IC inoculum; HS + IC + LYE: 0.625 mM + IC inoculum + 1/10 typical yeast extract.



6. Average rate of change of volatile fatty acid concentration (mmol C d^{-1}) of the growths with inorganic carbon medium. M1 test 1: 1st test, M1 inoculum; IC test 1: 1st test, IC inoculum; M2 test 2: 2nd test, M1 inoculum; IC test 2: 2nd test, IC inoculum; IC + HS; IC inoculum, high sulphide test; IC + HS + LYE: IC inoculum, high sulphide and 1/10 usual level of yeast test. Averages

	M1 Test 1	Std dev	IC Test 1	Std dev	M1 Test 2	Std dev
Acetic	0.0016	0.0164	0.1154	0.0775	-0.0132	0.0046
Propionic	0.0214	0.0302	0.0545	0.0547	-0.0266	0.0116
Butyric	0.1386	0.1954	0.0927	0.0177	0.0141	0.0835
Isovaleric	0.0006	0.0007	0.0273	0.0146	0.0038	0.0151
Valeric	0.0023	0.0033	0.0040	0.0024	-0.0085	0.0083

	IC Test 2	Std dev	IC + HS	Std dev	IC + HS + LYE	Std dev
Acetic	-0.0187	0.0187	-0.0767	0.1018	-3.1344	2.9934
Propionic	-0.0412	0.0534	0.1434	0.1764	-0.1040	0.1040
Butyric	-0.0179	0.0370	-0.0317	0.0249	0.1488	0.1505
Isovaleric	-0.0022	0.0023	0.0507	0.0439	-0.0310	0.0262
Valeric	-0.0191	0.0474	0.0976	0.0761	0.0000	0.0000

7. Total organic carbon, total carbon, and inorganic carbon average starting concentrations, mmol/L, averages of all tests by carbon staring conditions.

	TOC	stdev	TC	stdev	IC	stdev
IC (M1 inoculum)	36.00	9.39	50.95	1.25	14.95	9.78
IC (IC inoculum)	31.45	6.02	51.55	0.86	20.09	5.27
IC +LYE (IC inoculum)	30.33	2.27	30.83	2.25	0.49	0.02

8. Total organic carbon, total carbon, and inorganic carbon difference in medium at 0h and end of exponential stage, mmol/L, averages of all tests with similar conditions

	TOC	Std dev	TC	Std dev	IC	Std dev
0.313 (M1 inoculum)	-29.66	12.85	-16.31	0.79	13.34	12.07
0.625 (M1 inoculum)	-21.04	6.14	-12.51	0.20	8.54	6.33
0.313 (IC inoculum)	-11.74	5.27	-10.16	6.21	0.47	0.67
0.625 (IC inoculum)	-10.39	1.51	-7.29	1.88	3.11	0.36
0.625 LYE (IC inoculum)	-5.17	0.98	-5.44	0.78	-0.27	0.21