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Assessment of Nitrogen Sources for *Xanthobacter sp.* SoF1

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Abstract

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This study investigated different nitrogen sources for the cultivation of Xanthobacter sp. SoF1, which grows autotrophically with CO2 as the carbon source and hydrogen gas as the energy source under microaerobic conditions. The theory section compared ammonium hydroxide, nitrogen gas, urea, and nitrate to identify a safe, economical, and environmentally friendly nitrogen source alternative to ammonium hydroxide in the production of Solein® protein. On the basis of these comparisons, urea was selected as the nitrogen source for the experimental phase.

Three cultivations of Xanthobacter sp. SoF1 were conducted using a 10 L bioreactor. The first cultivation was a fed-batch process with 24.5 m-% ammonium hydroxide, providing reference data for the following experiments. The second cultivation was a batch process, in which urea was added to the reactor mixed with the cultivation medium. The third cultivation was a fed-batch process with urea. Microbial growth was monitored by measuring optical density, total dry mass and cell dry weight from bioreactor samples along with online measurements. Ammonium concentration was followed using rapid assays and external laboratory analysis.

The batch cultivation with urea was unsuccessful; therefore, urea was decided to be applied in a fed-batch cultivation. The fed-batch cultivation with urea showed a slight improvement compared to the reference cultivation with ammonium hydroxide, achieving the highest specific growth rate (0.037 h-1) and cell dry weight (21.9 g/L). The results indicate that urea is a potential alternative to ammonia for Solein® protein production. However, further research is required to confirm the results and assess the safety of the final product. This thesis was commissioned by Solar Foods Oyj as part of Hydrocow project funded by the European Innovation Counsil (EIC).

Keywords: *Xanthobacter sp*. SoF1, Solein, nitrogen source, urea, bioreactor cultivation

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Työssä tarkasteltiin Xanthobacter sp. SoF1 -bakteerin kasvua eri typenlähteillä, tavoitteena löytää turvallinen, taloudellinen ja ympäristöystävällinen vaihtoehto ammoniumhydroksidille Solein®-proteiinin tuotannossa. Kyseinen mikrobi kasvaa autotrofisesti käyttäen hiilidioksidia hiilenlähteenä ja vetykaasua energialähteenä mikroaerobisissa olosuhteissa. Teoriaosuudessa vertailtiin ammoniumhydroksidia, typpikaasua, ureaa sekä nitraattia ja näiden vertailujen perusteella urea valittiin kokeelliseen osioon typenlähteeksi.

Kokeellisessa osassa suoritettiin kolme kasvatusta 10 litran bioreaktorilla. Ensimmäinen kasvatus oli panossyöttöprosessi, jossa typenlähteenä käytettiin 24,5 m-% ammoniumhydroksidia. Tästä kasvatuksesta saatiin vertailuarvot seuraaviin kasvatuksiin. Toinen kasvatus oli panosprosessi, jossa urea lisättiin kerralla reaktoriin kasvatusalustan mukana. Kolmas kasvatus oli panossyöttöprosessi, jossa ureaa syötettiin reaktoriin manuaalisesti kasvatuksen aikana. Mikrobin kasvua seurattiin reaktorinäytteistä mitattavan optisen tiheyden (OD_{600}), kokonaiskuivamassan ja solukuivapainon avulla sekä online-mittauksilla. Ammoniumin määrää mitattiin pikatestillä sekä analyysillä ulkoisessa laboratoriossa.

Panoskasvatus urealla epäonnistui, ja tulosten perusteella päätettiin kokeilla panossyöttökasvatusta urealla. Panossyöttökasvatuksessa urealla saavutettiin paras spesifinen kasvunopeus (0,037 h^{-1}) ja korkein solukuivapaino (21,9 g/l).

Tulokset osoittavat, että urea on potentiaalinen vaihtoehto ammoniumhydroksidille Solein®-proteiinin tuotannossa, mutta lisätutkimuksia tarvitaan tulosten varmistamiseksi ja lopputuotteen turvallisuuden arvioimiseksi. Tämän opinnäytetyön toimeksiantajana toimi Solar Foods Oyj, ja työ oli osa Europan innovaationeuvoston (EIC) rahoittamaa Hydrocow-projektia.

Avainsanat: *Xanthobacter sp*. SoF1, Solein, typenlähde, urea, bioreaktorikasvatus

Contents

List of Abbreviations

CDW: *Cell dry weight.* The dry mass of cells per volume. Measured after removing all moisture, used as an indicator of microbial biomass.

DM: *Total dry matter content*. This analysis reveals the presence of all dry matter in the sample, including cells, minerals, and other components.

DO: *Dissolved oxygen*. The term refers to oxygen dissolved in the liquid phase measured using a probe inside a bioreactor.

HOB: *Hydrogen-oxidizing bacteria*. A group of bacteria that utilize hydrogen as an energy source in the presence of oxygen.

OD: *Optical density*. A measure of how much light is absorbed by a sample at a specific wavelength. It is often used to estimate cell concentration in microbial cultures.

PCA: *Plate Count Agar.* A microbiological growth medium used to estimate the number of viable bacteria in a sample.

PHB: *Polyhydroxybutyrate*. A type of biopolymer produced by certain bacteria as energy and carbon storage.

RO: *Reverse osmosis*. A water purification process.

SCP: *Single cell protein.* Protein derived from unicellular microorganisms.

SIP: *Sterilize in place*. A method used to sterilize bioreactors and other equipment without disassembly.

1 Introduction

The challenges of climate change and a growing global population demand innovative solutions to support traditional food production. In the future, arable land will not suffice to meet humanity's requirements, and here, cellular agriculture plays a significant role. For instance, transitioning from traditional beef and dairy production to microbial protein sources could save approximately between 15.9 m² and 35.6 m² of land per 100 grams of protein in Europe. [1.]

Products based on microbial protein already exist as food items. One of the most well-known meat alternatives, Quorn®, derived from *Fusarium venenatum,* was launched in 1985. [2.] Bioalbumen®, an ovalbumin product that mimics egg white, is produced using *Trichoderma reesei* as the host organism [3]. Pekilo®, originally produced from *Paecilomyces variotii* for feed use, is expected to be commercialised for human consumption by 2026 [4]. Solein®, a protein rich powder produced from *Xanthobacter sp.* SoF1 by Solar Foods, was launched in Singapore in 2023 and in the U.S. in 2024 [5].

Solar Foods is a Finnish biotechnology and food company that produces unique microbial-based single-cell protein (SCP) as a raw material for the food industry. The company aims to reduce the negative climate and environmental impacts of the food industry, with sustainability being a core part of its operations. Solar Foods' primary goal is to separate food production from traditional agriculture. [6.] This thesis has been conducted in collaboration with Solar Foods.

Currently, the *Xanthobacter sp*. SoF1 microbe used in Solein® protein production obtains its nitrogen source from aqueous solution of ammonium hydroxide, NH_4OH. The objective of this thesis was to find a safe, economical, and environmentally friendly alternative to ammonium hydroxide. Industrial ammonia is produced via the Haber-Bosch process, which causes significant environmental emissions, as the hydrogen used in the process is derived from fossil raw materials [7]. Additionally, ammonia is harmful to the environment,

animals, and humans, and its liquid or gaseous form is difficult to transport compared to solid nitrate or urea [8].

The aim of this thesis was to compare alternative nitrogen sources from the perspectives of cost, availability, and usability. In the experimental part, the key criterion was the growth rate of the microbe. Nitrogen gas, urea and potassium nitrate, alongside ammonium hydroxide, were selected as nitrogen sources for comparison in the theoretical section. If an alternative nitrogen source worked better in the experiments than ammonium hydroxide, it could be adopted in production.

This thesis was commissioned by Solar Foods and conducted as a part of the EU-project Hydrocow (number 101115118), funded by the European Innovation Council (EIC) under the Pathfinder Challenge: Carbon Dioxide and Nitrogen Management and Valorisation. Hydrocow project aims to produce milk protein beta-lactoglobulin using the *Xanthobacter sp.* SoF1 as a host and ammonium hydroxide is used as the nitrogen source in the project. The results of this thesis will be integrated into Hydrocow-project's reporting.

Solar Foods gained international recognition by winning the international category of NASA's Deep Space Food Challenge in 2024 [9]. The results achieved in this thesis could potentially be utilized in the future production of Solein® protein in space conditions, where safety and efficiency are primary criteria for raw material selection.

2 Theoretical Background

2.1 Production organism *Xanthobacter sp.* SoF1

Xanthobacter is a Gram-negative genus of bacteria from the Xanthobacteraceae family. *Xanthobacter sp.* SoF1 was found from Baltic Sea coastline in Naantali, Finland. The organism has been found to be suitable for human and animal consumption and has excellent nutritional properties. [10.] The dried protein powder, Solein®, consists of 75 % protein, 5 % (primary unsaturated) fats, 15 % dietary fibres and 5 % mineral nutrients. The powder contains all the necessary amino acids and provides iron, fibre and B vitamins. [5.]

Xanthobacter sp. SoF1 is a chemoautotrophic microorganism that can be grown in bioreactor conditions at limited oxygen conditions. The organism utilizes carbon dioxide as the only carbon source and Calvin-Benson-Bassham cycle is most likely used for carbon fixation. Energy for the organism is derived from hydrogen gas that is oxidized to H^+. Hydrogen for the process organism is produced by water electrolysis i.e. using electricity to split water to hydrogen and oxygen gases. Hydrogen and oxygen could be produced directly by placing electrodes inside the bioreactor. [10.]

The organism requires a nitrogen source e.g. ammonium hydroxide, ammonium salt (ammonium sulphate or chloride) urea, nitrate or nitrogen gas, and an addition of feed media containing minerals, such as phosphate, potassium, sodium and iron. [10.]

When cultivated under nutrient-limited conditions, the production organism, like many hydrogen-oxidizing bacteria (HOBs), might store carbon and energy in the form of polyhydroxybutyrate (PHB), which can accumulate up to 80 % of its cell weight in certain species [11]. In this experiment, the aim was to provide the cultures with an excess of nutrients to prevent protein yield-disrupting PHB accumulation. However, since the gas and nitrogen source feeds were adjusted manually, it may have influenced the PHB formation.

2.2 Nitrogen sources

2.2.1 Nitrogen gas

Nitrogen is one of the most essential elements in living organisms, playing a critical role in numerous biological processes. It exists in various forms within the biosphere, cycling between its inert state in the atmosphere and its vital role in living organisms. Nitrogen is converted from animal waste and decaying organic matter into molecular nitrogen (N_2) by specific microorganisms. Invertedly, atmospheric N_2 is fixed back to bioavailable forms for plants through the process of nitrogen fixation. Without a catalyst, a process to convert N_2 to NH_3, from an oxidation state of 0 to -3, has a large activation energy. Biological nitrogen fixation is very energy consuming, hydrolyzing minimum of 16 ATP molecules as explained in Equation 1. [12, s. 426.]

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i \qquad (1)$$

Since the hydrogen (H_2) for the cultivation of Xanthobacter sp. SoF1 at Solar Foods is produced using an electrolyzer, it is essential to determine the amount of hydrogen required for each reaction to form ammonia. This information is crucial for assessing the energy requirements associated with each nitrogen source. To convert the requirement of reducing power to the requirement of hydrogen, Equation 2 is used. To convert the requirement of ATP to the requirement of reducing power through oxidative phosphorylation, Equation 3 is used. Finally, the net reaction of biological nitrogen fixation in hydrogen oxidizing bacteria can be written as in Equation 4.

$$H_2 \rightarrow 2H^+ + 2e^- \qquad (2)$$

$$4ADP + 4P_i + 4H^+ + 4e^- + O_2 \rightarrow 4ATP + 2H_2O \qquad (3)$$

$$11H_2 + N_2 + 4O_2 \rightarrow 2NH_3 + 8H_2O \qquad (4)$$

This indicates that for every mole of NH_3, 5.5 moles of H_2 are required.

The conversion of atmospheric nitrogen to ammonia through biological fixation occurs under mild conditions and proceeds with significantly slower pace than industrially optimized methods [13]. Approximately 60 % of nitrogen fixation is facilitated by the enzyme nitrogenase, which is present in certain nitrogen-fixing bacteria [14, s. 650 & 1006]. Soil bacteria such as *Klebsiella* and *Azotobacter*, cyanobacteria and mainly one symbiotic bacterium, *Rhizobium,* have been proven to be able to fix nitrogen [12, s. 425]. *Xanthobacter sp.* are among the most extensively studied HOBs capable of utilizing nitrogen gas as a nitrogen source under low oxygen conditions (<5 mol %) in the absence of both organic carbon and combined nitrogen [15; 16]. In a previous research [10], *Xanthobacter sp.* SoF1 has been proven to be able to grow without a nitrogen source and to obtain its nitrogen via a gas mixture containing air, showing moderate growth in cultures.

Molecular nitrogen (N_2) is an inert, colourless, tasteless, and odourless gas, comprising 78 % of the Earth's atmosphere. Commercially, nitrogen is mostly obtained by fractional distillation of liquid air. [14, s. 650 & 1006.] In this process pure gases are separated from air by cooling them until they liquefy. Then, the components are selectively distilled at their respective boiling points. While this method yields gases of high purity, it is also a highly energy-intensive process. [17.] For the production of *Xanthobacter sp.* SoF1, nitrogen could be fed into the bioreactor either as N_2 or via a gas mixture containing air.

Due to its inert nature, nitrogen is extensively utilized across various industries, including the food industry, chemical production, and metal manufacturing. Liquid nitrogen is commonly employed as a rapid coolant. However, the majority of nitrogen produced is used in fertilizer manufacturing, where it is converted into fixed nitrogen forms such as ammonia (NH_3) and nitrate (NO_3^-) to boost agricultural yields. [14, s. 650 & 1006.]

2.2.2 Ammonia

Ammonia (NH_3) is a colorless, toxic gas with a distinctive, strong scent, composed of nitrogen and hydrogen. Most of the ammonia is produced as a source of nitrogen in agricultural fertilizers. [18.] Ammonia is also utilized in the production of solid nitrogen sources, such as ammonium nitrate and urea, which are primarily used as fertilizers in agriculture. Additionally, ammonia is commonly employed as a refrigerant gas, in cleaning products, and in various other chemical commodities. [19, s. 20–21.]

In 2021, the global production of ammonia was 150 million metric tonnes. Most of this production came from East Asia, with 64.6 million metric tonnes, and China alone produced approximately 43 million metric tonnes. [18.] Ammonia production has remained stable over the past decade. The largest exporters of ammonia in 2022 were Trinidad and Tobago, with exports valued at approximately 3,500 million U.S. dollars. The leading importer of ammonia in 2022 was the United States, with imports worth 2,300 million U.S. dollars, followed by India with 2,100 million U.S. dollars. [20.] Since most of the hydrogen used for ammonia production is derived from natural gas, the price of ammonia varies in relation to the natural gas prices. [7.]

Ammonia could also be produced using hydrogen that is made using renewable sources such as wind or solar power. Such ammonia is called green ammonia. [7.] The global market for green ammonia is projected to rise significantly between 2023 and 2031. In the agricultural sector, the market is expected to grow from 224,8 million U.S. dollars to 10,593 million U.S. dollars, reflecting an increasing awareness of the need for sustainable options. [20.]

2.2.3 Haber-Bosch Process for ammonia production

The most common way to manufacture ammonia is Haber-Bosch process [18]. Manufacturing of synthetic ammonia was first conducted by discovering Haber method. This process uses moderately high temperature and elevated pressure

and a contact catalyst to convert nitrogen and hydrogen mixture to ammonia. [21, s. 1069.]

Haber Process was first found by Fritz Haber, a German chemist in 1909. However, the process was too slow for industrial uses and therefore was sold to BASF, a German based chemical producer. A chemist Carl Bosch was set to scale the process suitable for profitable industrial scale and by 1913, the first industrial ammonia was produced. [19, s. 19.] The chemical equation for ammonia production is relatively simple, considering it to be one of the most important discoveries in the industrial revolution (Equation 5).

$$3H_{2\,(g)} + N_{2\,(g)} = 2NH_{3\,(g)} \quad (5)$$

In Equation 5, 1.5 moles of H_2 are required to form one mole of NH_3. What does not show from the reaction are the conditions required for the process. Gaseous nitrogen and hydrogen are mixed in high pressure (100–1000 atmospheres) and high temperature (400–600 °C). Iron oxide is used as a catalyst. [19, s. 20; 22.] Cooling the mixed product makes part of the ammonia condensate as liquid. Process is then continued, recycling the remaining gases in the process. [19, s. 20.]

Over 90 % of global ammonia is produced from fossil fuels. In the industrial ammonia manufacturing process via the Haber-Bosch method, over 96 % of the hydrogen is derived from fossil fuels, with only 4 % coming from electricity, which may also involve fossil fuels unless green energy is used. The Haber-Bosch process generates 2.16 tonnes of CO_2/tonne of NH_3 and consumes significant amounts of energy, exceeding 30 GJ/tonne of NH_3, due to the high temperatures and pressures required. [7].

2.2.4 Ammonium hydroxide

Ammonium hydroxide (NH_4OH), also called ammonia water, is an aqueous solution of ammonia gas. It is a widely used form of ammonia that has a

distinctive, strong scent. It primarily consists of ammonia (NH_3) and water (H_2O), with smaller amounts of hydroxide ions (OH^-) and ammonium ions (NH_4^+). [23.]

At Solar Foods, ammonium hydroxide is utilized in the production of *Xanthobacter sp.* SoF1 biomass as a nitrogen source. Additionally, it serves as a pH controller due to its properties as a weak base. It is convenient to use, as it can be directly fed into the reactor via the feed system without any prior preparation. As it dissolves, it readily forms ammonium ions, making it immediately available for cellular uptake. Ammonium can be used directly as a source of nitrogen by a variety of organisms, and it is considered to be the most bioavailable form of inorganic nitrogen. [24.]

The substance can cause burns to the skin and damage to the eyes. It also irritates the respiratory system. Due to its toxicity, it should be stored in a well-ventilated area, away from foodstuffs. It must be stored in a room without sewage or drainage facilities, because it is harmful to aquatic life. [8.]

2.2.5 Urea

Urea (NH_2CONH_2), also known as carbamide, is a white crystalline substance with distinctive odour. It has a high nitrogen content (46 %) relative to its molecular mass, which is why more than 90 % of the urea produced is used as a fertilizer either in its pure form or as an ingredient in fertilizer blends. Before industrial manufacturing, urea was made from animal waste. Synthetic urea was first produced by Friedrich Wöhler in 1828. Today, the Bosch-Meiser Urea process is used. In this process, ammonia and carbon dioxide in a form of dry ice are combined at high temperature and pressure to produce ammonium carbamate. In the second phase, urea and water are formed (Equation 6).

$$2NH_3 + CO_2 \rightarrow NH_2COO^-NH_4^+ \rightarrow NH_2CONH_2 + H_2O \quad (6)$$

The urea manufacturing process is exothermic. [19, s. 21.]

Urea is classified as a reduced form of nitrogen with a nitrogen oxidation state -3 [24]. Urea does not spontaneously degrade in solution and has a half-life of approximately 40 years at 25 °C, making it a very stable molecule. In the presence of the enzyme urease, a molecule of urea hydrolyzes into two molecules of ammonia (NH_3) and one molecule of carbonic acid (H_2CO_3), the half-time being only 20 ms at 25 °C. [25.]

The reaction catalyzed by urease to form ammonia is explained in Equations 7 and 8. There from urea, carbamate and ammonia are formed. Carbamate hydrolyzes spontaneously to form carbonic acid and a second molecule of ammonia.

$$NH_2CONH_2 + H_2O \xrightarrow{urease} NH_3 + NH_2COOH \quad (7)$$

$$NH_2COOH + H_2O \rightarrow NH_3 + H_2CO_3 \quad (8)$$

This results in a net increase in pH in solution. [26.] It is known that urease enzyme is present in *Xanthobacter sp.* SoF1, as urea has been used successfully as a nitrogen source in a previous cultivation experiment [10].

In 2022, 183,82 million metric tonnes of urea were produced worldwide [27]. In 2023, East Asia was the largest producer of urea globally, producing 40,37 million metric tonnes of nitrogen. The second largest producer was South Asia with 18,9 million metric tonnes. [28.]

The substance causes irritation to the skin, eyes, and respiratory system. It is not combustible but produces toxic or irritating gases when burning. When reacting with strong oxidants, nitrites, inorganic chlorides, chlorites and perchlorates generates a fire and explosion hazard. [29.]

2.2.6 Potassium nitrate

Potassium nitrate (KNO_3) is a water soluble, colorless or white to dirty gray crystalline salt consisting of nitrate and potassium ions. [30; 31.] Potassium

nitrate can for example be produced by oxidizing nitric acid at a high temperature and pressure. Nitric acid is produced in two phases from ammonia. First, the ammonia is produced through Haber-Bosch Process from nitrogen gas (N_2) and hydrogen (H_2) in high pressure and temperature in the presence of catalyst as explained above. Then, the ammonia is oxidized in several stages through Ostwald Process to form nitric acid. [32.]

Nitric acid can then be used to form many different kinds of nitrate salts by neutralizing it with bases such as sodium hydroxide, potassium hydroxide, calcium hydroxide, ammonium hydroxide and magnesium hydroxide. It has multiple uses in various fields from food additives to explosives and fertilizers. Potassium nitrate at high levels is toxic to humans. Still, it is used in moderation in food technology, for example to maintain red color in meat products. Almost 90 % of the potassium nitrate is produced to be used as fertilizer in the United States. Global production is about 30 million tonnes annually, Russia being the largest producer with half of the production capacity. [31.]

It is not combustible itself, but it can enhance the burning of combustible materials. In fire, toxic oxides of nitrogen are produced. Thus, it is important to store it properly, separately from reducing agents and combustible substances. The substance causes irritation to the skin, eyes, and respiratory system. After handling, contaminated clothing should be washed with water to prevent fire hazards. [33.]

Potassium nitrate (KNO_3) dissociates into potassium ions (K^+) and nitrate ions (NO_3^-) when dissolved in water. Most plants and microorganisms are capable of reducing nitrate ions (NO_3^-) to nitrite ions (NO_2^-) and further to ammonium (NH_4^+), making nitrogen available for microbial use once again. [12, s. 424.] In the nitrogen cycle, nitrogen undergoes various oxidation states, nitrate representing its highest oxidation state (+5). [14, s. 1006.] The reduction of nitrate to ammonium requires two sets of nitrate and nitrite reductase enzymes (Nap/Nrf and Nar/Nir) and various electron carriers. During this process, the oxidation state of nitrogen changes from +5 to -3, converting the most oxidized form of nitrogen

to the most reduced form of nitrogen (Equation 9). The energy required for this reduction depends on the environmental conditions. [34]. Furthermore, by converting the requirement of reducing power to the requirement of hydrogen using Equation 2, the nitrate to ammonia reaction can be written as in Equation 10.

$$NO_3^- + 10H^+ + 8e^- \rightarrow NH_4^+ + 3H_2O \quad (9)$$

$$4H_2 + HNO_3 \rightarrow NH_3 + 3H_2O \quad (10)$$

In this case, for each mole of NH_3, 4 moles of H_2 are required.

2.2.7 Comparison of nitrogen sources

The cost comparison focuses on both the price per kilogram of the chemical and the price per kilogram of nitrogen contained in the chemical. Table 1 provides a detailed price breakdown as well as the nitrogen content for each chemical. Prices have been compiled from various companies' price lists [35].

Table 1. The price list of nitrogen containing chemicals.

Chemical	**Nitrogen content (m-%)**	**Price [€] / kg**	**Price [€] / 1 kg N**
Ammonium hydroxide 24.5 m-%	20	0.55	2.73
Urea	46	2.40	5.22
Potassium nitrate	14	4.53	32.69

As seen in Table 1, ammonium hydroxide is the most cost-effective nitrogen source, both per kilogram of the product and per kilogram of nitrogen. Urea is moderately priced but offers a much higher nitrogen content (46 %) compared to ammonium hydroxide, making it the most nitrogen-dense option. Potassium

nitrate is the most expensive choice, particularly in terms of nitrogen cost, which significantly limits its feasibility for large-scale use.

Nitrogen gas (N_2) has been excluded from the table. In a previous experiment, some of the cultivations were left without an external nitrogen source, and the nitrogen was supplied to the reactor via aeration only. Growth with air containing nitrogen gas was slower than growth with external nitrogen sources. [10.] In such a scenario, where nitrogen is fixed directly from the air (78 % of N_2), the cost of nitrogen gas would be considerably low.

Comparing the energy consumption of different nitrogen conversion pathways to ammonia, biological N_2 fixation is the most energy-intensive process, requiring 0.55 kWh/mol (NH_3). This is 1.9 times higher than ammonia production via the Haber-Bosch process which requires 0.29 kWh/mol (NH_3), and 1.4 times higher than biological nitrate reduction, which requires 0.4 kWh/mol (NH3). In all cases, the total energy demand is determined by the hydrogen requirement of the reaction, with the energy requirement calculated based on H_2 production via NEL electrolyzer. [36.] However, for the Haber-Bosch process, the additional energy required to operate the process itself is also included in the total hydrogen energy calculation.

Urea and ammonia share the same nitrogen oxidation state (-3), meaning their direct conversion does not involve redox changes. Additionally, enzymatic hydrolysis of urea to ammonia, catalyzed by urease, is the least energy demanding pathway among the nitrogen conversion processes analyzed. A detailed breakdown of the calculations is provided in Appendix 1.

Nitrogen gas (N_2) is an intriguing option among the nitrogen sources selected for the study. Nitrogen gas would be a practical choice for remote locations, as it can be directly extracted from the air, thereby reducing the requirement for raw material deliveries. However, the consumption rate of gaseous nitrogen is likely slower than that of urea or nitrate due to its higher demands for reducing power (electrons) and energy input (ATP). This slower consumption rate could also pose

challenges on a larger production scale, where maintaining high productivity is crucial.

Ammonium hydroxide's liquid form complicates transport and storage, and its toxicity and environmental hazards pose additional challenges despite its low cost. Urea, in contrast, is a solid chemical that is safer to handle, transport, and store. It does not present significant fire or explosion risks, and its breakdown into ammonium, which requires urease, is known to be achievable within the *Xanthobacter* sp. SoF1 cell. Potassium nitrate, while also a solid, has significant drawbacks, including fire-accelerating properties and a short shelf life as it is usually sold without anti-caking agents. Its high cost further cuts down its practicality.

Both urea and potassium nitrate are derived from ammonia meaning their production processes are not significantly more environmentally friendly than ammonium hydroxide's. However, their solid forms reduce transport-related hazards and costs and improve environmental safety. Additionally, their storage and handling present fewer risks compared to ammonium hydroxide. However, if urea or nitrate were to be selected to be used in the production of *Xanthobacter sp*. SoF1, more environmentally friendly options made of nitrogen side streams would be considered.

In the previous experiment with *Xanthobacter sp.* SoF1, it was cultivated with ammonia, urea, potassium nitrate and one of the cultivations was left with air as a sole source of nitrogen. The cultivations were conducted in a 15-vessel parallel bioreactor system at 200 mL volume. Growth with nitrate and nitrogen gas was considerably slower compared to ammonia and urea that showed similar growth rates. By the end, nitrate exceeded nitrogen gas in growth rate. Despite this, cultures utilizing only air as their nitrogen source showed signs of growth, confirming that *Xanthobacter sp*. SoF1 can fix nitrogen. [10.]

On the basis of the comparison, urea was the most promising nitrogen source to proceed with in cultivating *Xanthobacter sp*. SoF1 in the experimental part of this

thesis. Its high nitrogen content, safety, ease of transport, and moderate cost make it the optimal choice for large-scale production. While ammonium hydroxide is the cheapest option, its safety and environmental drawbacks are substantial. Potassium nitrate, with its high cost and handling challenges, is the least practical option. Nitrogen gas, when fixed from the air, is the most cost-effective option. However, the rate of nitrogen fixation is significantly slower compared to the hydrolysis of urea, for example. Urea emerges as the best balance between cost, practicality, and environmental considerations, making it the preferred nitrogen source for further testing and potentially to be used in the Solein® process.

3 Materials and Methods

3.1 Work plan

The experimental part of this thesis was conducted in a 10 L laboratory-scale bioreactor. The first cultivation was conducted in a fed-batch mode, where a 24.5 m-% ammonium hydroxide was fed into the bioreactor, simultaneously serving as a pH regulator. This cultivation served as the reference point. The amounts of nitrogen sources and calculations are presented in section 3.2.2. In the second and third experiment, urea was used as the nitrogen source, requiring a separate pH adjustment mechanism. The second cultivation was a batch process, where urea was added to the bioreactor in a single dose alongside with the initial cultivation medium. The amount of urea was calculated to match the nitrogen content provided by 24.5 m-% ammonium hydroxide in the reference cultivation. The third cultivation was a fed-batch process, where 24.5 m-% urea solution was fed to the bioreactor. The feed rate of nitrogen source in the fed-batch cultivations with ammonium hydroxide and urea, was adjusted manually and the total usage of nitrogen sources was monitored measuring the weight of the feed bottles. Each cultivation lasted 1+6 days, 1 being the day of the inoculation which will be later marked as day 0. The primary research question in the experimental part was that how effectively *Xanthobacter sp*. SoF1 can utilize urea as a nitrogen source. The key outcomes of the experiments were the observed growth rates achieved with 24.5 m-% ammonium hydroxide and urea in the cultivations.

3.2 Raw materials

3.2.1 Culture media for shake flask cultivations

For the autotrophic shake flask cultivation, modified DSMZ81 media with 10-fold of phosphates was used [37]. $NaVO_3$ x H_2O and vitamin solution were excluded from the Fresh Solution B. To make the cultivation medium, four solutions were prepared: Stock Solutions A and C, trace elements solution and Fresh Solution

B. Compositions of Solutions A, C, trace elements solution and preparations of Fresh Solution B are presented in Tables 2–5.

Table 2. Composition of Stock Solution A.

Chemical	**Amount [g]**	**Brand**	**Batch**
KH_2PO_4	46.0	Algol	3-02181-11
Na_2HPO_4 x 2 H_2O	58.0	Algol	24-02-24/269-916

KH_2PO_4 and Na_2HPO_4 x 2 H_2O were dissolved into reverse osmosis (RO) water to make a final volume of 1 L.

Table 3. Composition of Stock Solution C.

Chemical	**Amount [g]**	**Brand**	**Batch**
$FeSO_4$ x 7 H_2O	1.25	Cambridge Commodities	230382

$FeSO_4$ x 7 H_2O was dissolved into RO water to make a final volume of 500 mL.

Table 4. Composition of trace elements solution.

Chemical	**Amount [g]**	**Brand**	**Batch**
$ZnSO_4$ x 7 H_2O	0.0500	Cambridge Commodities	23P0569
$MnSO_4$ x H_2O	0.0150	Cambridge Commodities	2024022941
H_3BO_3	0.1500	Algol	01082018
$CoSO_4$ x 7 H_2O	0.1000	Algol	12152
$CuSO_4$ x 5 H_2O	0.0075	Cambridge Commodities	D0154
$NiSO_4$ x 6 H_2O	0.0120	Algol	A32282
$NaMoO_4$ x 2 H_2O	0.0130	Cambridge Commodities	P1001C-24-27

$ZnSO_4$ x 7 H_2O, $MnSO_4$ x H_2O, $CoSO_4$ x 7 H_2O, $CuSO_4$ x 5 H_2O, $NiSO_4$ x 6 H_2O and $NaMoO_4$ x 2 H_2O were dissolved into RO water to make a final volume of 500 mL.

Table 5. Composition of Fresh Solution B preparations.

Chemical	Amount [g]	Brand	Batch
$MgSO_4$ x 7 H_2O	0.5	K plus S	2124000028
$CaSO_4$ x 2 H_2O	0.012	Magnesia	D022018
$MnSO_4$ x H_2O	0.0053	Cambridge Commodities	2024022941
24.5 % NH_4OH	1.32	Algol	14062024

To prepare the Fresh Solution B, $MgSO_4$ x 7 H_2O, $CaSO_4$ x 2 H_2O and $MnSO_4$ x H_2O were dissolved into 800 mL of tap water. Ammonium hydroxide was added to the solution. pH of the solution was measured before and after adding the ammonium hydroxide. pH of the solution was adjusted with 9.5 % H_2SO_4, until the target pH 6.8-7 was reached.

Solutions A and C, trace elements solution and Fresh Solution B were sterilized in an autoclave (Systec, DX-200, Germany) in 121 °C for 20 min and were left to cool down in a laminar flow cabinet (Esco, Sentinel Gold, Germany). Fresh solution B was completed by adding Stock Solutions A & C, trace elements solution and sterile tap water. Amounts are shown in Table 6. All work was conducted aseptically.

Table 6. Composition of a completed Fresh Solution B.

Stock Solution	Volume (mL)
Stock Solution A	50 mL
Stock Solution C	16 mL
Stock solution trace elements	5 mL

Stock Solution	Volume (mL)
Sterile tap water	129 mL

Stock Solutions A and C, trace elements solution and sterile tap water were added to the Fresh Solution B in a laminar flow cabinet using sterile serological pipette tips. The completed Fresh Solution B was agitated carefully and utilized to prepare pre-culture shake flasks for the first phase of fermentation. The composition of the pre-cultures is explained further in chapters 3.3.1 and 3.3.2. Solutions A & C, trace elements solution and Fresh Solution B were stored in a dry place. A new Fresh Solution B was prepared for each cultivation to ensure that it would not get contaminated.

3.2.2 Nitrogen source preparations for the bioreactor cultivations

For the ammonium hydroxide fed-batch cultivation, 500 mL of 24.5 m-% ammonium hydroxide was sterilized in an autoclave 121 °C for 20 min. For the urea batch and fed-batch cultivations, the required quantity of urea was calculated to match the amount of nitrogen in 24.5 m-% ammonium hydroxide used in the reference fed-batch cultivation. The amounts of nitrogen sources are explained in Table 7.

Table 7. The nitrogen sources used in the cultivations.

Cultivation	Nitrogen source	Nitrogen (g)	Amount of chemical/ solution (g)	Added to the reactor via
Ammonium hydroxide fed-batch	24.5 % NH_4OH	33.45	166.00	Feed line
Urea batch	Urea (NH_2CONH_2)	33.45	71.70	Batch medium
Urea fed-batch	24,5 % Urea (NH_2CONH_2)	33.45	364.35	Feed line

In the ammonium hydroxide reference fed-batch cultivation, 166 g of 24.5 % ammonium hydroxide was used as the nitrogen source. There is 33.45 g of nitrogen in 166 g of 24.5 % ammonium hydroxide; thus, for the second cultivation, 71.70 g of urea was mixed to the batch medium and fed to the reactor in the beginning. For the third cultivation, 24.5 % urea solution was prepared by adding 71.70 g of urea and mixing it with 292.65 g of tap water.

3.2.3 Culture medium for bioreactor cultivations

The cultivation medium was an adaptation of a substrate previously demonstrated to be effective in cultivation of the *Xanthobacter sp.* SoF1. The composition of the medium is presented in Table 8.

Table 8. Composition of the batch medium for the bioreactor cultivations. Medium was identical, except for the second cultivation, where urea was added to the medium.

Chemical	**Concentration [g/L]**	**Brand**	**Batch**
$CaCl_2$ x 2 H_2O	0.0200	Algol	C5457
$MnSO_4$ x H_2O	0.0012	Cambridge commodities	2024022941
Citric acid	0.4600	Algol	1295951
H_3BO_3	0.0012	Algol	01082018
$ZnSO_4$ x H_2O	0.0019	Cambridge Commodities	23P0569
$CoSO_4$ x 7 H_2O	0.0009	Algol	12152
$CuSO_4$ x 5 H_2O	0.0003	Cambridge Commodities	D0154
$NiSO_4$ x 6 H_2O	0.0002	Algol	A32282
Na_2MoO_4 x 2 H_2O	0.0002	Cambridge Commodities	P1001C-24-27
$FeSO_4$ x 7 H_2O	0.1069	Cambridge Commodities	230382

Chemical	Concentration [g/L]	Brand	Batch
$MgSO_4$ x 7 H_2O	0.7000	K plus S	2124000035
KH_2PO_4	0.5570	Algol	3-02230-11
Na_2HPO_4 x 2 H_2O	0.7000	Algol	24-02-24/269

The components were measured to a 10 L carboy and filled with tap water to a final volume of 10 L. For the ammonium hydroxide and urea fed-batch cultivations, the medium was prepared as stated above. For the urea batch cultivation, 7.17 g/L NH_2CONH_2 (18F2856900, VWR) was added to the medium.

3.3 Pre-cultures of *Xanthobacter sp*. SoF1

3.3.1 First pre-culture shake flasks

For the pre-culture, two 250 mL baffled shake flasks (coded by numbers 1 & 2) with membrane caps were sterilised in an autoclave 121 °C for 20 min. After cooling, 30 mL of sterile Fresh Solution B was added to each flask. One glycerol stock of *Xanthobacter sp*. SoF1 was defrosted for 30 min prior to inoculation. In this experiment, isolated bacterial strain VTT-E-193585 from VTT culture collection was used. Shake flasks were placed in an incubator in a sealed container and maintained under autotrophic conditions (35 mL/min H_2, 5.52 mL/min O_2 and 5.52 mL/min CO_2) in 30 °C and agitated at 120 rpm. Shake flasks were incubated for 96 hours.

3.3.2 Second pre-culture shake flasks

After cultivating the first pre-culture flasks for 96 h, 13 sterilized 250 ml baffled shake flasks (coded by numbers 3–15) with membrane caps were prepared by adding 38 mL of sterile Fresh Solution B to each flask. 2 mL from the first pre-culture shake flask 1, was used to inoculate each of the 13 shake flasks. Shake flasks were placed in an incubator in a sealed container and maintained under autotrophic conditions (35 mL/min H_2, 5.52 mL/min O_2 and 5.52 mL/min CO_2) 30

°C and agitated at 120 rpm. Shake flasks were incubated for 72 h. After 72 h of cultivation, cell suspensions (OD_{600} > 1.0) from the shake flasks were combined into a sterilized 1 L bottle and used for inoculation of the 10 L bioreactor cultivation. Separate shake-flask pre-cultures were made for each bioreactor cultivation.

3.4 Bioreactor cultivations

Cultivations were conducted in a 10 L stirred tank bioreactor (Figure 1) made in-house (Solar Foods, Finland).

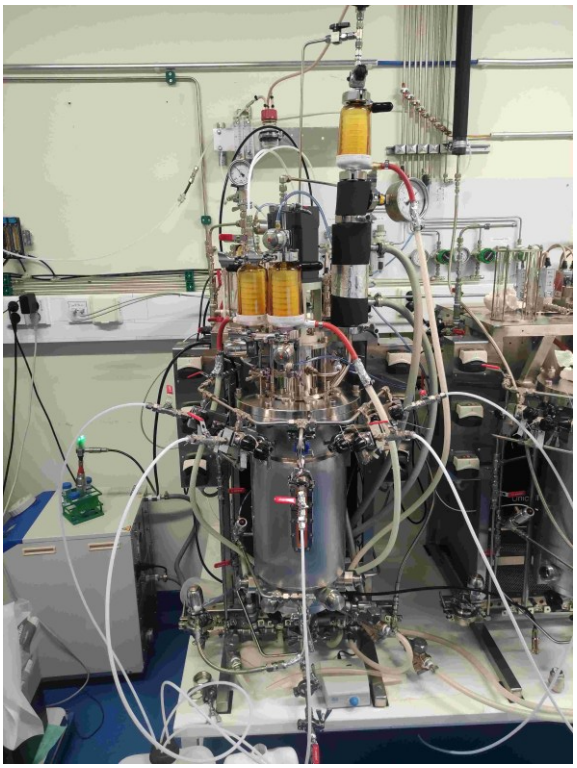

Figure 1. The 10 L bioreactor used in the bioreactor cultivation experiments.

The bioreactor and the gas filters were steam sterilized in place (SIP) 121 °C for 20 min. All detachable feed lines were sterilized separately in an autoclave in 121 °C for 20 min. 9.5 L of culture medium was fed to the reactor via feed line, through a sterile filter (Pall Corporation, Mini Kleenpak™ Capsule Filters 0.2 µm, UK). In the beginning, the pH was adjusted to 6.3–6.6 with sterile 24.5 m-% ammonium hydroxide in the ammonium fed-batch cultivation or with sterile 1 M NaOH in the

urea batch cultivation or with sterile 1 M KOH in the urea fed-batch cultivation. KOH was selected for the third cultivation to replace NaOH due to certain complications, which are discussed later in the text. 500 mL of inoculum (OD_{600} >1.0) from the shake flask cultivations (second pre-culture) was pumped to the reactor. Optical density from the initial reactor sample was aimed to be >0.1.

As a main source of carbon and energy, a gas mixture consisting of 0.18-0.98 L/min H_2, 0.03- 0.34 L/min O_2 and 0.06-0.17 L/min CO_2 was supplied constantly to the bottom of the reactor through a pipe with an outer diameter of 6 mm and an inner diameter of 4 mm. Gases were adjusted manually.

Mixing of the bioreactor was performed with Rushton-type impellers. Agitation was raised manually up to 800 rpm during the batch cultivation to maintain the dissolved oxygen (DO) at 0.0 ± 0.2 %. Temperature was maintained at 30 °C with a temperature control system. pH was maintained manually at 6.4–6.8 either with 24.5 % ammonium hydroxide in the ammonium hydroxide fed-batch cultivation, 1 M NaOH in the urea batch cultivation or 1 M KOH in the urea fed-batch cultivation. pH adjustments were conducted manually, unless stated otherwise. 20-fold diluted antifoam (AF) (SB 2121/Struktol, Schill + Seilacher) was sterilized in an autoclave 121 °C for 20 min and added manually to the reactor via feed line when foaming occurred. DO was maintained at 0.0 ± 0.2 % by adjusting manually the gas flows and agitation.

3.5 Fermentation Analysis

3.5.1 In-house analysis

Cultivations were monitored by using online measurement devices to continuously track temperature (Pt 100), pH (Mettler Toledo, In Pro 3100i SG 120, Switzerland), optical density (OD) (Hamilton Company, Dencytee RS485 120, Switzerland), and dissolved oxygen (DO) (Mettler Toledo, In Pro 6860i, Switzerland) within the bioreactor.

Additionally, bioreactor cultivations were monitored from daily reactor samples. Samples were manually collected from the reactor every 24 hours. However, due to various factors, the exact sampling times occasionally varied. From all daily samples, 0.2 ml was pipetted onto a plate count agar (PCA) petri dish to monitor for contamination and assess the purity of the cultivations. Cell growth was assessed from the daily samples by measuring optical density (OD_{600}) of the cultures at 600 nm with a spectrophotometer (Genesys 30/Visible Spectrophotometer, Thermo Scientific, China). Cell growth was assessed from daily samples using a microscope (Motic, Panthera, China). pH was also measured from the daily samples (Thermo Scientific, Orion Star A211, Indonesia). Outflow gases were monitored with a gas chromatograph (Thermo Scientific, Prima BT, UK).

Growth of the cultivations was assessed by calculating the specific growth rate (μ) and maximum specific growth rate (μ_{max}) using the natural logarithm of the measured optical density at 600 nm (OD_{600}). Specific growth rate (μ) was calculated for the duration of the entire cultivation period using Equation 11.

$$\mu = \frac{(\ln X_2 - \ln X_1)}{t_2 - t_1} \tag{11}$$

In Equation 11, *X* represents optical density (OD_{600}), and *t* refers to the cultivation time. [38.] The maximum specific growth rate (μ_{max}) was calculated with the same equation, but only during the exponential growth phase.

Cell growth was further analysed through a total dry matter content (DM) of the cultivation samples. This analysis revealed the presence of all dry matter in the sample, including cells, minerals, and other components. For this, 1.5 g of the bioreactor sample was measured to each of two separate moisture analyzers (Precisa, EM 120- HR & XM 60 HR, Switzerland). DM was measured a few times per cultivation, primarily towards the end, to assess the growth.

Cell dry weight (CDW: g/L) was determined from washed samples to provide a more accurate measurement of the dry matter content. CDW was measured only

once during the cultivation, from the final sample on day 6. For the CDW analysis, three Eppendorf tubes were pre-dried in an oven (VWR, DRY-Line 56 Prime, Poland) at 105 °C for 72 hours, and after cooling, their accurate weights were recorded. Then, 1.9 mL of sample were measured into each tube and centrifuged (Eppendorf, Centrifuge 5430 R, Germany) at 7830 rpm for 20 minutes. The supernatants were carefully removed, and the tubes were refilled with distilled water to the original volume. This washing process was repeated twice. After washing, the tubes containing only the cells were dried in an oven at 105 °C for 36 hours, and their final accurate weights were recorded after cooling. CDW was then calculated by subtracting the mass of the tube from the mass of the tube containing cells and divided by the original sample volume. CDWs were analyzed at approx. 140 h from the beginning of the cultivations.

To ensure sufficient nitrogen availability in the bioreactor, ammonium (NH_4^+) concentration was analyzed by using rapid ammonium test strips (Dosatest/Ammonium Test Strips, VWR Chemicals). Bioreactor sample and distilled water were measured to the measuring vessel to a desired dilution. 10 drops of sodium hydroxide solution was added to the diluted sample. The test strip was dipped into the shaked test solution for 5 seconds. The color of the test strip was compared to the color scale.

3.5.2 Outsourced analysis

Ammonium concentration was confirmed through an external laboratory to verify the reliability of the rapid test kit. 40 mL of sample was centrifuged at 7830 rpm for 20 minutes or until two distinct phases were visible. 10 mL of the supernatant was transferred to a 15 mL Falcon tube, stored in a freezer, and analyzed for ammonium content using method SFS-EN ISO 7150-1.

4 Results and discussion

4.1 Reference cultivation on ammonium hydroxide

In the shake flask cultivations, it was noted that the growth was weak. The shake flasks were transferred to another incubator due to suspected issues with the gas supply in the first incubator. Following the transfer, cell growth in the bottles improved, and it was possible to inoculate the bioreactor. The sample from the shake flasks had an OD of 7.01 at the moment of inoculation. However, microscopy examination revealed that the cells had formed clusters, indicating that growth conditions were not ideal (Figure 2).

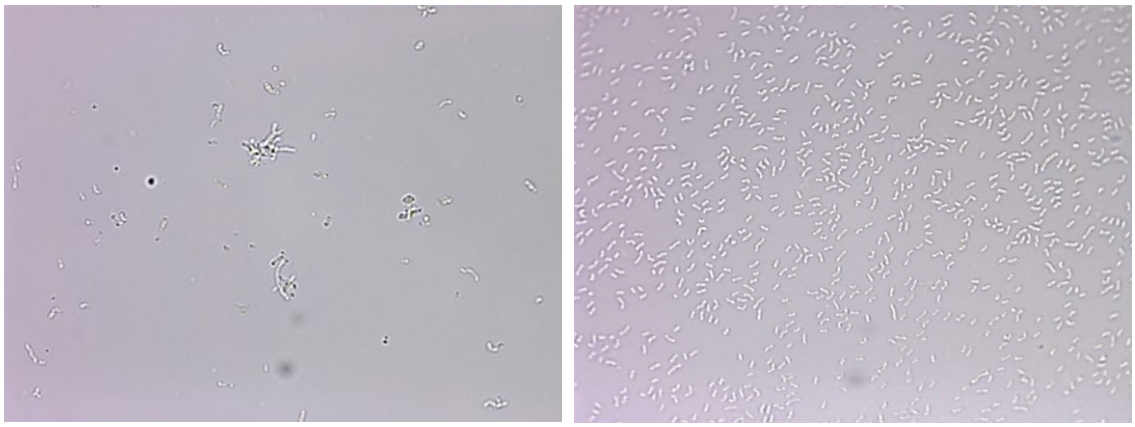

Figure 2. On the left: Microscopy image from the second pre-culture sample taken at the moment of inoculation of the first bioreactor cultivation. The image shows clusters and irregularly formed cells, indicating poor cell conditions. On the right: Example of normal, healthy-looking cells of *Xanthobacter sp*. SoF1.

The bioreactor cultivation began by adding 9.5 L of medium into the reactor through the feed line. A gas mixture was introduced at the bottom of the bioreactor through a pipe at the following rates: 0.18 L/min H_2, 0.03 L/min O_2, and 0.06 L/min CO_2. N_2 was supplied to the headspace at 0.40 L/min to dilute the gas composition of the headspace. Agitation was set to 400 rpm. The medium pH was then adjusted to 6.46 using 24.5 m-% ammonium hydroxide. The bioreactor was inoculated with 500 mL of cell mass from the second pre-culture. Gas flow

rates and agitation were increased gradually, and the DO sensor reading was maintained near zero.

The pH of the daily samples ranged from 6.42 to 6.82, with a slight increase towards the end of the cultivation, indicating that the culture was transitioning into the stationary phase of growth. At the final measurement point (day 6, 143 h), DM, the maximum cell density, calculated from CDW, and OD were 1.631 %, 16.02 g/L and 29.5 respectively (Table 9).

Table 9. pH, total dry matter content, cell dry weight, optical density and growth rate measured from the daily reactor samples of bioreactor cultivation with 24.5 m-% ammonium hydroxide. The maximum specific growth rate (μ_{max}) is highlighted in the table.

	t (h)	pH	DM (%)	CDW (g/L)	OD600	Growth rate (μ)
Day 0	0	6.42	-	-	0.346	-
Day 1	26	6.77	-	-	1.134	0.047
Day 2	49	6.43	0.215	-	4.613	**0.063**
Day 3	71	6.60	0.369	-	7.825	0.023
Day 4	98	6.45	-	-	13.450	0.020
Day 5	121	6.78	1.301	-	22.350	0.021
Day 6	143	6.82	1.631	16,02	29.500	0.013

The specific growth rate (μ) of the cultivation and the maximum specific growth rate (μ_{max}) at 26–49 h of cultivation were calculated to be 0.031 h^{-1} and 0.063 h^{-1} respectively.

The ammonium concentration measured from the reactor sample on days 5 and 6 was 250 mg/L on both days. Microscopy observations conducted during the cultivation revealed that on day 2, there were still similar elongated cells and clusters that were noted in the shake flasks after the pre-cultivation period. From day 3, the cells appeared healthy and seemed to be in good condition (Figure 3).

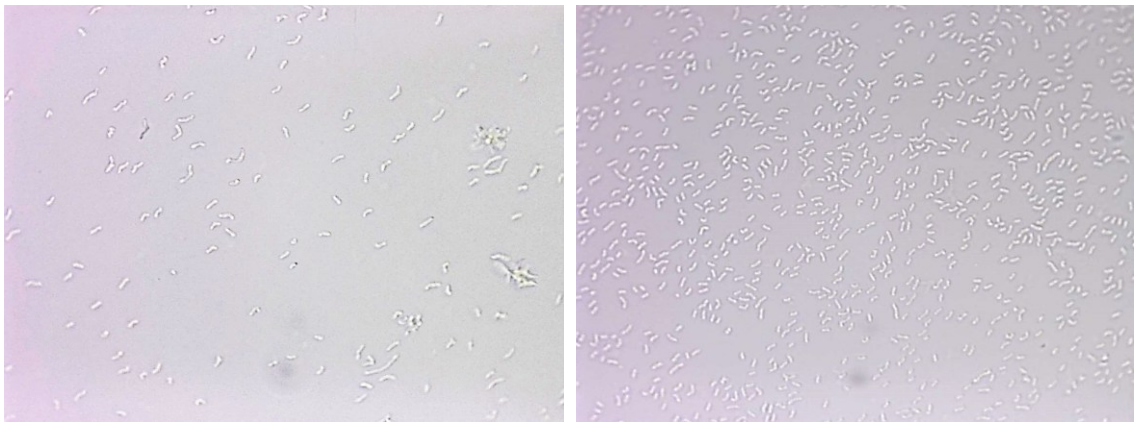

Figure 3. On the left: On day 2 of cultivation with ammonium hydroxide there was still elongated and branched cells but also some normal cells, which indicated that the cells were adjusting to new growth conditions. On the right: On day 5, the cells appeared normal.

Total of 166 g of 24.5 m-% ammonium hydroxide was fed to the reactor throughout the cultivation. The petri dishes remained uncontaminated throughout the cultivation period. A decrease in growth was indicated by an increase in outlet gas concentrations in mass spectrometer data on day 5, suggesting that the microbes were no longer utilizing the gases as efficiently as before. This likely indicated the depletion of some components in the cultivation medium.

4.2 Cultivations on urea

4.2.1 Urea batch

The overall growth in the pre-culture shake flasks was not optimal. The best growth was observed from the second pre-culture flasks located either under the inlet or outlet gas lines of the incubator as seen in Figure 4.

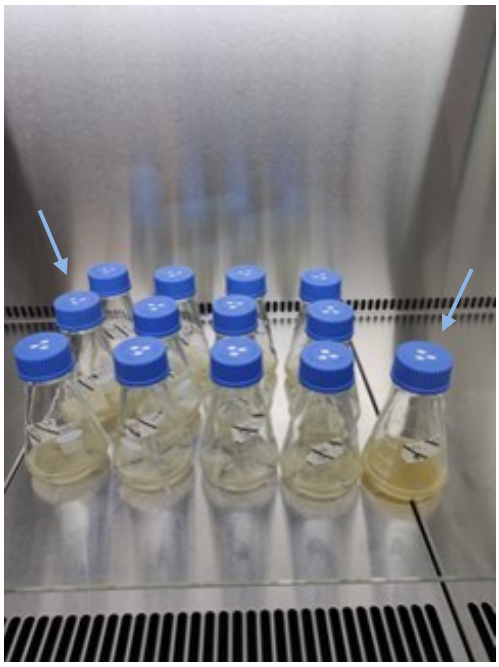

Figure 4. In the second pre-culture shake flasks of urea batch cultivation, the cell growth was better in the bottles situated next to the gas inlet/outlet in the incubator. In the figure, flasks are placed in the laminar flow cabinet in the same order as they were in the incubator. Flask on the left and right sides (marked with arrows) showed better cell growth compared to the flasks in the middle.

It was later determined that the incubator had a malfunction in the gas distribution system. As a result, the cells appeared unhealthy from the beginning of the bioreactor cultivation. In the first sample (Day 0), only a few normal looking cells were detected under the microscope, and most of the cells appeared irregular in shape (Figure 5).

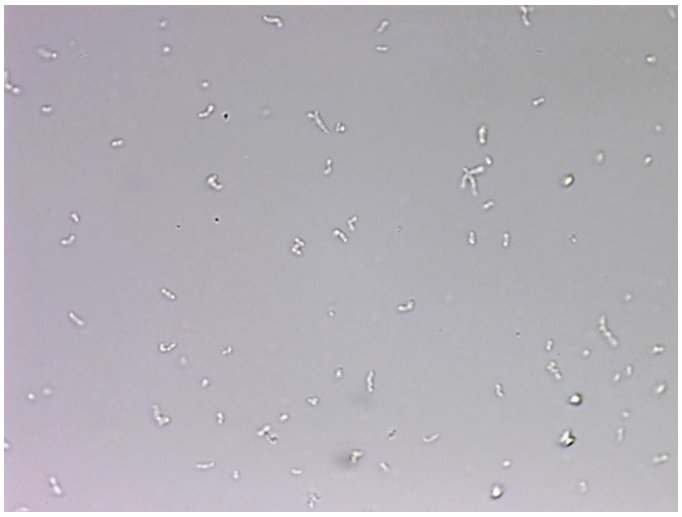

Figure 5. A microscopy image from second pre-culture shake flasks of urea batch cultivation. Most of the cells were irregular by shape in the initiation of the bioreactor cultivation.

The contents of the shake flasks were combined into a steam sterilized 1 L glass bottle (Figure 6) in the final volume of 500 mL, from which a sample was taken. The pH of the inoculum was 6.55. The OD_{600} was 1.150, which was significantly lower than the 7.010 measured in the pre-culture of the first cultivation on ammonium hydroxide. Despite this, OD was >1.0 and it was decided to proceed with the bioreactor cultivation using this inoculum due to time limitations.

Figure 6. Inoculum of urea batch cultivation.

The cultivation was initiated similarly as the baseline cultivation with ammonium hydroxide. To adjust the pH to the desired level, 1 M NaOH was used as the base. After approximately 100 g of 1 M NaOH had been added and the pH of the reactor was 6.30, the CO_2 levels in the exhaust gases rapidly decreased. This could be due to a reaction that bound the CO_2, possibly forming sodium carbonate or other solids in the reactor. Simultaneously, the online OD measuring probe showed a slight rise in the curve, which could also indicate the formation of some solids (Figure 7).

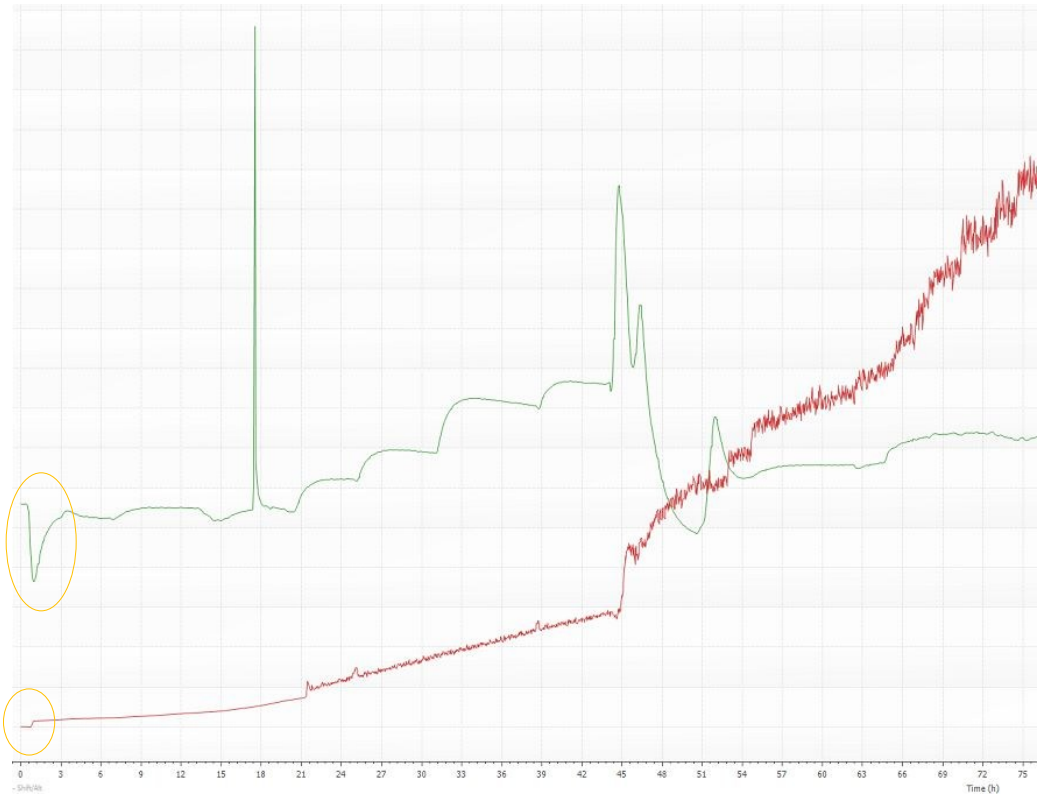

Figure 7. The outlet gases and OD were monitored with an online system. At the initiation of urea batch cultivation, the outlet flow of CO_2 (green) momentarily decreased, while the OD curve (red) showed a slight rise.

After inoculating the bioreactor with 500 mL of cell suspension from the second pre-culture, an automatic pH control was activated and set to a target pH value of 6.60. However, the automatic pH controller required further adjustment as it continued to pump the base even after the pH value exceeded the set point, reaching a pH value of approximately 6.80. As the pH value continued to rise, attempts were made to stabilize it manually by gradually increasing the CO_2 flow

rate from 0.06 to 0.11 L/min. This worked temporarily, but the pH value began to rise again. To lower the pH, 25 % phosphoric acid (H_3PO_4) was introduced into the pH control system. By this time, the pH value had already increased to approximately 7.2. The CO_2 flow was returned to its initial rate, and a total of approximately 1100 g of H_3PO_4 was added to the reactor, reducing the pH value to 6.94. The addition of H_3PO_4 caused a sharp increase in CO_2 levels in the exhaust gases, indicating that if there was any previously bound CO_2, it was now being released (Figure 8).

Figure 8. In the urea batch cultivation, adding H_3PO_4 to adjust pH to desired level resulted in a rise in the exhaust CO_2 levels. The spikes are visible in the middle of the image marked with yellow circles.

Manually increasing the mixing rate reduced the CO_2 levels in the exhaust gas flow, but increasing the acid flow caused the CO_2 levels to rise again. Stopping the acid pump lowered the CO_2 concentration in the exhaust gases, but the concentration rose again when the pump was restarted. The cultivation medium experienced significant fizzing that was visible in the sight glass of the bioreactor. Despite the continued addition of phosphoric acid, the pH remained around neutral and slightly increased until the cultivation was terminated. In the daily

samples, there was a visible layer of white solids forming in the bottom of the falcon tube (Figure 9).

Figure 9. One of the daily samples taken from the batch cultivation with urea, showing some unrecognized solids settling at the bottom of the falcon tube

The pH of the daily samples ranged from 6.39 to 7.27, showing a slight increase throughout the cultivation. At the final measurement point (day 6, 140 h), DM, the maximum cell density calculated from CDW, and OD were 0.720%, 1.44 g/L, and 3.295, respectively, indicating poor overall growth (Table 10).

Table 10. pH, total dry matter content, cell dry weight, optical density and growth rate measured from the daily reactor samples of batch cultivation with urea. The maximum specific growth rate (μ_{max}) is highlighted in the table in bold text

	t (h)	pH	DM (%)	CDW (g/L)	OD600	Growth rate (μ)
Day 0	0	6.39	-	-	0.067	-
Day 1	19	6.84	-	-	0.262	**0.079**
Day 2	45	6.96	-	-	1.061	0.053
Day 3	66	7.27	-	-	2.478	0.041

	t (h)	pH	DM (%)	CDW (g/L)	OD600	Growth rate (μ)
Day 4	89	7.25	0.682	-	3.575	0.016
Day 5	-	-	-	-	-	-
Day 6	140	7.13	0.720	1.44	3.295	-0.002

The CDW was probably even lower since some of the dry matter was still non-cellular (possibly solids), which can be seen as a lighter arrow-like pattern in Figure 10.

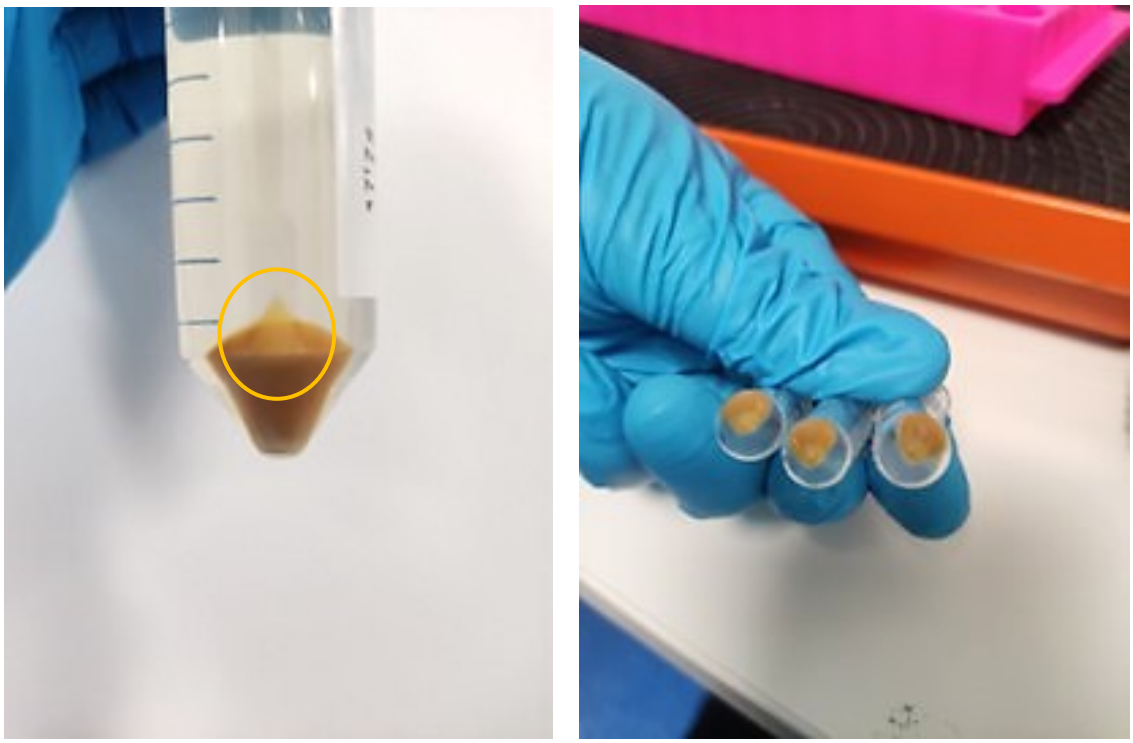

Figure 10. Samples taken on day 6 from batch cultivation with urea. Centrifuged test tubes showed darker cell mass along with lighter solids. On the left, an arrow-like shape had formed in addition to the darker cell mass. The material in the Eppendorf tubes (right) had been rinsed twice with deionized water; yet, some of the lighter-colored solids remained.

The specific growth rate (μ) of the cultivation and the maximum specific growth rate (μ_{max}) at 0–19 h of cultivation were calculated to be 0.028 h^{-1} and 0.079 h^{-1}, respectively. During the exponential growth phase (0–19 h), OD increased from 0.067 to 0.262. Given the generally poor growth observed, the calculated maximum growth rate is not considered comparable to other cultivations.

The daily samples were analyzed with a microscope. The number of cells increased from the pre-culture phase, but in addition to normally shaped cells, some abnormal cells were observed, including elongated, thick, and multi-branched forms (Figure 11). Overall, microscopy observations confirmed that the growth was abnormal, and the cells were not in a healthy state. The petri dishes remained uncontaminated throughout the cultivation period.

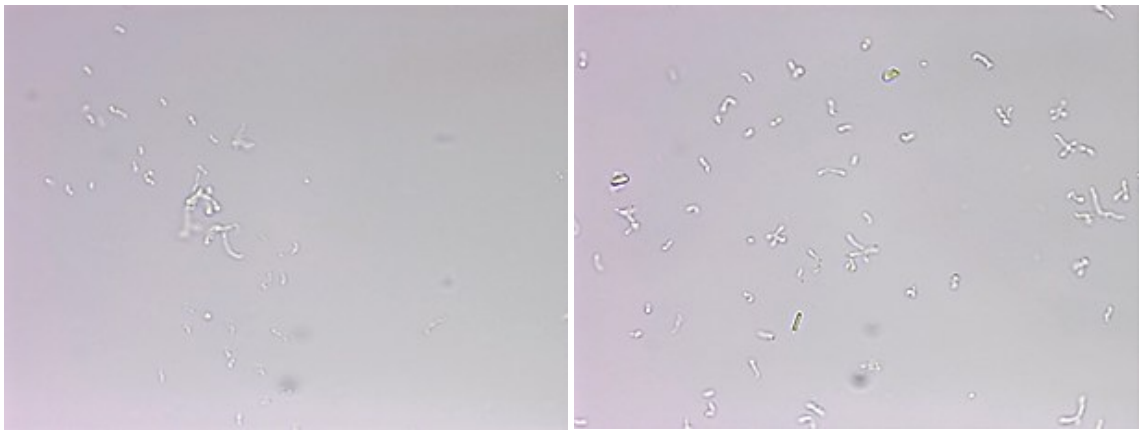

Figure 11. Irregular shaped cells of urea batch cultivation, days 1 (left) and 6 (right).

Ammonium measurements using the rapid test kit produced inconsistent and unreliable results. The tests were conducted with 1-fold, 2-fold, and 10-fold dilutions at 140 h, yielding results of 75–200 mg/L, 300–600 mg/L, and 1750–3000 mg/L, respectively. Interestingly, the measured ammonium concentration increased with greater dilutions, indicating that some factor interfered with the analysis.

To verify the results of the rapid test, ammonium concentration was also analyzed at an external laboratory from a sample taken at 140 h. The measured value was 3010 mg/L ± 240, which closely aligns with the results obtained using the rapid test kit with 10-fold dilution. Compared to the 24.5 m-% ammonium hydroxide reference cultivation with a measured ammonium content of 250 mg/L, this suggests that ammonium ions were present in excess within the reactor. Additionally, the value was above the range of the rapid test kit (10–400 mg/L) which could explain the inconsistent results.

Troubleshooting

It is possible that ions, such as Na^+, NH_4^+ or Ca^{2+} reacted with CO_2 fed in the reactor, forming carbonates in the beginning of the bioreactor cultivation. The Na^+ ions may have originated from NaOH used as a pH controller. Thus, NaOH was replaced with KOH in the third cultivation. NaOH could have formed sodium carbonates in the reactor (Equation 12):

$$2\ NaOH + CO_2 \rightarrow\ Na_2CO_3 + H_2O \quad (12)$$

The addition of phosphoric acid may have temporarily formed areas with more acidic pH, dissolving carbonates and releasing CO_2, resulting in a significant increase in the outflowing CO_2. Releasing of CO_2 then potentially raised the pH and made its regulation more challenging. Equation 13 demonstrates how sodium carbonate reacts with phosphoric acid and releases sodium phosphates, water and carbon dioxide which escapes the solution as a gas:

$$Na_2CO_3 + H_3PO_4 \rightarrow\ Na_3PO_4 + H_2O + CO_2 \quad (13)$$

Gunnarsson *et al.* noted in their study that a NaOH (aq) solution containing urea absorbed more CO_2 than a NaOH (aq) solution without urea. Their research demonstrated that urea could catalyze the binding of CO_2 and act as a buffer, potentially aiding in stabilizing pH fluctuations. The experiment revealed simultaneous interactions of urea with both CO_2 and CO_3^{2-}, leading to enhanced CO_2 binding and buffering effects. This could possibly explain the observed difficulty in lowering the pH during the process. The study also stated that the addition of urea to NaOH solution increases the pH because urea exponentially decreases proton activity as the concentration of urea increases. [39.]

In the cultivations of this thesis, DSMZ medium with a 10-fold increase in phosphate concentration, which as been proven effective in previous cultivations with *Xanthobacter sp.* SoF1, was used. This may have contributed to the solid's formation and buffering effect. In the references used for this work, the phosphate

concentrations of the media were notably lower [10; 11]. No similar solids were observed in these reference studies when 8 M NaOH and 3,6 M H_3PO_4, and 1 M KOH was used for the pH control [10; 11]. Additionally, it is possible that phosphoric acid used as a pH control agent, formed phosphate compounds, which may have acted as a buffer and contributed to the observed pH stabilization.

Similar solids have been observed previously when the ammonium concentration in the cultivation of *Xanthobacter sp.* SoF1 has been too high [40]. However, further analysis would be required to confirm if the solids were formed due to high ammonium concentration.

4.2.2 Urea fed-batch

Due to the issues with the incubator, no observable growth was detected in the pre-culture shake flasks this time. For the bioreactor cultivation, 150 mL cell suspension (OD_{600} 28.9) was taken from the 20 m^3 production reactor (Solar Foods, Vantaa) to inoculate the 10 L reactor to the same initial optical density (0.346) as in the reference cultivation with ammonium hydroxide. To adjust the pH to the desired level, 1 M KOH was used as the base. KOH was added into the bioreactor through the feed line until a pH of 6.30 was reached.

The pH of the daily samples ranged from 6.21 to 6.89, with a slight increase towards the end of the cultivation, indicating that the culture was transitioning into the stationary phase of growth. At the final measurement point (day 6, 140 h), DM, the maximum cell density calculated from the CDW, and OD were 2.530 %, 21.93 g/L and 55.4 respectively (Table 11).

Table 11. pH, DM, CDW, OD and growth rate measured from the daily reactor samples of fed-batch cultivation with urea. The maximum specific growth rate (μ_{max}) is highlighted in the table in bold.

	t (h)	pH	DM (%)	CDW (g/L)	OD600	Growth rate (μ)
Day 0	0	6.21	-	-	0.346	-
Day 1	18	6.41	-	-	0.773	0.047
Day 2	42	6.50	-	-	3.595	**0.064**
Day 3	75	6.52	0.612	-	13.15	0.039
Day 4	98	6.64	1.154	-	19.90	0.017
Day 5	118	6.75	1.848	-	31.8	0.024
Day 6	140	6,89	2,530	21,93	55,4	0,026

The specific growth rate (μ) of the cultivation and the maximum specific growth rate (μ_{max}) at 18–42 h of cultivation were calculated to be 0.037 h^{-1} and 0.064 h^{-1} respectively.

24.5 % urea solution was filtered using a membrane dry vacuum pump (VWR, VCP 130, USA) and 500 mL filtration unit with 0.20 μm pore size (VWR, Complete filtration unit). In the initiation, 12 g of 24.5 % urea solution was added to the bioreactor through the feed line. After that, the urea solution was continuously pumped to the bioreactor at a rate of 1–7 mL/h. The addition of urea was found to increase the pH of the cultivation medium. Thus, the feeding rate of urea had to be adjusted according to pH of the medium. The pH of urea solution was measured to be 8.00.

Total of 336.50 g from the prepared 364.35 g of 24.5 % urea solution was fed to the reactor through the feed line along the cultivation. During the cultivation period, ammonium concentration was measured with ammonium test strips. On day 1, the analysis showed an ammonium concentration of 50 mg/L, and the urea feed was maintained at 1 mL/h. In the following days, the ammonium concentration decreased to <10 mg/L. Consequently, the urea feed rate was raised to 3 mL/h and then to 5 mL/h, but the ammonium concentration remained

around 10 mg/L. On day 5, the feed rate was further increased to 7 mL/h, resulting in an ammonium concentration of >50 mg/L. On day 6, the feed rate was decreased to 5 mL/h, and the measured ammonium concentration ranged from 100 to 300 mg/L, with less linear results indicating potentially higher ammonium concentrations in the medium. This might suggest that the cultivation was transitioning towards the stationary growth phase.

To verify the results of the rapid test, ammonium concentration was also analyzed at an external laboratory from samples taken on days 3, 5 and 6. The measured ammonium value on day 3 was <50 mg/L, being in line with the previous measurements with the test strips. On days 5 and 6, the results were 122 (± 9.8) mg/L and 639 (± 51) mg/L respectively, indicating possibly the end of the cultivation. The final ammonium concentration was above the rapid kit's range (10–400 mg/L) explaining the inconsistent results.

Microscopy observations conducted during the cultivation revealed that on day 2, there were healthy looking cells with some crystalline structures present. On day 3, some filamentous structures and longer cells were observed in addition to healthy looking cells (Figure 12)

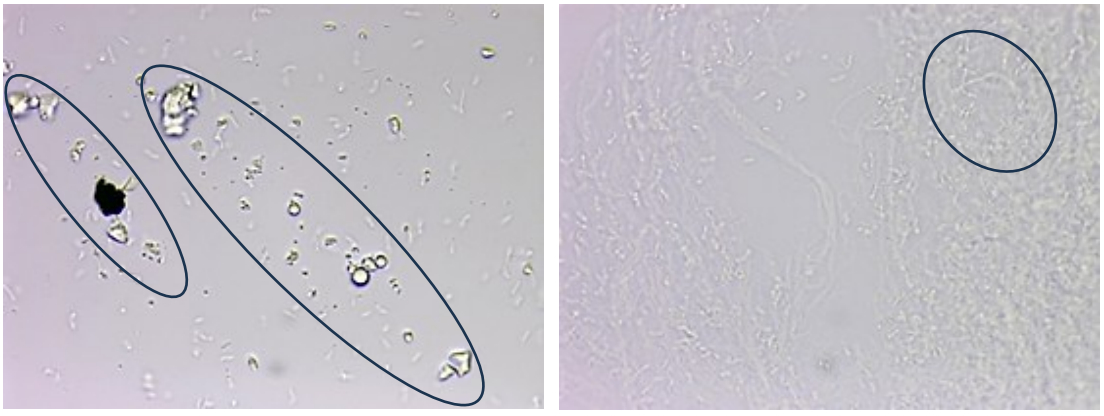

Figure 12. On day 2 (left), there were some crystalline objects visible in the microscopy image. The crystals are circled on the image. On day 3 (right), some filamentous structures and longer cells were found. A long cell is circled on the image. The filaments covered large areas in the microscopy images.

The petri dishes remained uncontaminated throughout the cultivation period. The filamentous structures disappeared on days 4 and 5, until they reappeared on day 6. The longer cells could possibly indicate the presence of PHB, suggesting suboptimal growth conditions. Suzuki, T. *et al* proposed that in nitrogen-limited cultivation, microbes accumulate PHB in high concentrations inside the cells. When there was an excess of nitrogen present, it reduced activity of microbial PHB synthesis and caused degradation of already accumulated PHB. [41.] The low measured ammonium concentration might explain the longer cells observed on day 3, indicating possible PHB formation. Further PHB analysis would be required to confirm this theory. Filamentous structures could suggest the formation of extracellular polysaccharides. This hypothesis would also require additional analysis.

On day 3, gas consumption was high, indicating rapid growth of the cells. Biomass accumulated rapidly in the reactor, requiring the removal of 400 mL and 550 mL of sample on days 3 and 4, respectively, in addition to the normal daily samples, to maintain the liquid level at 10 L.

Supernatant from the final reactor sample (day 6) exhibited an unusual odour similar to acetone. At this stage, the culture was already in the deceleration and moving towards the stationary phase of growth. Conducting a continuous cultivation could help to determinate whether a similar odour would be present when sufficient nutrients are provided throughout the cultivation. Additionally, it would require further investigation to confirm if the product would pose any risk to consumers.

On day 6, a decrease in growth was indicated by an increase in outlet gas flows, suggesting that the microbes were no longer utilizing the gases as efficiently as before. This likely indicated that the cultivation was transitioning to the stationary phase.

4.1 Comparison of cultivations

Initial growth of the *Xanthobacter sp.* SoF1 in fed-batch cultivations with ammonium hydroxide and urea were similar. The growth curves were plotted using the OD_{600} and the natural logarithm of OD_{600} measured from the daily samples (Figures 13 & 14).

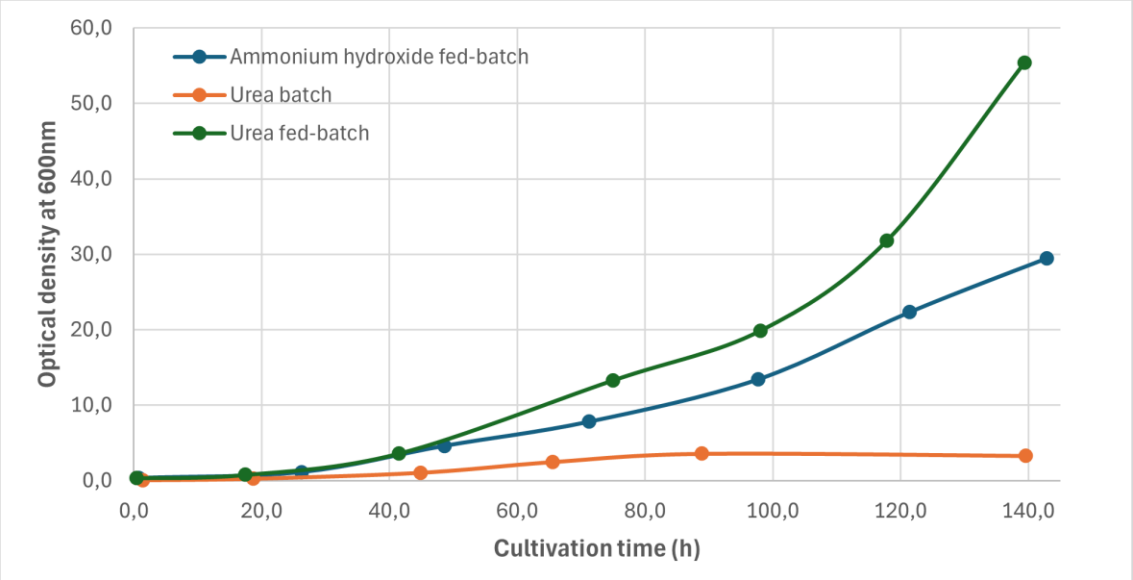

Figure 13. Optical density of fed-batch cultivations with ammonium hydroxide and urea and batch cultivation with urea at 600 nm (OD_{600}).

Towards the end of the cultivations, growth on urea surpassed the growth of the one with ammonium hydroxide. Both curves (Figures 13 and 14) show that urea batch cultivation was less successful than fed-batch cultivations with ammonium hydroxide and urea.

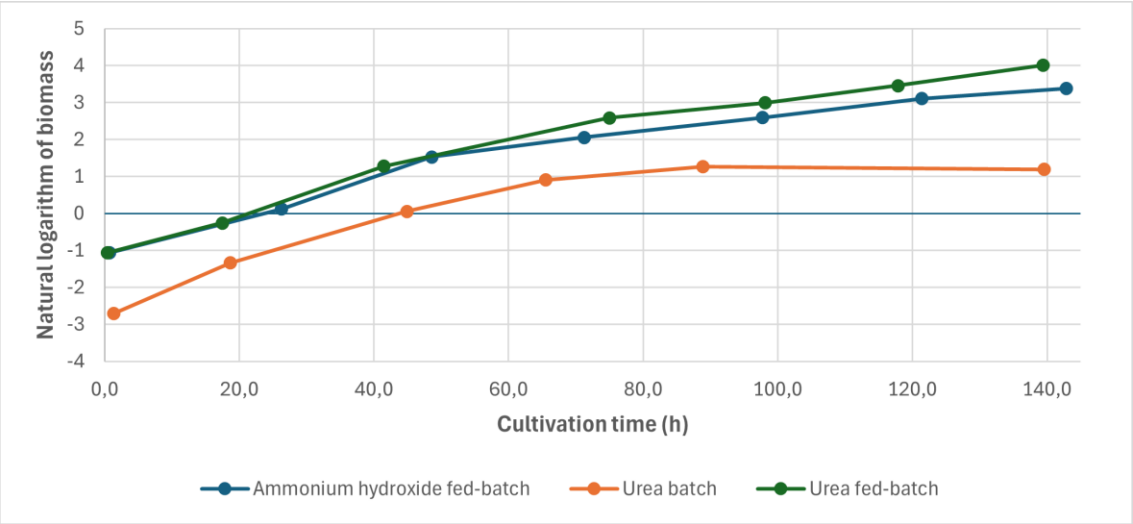

Figure 14. Natural logarithm of fed-batch cultivations with ammonium hydroxide and urea, and batch cultivation with urea calculated from optical density at 600 nm.

In the plotted growth curve of the natural logarithm of OD_{600}, fed-batch cultivations with ammonium hydroxide and urea were comparable. The maximum cell density calculated from CDW, along with the specific and maximum specific growth rates (μ & μ_{max}), indicated that urea fed-batch cultivation was the most successful (Table 12).

Table 12. Comparison of cultivations.

Cultivation	**CDW (g/L)**	**μ (h^{-1})**	**μ_{max} (h^{-1})**
Ammonium hydroxide fed-batch	16.02	0.031	0.063
Urea batch	1.44	0.028	0.079
Urea fed-batch	21.93	0.037	0.064

In urea batch cultivation, μ_{max} was reached in the earliest phase (0–19 h), after which growth slowed down and turned negative in 89–140 h of cultivation. Fed-batch cultivations with ammonium hydroxide and urea reached their maximum specific growth rates later (26–49 h & 18–42 h, respectively) but were able to maintain cell growth until the end of cultivations, indicating more favorable growth conditions were present during the cultivations.

5 Conclusion

The experiments provided valuable insights into the feasibility of using urea as a nitrogen source for the cultivation of *Xanthobacter sp*. SoF1. The results indicate that, when supplied gradually, urea could serve as a viable alternative for further production testing. Moreover, urea is safer and more convenient to handle compared to ammonium hydroxide that is being currently utilized in the Solein® process.

The urea batch cultivation faced several challenges, likely due to multiple factors. The high initial urea concentration in the reactor may have inhibited microbial growth. Additionally, pH adjustments with phosphoric acid were not performed quickly enough, as it was not anticipated to be necessary based on previous *Xanthobacter sp.* SoF1 cultivations. Moreover, the inoculum used in this experiment exhibited weaker growth compared to previous cultivations, which may have affected the overall outcome. To conclusively determine whether a stronger inoculum would have led to successful cultivation, further experiments are required.

The irregular cultivation initiation times posed logistical challenges, as manual parameter adjustments (e.g., gas flow, mixing, and nitrogen sources) could not follow a consistent schedule. For example, nightfall occasionally interrupted manual adjustments, potentially slowing down microbial growth. Therefore, utilizing an automatic control system for gases and agitation would be beneficial. Challenges during the pre-culture shake flask phase impacted the initial conditions of bioreactor cultivations. If all bioreactor cultivations had begun from a uniform baseline, the results might have been different. Duplicate runs should ideally have been conducted for all cultivations, including the reference cultivation using ammonium hydroxide, to ensure more reliable comparison of the results. However, due to time constraints, this was not feasible within the timeframe of this study.

Further investigations could focus on verifying the growth rate in urea fed-batch cultivation. While this study indicated strong growth in fed-batch cultivation using urea, it might also indicate for instance, PHB accumulation, since the cell growth was calculated based on OD and CDW. The actual cell growth should be examined through repeated cultivation and additional analyses.

Further testing would be required to assess the safety of the final product, including the potential formation of harmful compounds and any changes in the taste of the Solein® product. From an environmental perspective, urea derived from conventional ammonia might not be a superior alternative. However, as the demand for green ammonia increases, more sustainable urea production methods may emerge. If urea can be synthesized from recycled nitrogen sources, it could become a more environmentally feasible option.

Overall, the experiments and findings of this thesis provide a foundation for advancing future research on urea as a nitrogen source. Furthermore, based on the knowledge gained in this work, it is possible to proceed to the experimental phase with other nitrogen sources for the cultivation of *Xanthobacter sp*. SoF1.

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Energy requirements of reactions

Ammonia from Haber-Bosch process

$3H_2 + N_2 \rightarrow 2NH_3$

n (H_2)	3	mol
n (NH_3)	2	mol
n (H_2) / n (NH_3)	1.5	mol (H_2) / mol (NH_3)
M (NH_3)	17.0	g / mol
M (H_2)	2	g / mol

Energy requirement to maintain Haber-Bosch temperature and pressure [7].

30	GJ / tonne of NH_3
30	MJ / kg
30	kJ / g
3600	kJ / kWh
0,008	kWh / g
0.14	**kWh / mol (NH_3)**

NEL electrolyser efficiency for hydrogen generation

50	kWh / kg of hydrogen gas [36].
0.1	kWh / mol (H_2)
0.15	**kWh / mol (NH_3)**

Energy required to run Haber-Bosch added to electrolyser power requirement

0.29	**kWh / mol (NH_3)**

Biological N_2 fixation to ammonia

$11H_2 + N_2 + 4O_2 \rightarrow 2NH_3 + 8H_2O$

n (H_2)	11	mol
n (NH_3)	2	mol
n (H_2) / n (NH_3)	5.5	mol (H_2) / mol (NH_3)

Electrolyser power requirement

0.55	**kWh / mol (NH_3)**
1.9	Biological fixation / Haber-Bosch

Biological nitrate reduction to ammonia

$4H_2 + HNO_3 \rightarrow NH_3 + 3H_2O$

n (H_2)	4	mol
n (NH_3)	1	mol
n (H_2) / n (NH_3)	4	mol (H_2) / mol (NH_3)

Electrolyser power requirement

0.4 kWh / mol (NH_3)

1.4 Nitrate reduction / Haber-Bosch

1.4 Biological fixation / Nitrate reduction