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Yeast as single cell protein – optimization of medium compositions and process parameters

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Abstract

There is an increasing global demand for protein, due to an increased aging population and changing diets favouring increased consumption of animal protein. Using agriculture to produce animal protein requires large areas of farmland and is associated with high emissions of greenhouse gases. Single cell protein (SCP) is microbial protein produced for use in food or feed and could be promising as an alternative protein source due to its high protein content and low fat content, short harvest cycles and being independent from seasons and climate. Efficient production of SCP is highly dependent on process and media design. Yeasts are promising candidates for producing SCP with a near optimal amino acid composition, practical cell size and flocculation abilities which facilitates harvesting.

In this study, the yeasts *Blastobotrys adeninivorans, Cyberlindnera jadinii* and *Wickerhamomyces anomalus* were grown on glucose as the main carbon source in a variety of scales and media compositions to assess cell growth by batch cultivation. Additionally, *W. anomalus* was used for a continuous cultivation experiment. The highest cell dry weight (CDW) obtained during batch cultivation was achieved at 40.23 g/L using *W. anomalus* with the addition of a vitamin and trace solution to a media containing 100 g/L glucose with yeast extract and meat peptone in a 1.5 L bioreactor. Unwanted ethanol production was prevented in *W. anomalus* and *C. jadinii* cultivations by shortening inoculum incubation time, however some unidentified side products were still produced by *W. anomalus* under some conditions. The continuous cultivation experiment demonstrated that when using a feed rate of 19.062 mL/h the oxygen demand became too high for the bioreactor system to maintain. Achieving a continuous steady-state for this set-up may therefore require using a lower feed rate to increase yield.

Sammendrag

Det er en økende global etterspørsel etter protein, grunnet en økt aldrende befolkning og forandrede dietter med økt inntak av animalsk protein. Å bruke jordbruk til å produsere animalsk protein krever store områder med dyrket mark og er assosiert med høye utslipp av klimagasser. Encelleprotein er mikrobielt protein produsert for å brukes i mat eller fôr og kan være lovende som en alternativ proteinkilde grunnet dets høye protein og lave fett nivåer, korte innhøstingssykluser og å være uavhengig av sesong og klima. Effektiv produksjon av encelleprotein er svært avhengig av prosess- og mediedesign. Gjær er lovende kandidater for produksjon av encelleprotein med en nær optimal aminosyrekomposisjon, praktisk cellestørrelse og med flokkuleringsevner som hjelper med høsting.

I dette studiet, ble gjærartene *Blastobotrys adeninivorans, Cyberlindnera jadinii* og *Wickerhamomyces anomalus* grodd på glukose som hoved karbonkilde på varierende skalaer og mediakomposisjoner for å vurdere cellevekst fra batch kultivering. I tillegg ble *W. anomalus* brukt i et kontinuerlig kultiveringseksperiment. Høyeste oppnådde tørrvekt av celler gjennom batch kultivering var 40,23 g/L ved å bruke *W. anomalus* med en tilsatt vitamin og sporstoffløsning i et medie med 100 g/L glukose med gjærekstrakt og pepton fra kjøtt i en 1,5 L bioreaktor. Uønsket etanolproduksjon var forhindret i kultivering av *W. anomalus* og *C. jadinii* ved å forkorte inkuberingstiden for inokulumet, men *W. anomalus* produserte fortsatt andre uidentifiserte sideprodukter ved noen tilfeller. Kontinuerlig kultiveringseksperimentet demonstrerte at ved å bruke en matehastighet på 19,062 mL/t ble oksygenkravet for høyt for bioreaktoren å opprettholde. For å oppnå en stasjonærtilstand for dette oppsettet kan det måtte brukes en lavere matehastighet, for å øke utbyttet.

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1. Introduction

1.1 Global protein demand

The global demand for food is rapidly increasing due to an increasing world population. By 2050, the global population is estimated to reach 9.7 billion (Kumar et al., 2024). To satisfy this growing demand, there is a need for new and alternative food sources. Additionally, over 1 billion people globally are malnourished (Sekoai et al., 2024), and in around half of African countries there is already a protein source deficiency causing public health problems (Jach et al., 2022). From 2000 to 2018 the global protein demand increased by 40% (Kumar et al., 2024). In particular, the demand for animal protein is increasing due to an increasing global population, with an increasingly high number of older people who have a greater requirement for protein. Moreover, growing incomes leads to higher consumption of meat and dairy, and diets in developing countries are changing towards higher inclusion of animal products and away from protein sources like cereals (Boland et al., 2013; Godfray et al., 2010; Smith et al., 2024). In 2022 the global meat production was estimated at 347 million tonnes, with a predicted increase to 382 million tonnes by 2032. For dairy products within the same time period, production is predicted to increase from 897 million tonnes to 1039 million tonnes (OECD, 2023).

1.2 Animal/plant protein and agriculture

Currently, animal proteins represent 18% of the calories and 25% of the proteins being consumed worldwide (Kim et al., 2019). To produce animal proteins, plant proteins, like soybean meal, are often used in animal diets. The production of these plant proteins requires enormous areas of farmland and may compete with farmland that has the potential to be used directly for human food production. Also, the conversion of plant protein to animal protein is inefficient, as ~6 kg of plant biomass is required to produce 1 kg of animal protein (Murali Sankar et al., 2023). When looking at per capita demand for calories and proteins from crops being used in both plant and animal protein production, it was found that crop production

would need to be doubled between 2005 and 2050 for it to reach the estimated global food demands (Tilman et al., 2011). When using soybean as fish feed, it was found that 6.7 m^2 of land was required for 1 kg of protein (Bertasini et al., 2022). Fish meal, a by-product from the fish industry, is an animal protein source being used as feed for both livestock and farmed fish (Bimbo & Crowther, 1992), however fish meal is also becoming a limited supply and is frequently partly replaced by vegetable sources (Magnani et al., 2023).

Every year, the average person consumes 43 kg of meat and 80 billion animals are slaughtered for meat globally (Jach et al., 2022). The overconsumption of meat is not only a concern in terms of shortage, but also because it has a negative impact on the environment and the general population health (Onwezen et al., 2021). Agriculture poses a serious environmental threat because of land clearing, the effect of fertilizers on several different ecosystems and greenhouse gas emissions (Tilman et al., 2011). In the European Union, 10.3% of all greenhouse gas emissions are derived from the agriculture industry (Bertasini et al., 2022), and from 2007 to 2013 it was shown that ~35% of the global anthropogenic greenhouse gas emissions were derived from the food production industry (Jain et al., 2023).

1.3 Alternative protein sources

Because of the environmental impacts and supply limitations related to animal protein as previously discussed, alternative protein sources have been explored since the early fifties (Suman et al., 2015). Both plant and non-plant protein sources, such as insects, have been investigated for use as animal feed or human consumption. Insect meals can provide high concentrations of protein, even higher than fishmeal and soybean meal (Magnani et al., 2023). Even though insects are included in the diets of several developing countries, the interest in the western world is still very low (Giotis & Drichoutis, 2021). Another example, being targeted as a meat substitute, is lab-grown meat in which stem cells (from e.g. livestock or fish) are harvested and cultivated in vitro. However, much technological development is still needed (Broucke et al., 2023).

1.4 Microbes in food

Using microbes as a part of our diets have been done for thousands of years. Already in the year 2500 B.C., yeast belonging to the *Saccharomyces* genus was found to have been used as a leavening agent for bread (Upadhyaya et al., 2016). From there on, and still to this day, other products involving microbes such as alcoholic beverages, cheeses, and yoghurt, have been a huge part of different food cultures around the world. Also, different fermented foods have been shown to be beneficial in terms of alleviating some life style disorders, such as irritable bowel syndrome and Crohn's disease (Yadav et al., 2024).

During World War I, the yeast *Saccharomyces cerevisiae* was cultivated on molasses and used as a protein supplement because of a lack of conventional protein sources. This is the first known instance of growing microbial cells for the purpose of consumption (García-Garibay et al., 1999). Since the 1950s, because of advances in plant breeding and agriculture, plant protein was in high abundance and cheaply available, and was therefore able to outcompete edible microbes as a protein source (Puyol et al., 2017).

1.5 Single cell protein

Single cell protein (SCP) refers to either microbial protein in the form of whole cells or extracted microbial protein produced for use in food or feed (Bratosin et al., 2021). In the case of microbial cells, the term SCP does not solely describe the protein, but also the other parts included in the microbial biomass, such as carbohydrates and minerals (Severo et al., 2024). An optimal microbial species or strain used for the production of SCP has a fast growth rate and low nucleic acid content, while also meeting industrially acceptable requirements, such as for nutrients and temperature (Salazar-López et al., 2022). SCP shows great potential for use in food and feed because of its high protein content, including the essential amino acids lysine and methionine, several vitamins like thiamine and riboflavin and low fat content (Bratosin et al., 2021).

When producing SCP it is generally considered acceptable if the biomass contains 30% or more protein (Abdullahi et al., 2021), however depending on substrate and microbe species protein contents above 50% are often achieved (Jach et al., 2022). To compare, the protein content of soybean meal and fishmeal is 43%-48% and 50%-70%, respectively (Z. Zhang et al., 2024). One of the main hurdles when using SCP as a food have been high nucleic acid content, which may cause problems like kidney stone formation. For human consumption the nucleic acid content must therefore be reduced to below 2% (Nasseri et al., 2010). Also, microbial toxins formed during production which may cause allergic reactions or even be immunosuppressive or carcinogenic is being actively looked into (Ribeiro et al., 2023).

Different types of microbes have been investigated for use as SCP, such as yeast, microalgae, and bacteria (Ritala et al., 2017). With these types of microbes, and the countless different species within these groups comes different requirements and growth potentials in terms of the SCP production process. Some unique differences include the cultivation strategy. Photoautotrophic microalgae have the capacity to grow in seawater and convert CO_2 to biomass using energy from sunlight (Barbosa et al., 2023), while some chemoautotrophic bacteria are able to use energy from oxidizing inorganic compounds, such as H_2, to convert CO_2 to biomass. Heterotrophic microbes, such as yeast and some microalgae, obtain energy and carbon from organic matter, and are the most commonly used microbes in SCP production due to their high growth (Sobhi et al., 2023).

1.6 Single cell protein versus other proteins sources

Compared to animal and plant proteins, SCP is considered to have a high nutritional value (Bajić et al., 2023), and compared to common plant proteins, a higher amino acid content, indicating a lower content of non-protein nitrogen, and a more complete amino acid composition (C. Ma et al., 2024). Other advantages are short harvest cycles and being independent from seasons and climate (Zeng et al., 2022). Also, microbes convert nutrients, such as nitrogen and phosphorous, more efficiently than terrestrial plants (Rodero et al., 2024).

A life cycle assessment done in 2024 looked at the environmental impact following a global transition from agriculture to “cellular agriculture” from 2020 to 2050. The results were

based on SCP replacing meat varieties such as cattle, pork, and poultry. In addition, eggs and dairy products were replaced by ovalbumin production using the fungi *Trichoderma reesei*. The production processes would be powered by wind and solar power, taking into account the global capacity of the green energy technologies by 2050. The carbon source for the eggs and dairy replacement products would be glucose from maize. The authors calculated that the glucose production for this transition would only need to utilize up to 6% of the total arable land in 2050. For the SCP production, hydrogen-oxidizing bacteria would be used with CO_2 as a carbon source, and therefore not requiring any agricultural land. The results showed an overall reduction in greenhouse gas emissions and land use. They also looked at demands for critical materials, like phosphorous and nickel, related to this transition. Only one out of the 16 assessed materials, tellurium, would exceed the production capabilities by 2050. However, the demand for all the critical materials would still increase, expect for phosphorus, where the demand would actually decrease. Overall, the study showed promise for the sustainable development of cellular agriculture on a global scale. However, certain aspects of this transition was not considered, such as utilizable by-products from animal production no longer being available or that the majority of SCP studies are done at the laboratory scale (El Wali et al., 2024).

Compared to using insects for protein production, cultivation of SCP in bioreactors is a faster process. Insects grow slowly and produce large amounts of faeces which increases the production workload (Yang et al., 2022). One study showed reduced growth and lower feed utilization when using insect meal as compared to SCP as fish feed (Carvalho et al., 2023). A life cycle assessment study from 2015 found the environmental impact of SCP to be higher than that of insect-based substitutes, but lower than lab-grown meat (Smetana et al., 2015). Comparing SCP to other novel alternative protein sources reveals both advantages and disadvantages, and much technological development is still needed to grasp its full potential.

1.7 Substrate and nutrients

SCP production is highly dependent on a well-balanced growth medium. To maintain growth and cell functions, microbes require water, carbon, nitrogen, minerals, and vitamins. For aerobic microbes oxygen is also required. The amounts of these compounds and their ratios

are crucial in optimizing the SCP production process (Reihani & Khosravi-Darani, 2019). The carbon substrates used should be nontoxic, renewable, and cheap. Other major factors influencing the choice of substrate is availability and proximity to the production site (Raziq et al., 2020).

Substrates can be divided into first-generation which already have high commercial value, second-generation which include waste materials from other industries (Aidoo et al., 2023) and third-generation using atmospheric CO_2 (Liu et al., 2020).

1.7.1 Carbon source

1.7.1.1 First-generation

First-generation feedstocks include sugars obtained from food ingredients such as corn and rice flour, but it is in competition with the direct use as food (Aidoo et al., 2023).

High energy resources such as the petrochemicals ethanol and methanol are unconventional sources of carbon, but can still be used for SCP production (Suman et al., 2015). For these types of substrates its disadvantages are high volatility and costs. Advantages include being nontoxic and highly soluble in water. Also, when compared to petroleum hydrocarbons such as methane, ethanol and methanol are partially oxidized and therefore requires less oxygen (Tawfik et al., 1981; Watteeuw et al., 1979).

1.7.1.2 Second-generation

Using first-generation substrates for SCP production is gradually losing interest as these compounds are highly sought after (Aidoo et al., 2023). Because of the growing global population there is also an increase in waste generation (Jomnonkhaow et al., 2024) and one of the benefits of SCP production is the potential for adding value to these waste products by using them as growth substrates (Suman et al., 2015). Because of the ability of many microbes to grow on cheaper carbon sources, the production of SCP is more economically efficient than conventional protein production from plants and animals (Kumar et al., 2024). For some waste substrates it might be necessary to add nutrients, such as additional nitrogen

from simple sources like ammonium salts or urea or from complex sources like yeast extract (Jach et al., 2022). Also, the high complexity of some waste substrates may require an additional processing step in the form of physical, chemical or enzymatic pretreatment methods (Reihani & Khosravi-Darani, 2019).

Microbes for SCP are most often grown on waste from the food, forestry or agricultural industry (J. Ma et al., 2023). Straw-based biomass, like corn stover and wheat straw, are by-products from food crop production and are most often burned or discarded which in turn causes environmental pollution. After certain treatments, the biomass can be used as feed for ruminants, but its protein content (<15%) is less than optimal. Producing SCP using straw-based biomass as a substrate for microbes with lignocellulose degrading capabilities has the potential to avoid this type of resource waste (Z. Zhang et al., 2024). In aquaculture, efforts to improve recovery from nitrogen losses are being made, including using these losses as substrate to produce SCP which in turn can be reincorporated into fish feed (Deng et al., 2021). Overcoming the recalcitrance and complexity of lignocellulosic biomass may prove spruce wood to be a good candidate as an alternative carbon source with its rich sugar contents (Lapeña, Kosa, et al., 2020).

Date fruit waste in tropical climate countries has also been investigated, where sub-standard dates are discarded (Hashempour-Baltork et al., 2020). The global pulse industry has increased drastically the last two decades, and with it, big amounts of waste are generated. In particular, pea starch from these wastes has great potential as a substrate for SCP production (Aidoo et al., 2024).

1.7.1.3 Third-generation

When producing SCP from first-generation or second-generation substrates, the economic challenge often lies with the cost of feedstock. CO_2 is the most abundant carbon source on earth, and this, coupled with its drastic increase over the past 50 years causing great environmental concern, makes utilization of CO_2 highly desirable. The challenges that must be overcome to use this substrate are achieving efficient CO_2 capture and subsequently high production of SCP (Liu et al., 2020).

The use of third-generation substrates is a very recent approach and rapid progress is currently being achieved. Renewable energy company Drax based in England is planning to

use CO_2 captured from their own power plant emissions as substrate to grow SCP, completely removing costs and emissions concerned with feedstock transportation. However, the emissions also contains compounds toxic to cells, like CO and NO_x, and are emitted at temperatures above 1200°C unsuitable for cells (Banks et al., 2022). One promising approach to converting CO_2 into SCP is by using a two-stage fermentation system where first, acetogenic microbes are used to produce acetate from CO_2, and then using the acetate as a carbon source to produce SCP in a secondary fermentation (Molitor et al., 2019).

1.7.2 Nitrogen source

Nitrogen is necessary for microbes to grow. There are many different nitrogen sources with the potential for use in SCP production (Reihani & Khosravi-Darani, 2019). For some second-generation substrates, a sufficient nitrogen source may already be provided from the waste. Some nitrogen-rich wastes, such as poultry by-products, can be enzymatically hydrolysed and used in addition to a carbon source (Lapeña et al., 2020). These complex nitrogen sources may also be used in combination with simple and cheap nitrogen compounds, such as ammonium salts, nitrates, and urea. These simple nitrogen compounds may also be utilized as the sole source of nitrogen (Lapeña et al., 2020). Complex commercial nitrogen sources like yeast extract are generally associated with more rapid and efficient fermentation processes because they include more nutrients, like amino acids, reducing the amount of compounds the cells has to synthetize themselves, however, they are usually more costly (Kampen, 2014).

The concentration of nitrogen relative to the concentration of carbon is an important factor for SCP production. This carbon to nitrogen ratio (C/N ratio) in the media is often adjusted to match the C/N ratio within the microbes, which will vary between different microbes. For SCP production, the C/N ratio should not be too high so that the nitrogen is consumed before all the carbon is consumed, and not too low to avoid wasting the nitrogen (Sharif et al., 2021).

1.7.3 Gas fermentation

Using microbes to produce SCP from oxidizing gas is a novel fermentation strategy. The two most promising processes involve using bacteria which oxidizes hydrogen or methane gas (Woern & Grossmann, 2023). Demand for natural gas has very recently increased, and it is difficult to obtain sufficient sustainable biogas supply for large scale SCP production, highlighting the main challenge of using methane as a substrate. Still, the company Calysta, which is at the forefront of this gas fermentation technology, is heavily funded and is planning to produce 20.000 metric tons a year of SCP from methane in their new production plant in China (Banks et al., 2022). Hydrogen oxidizing bacteria is able to produce SCP by using energy from hydrogen gas obtained from electrolysis of water powered by solar or wind energy, and CO_2 as a carbon source (Angenent et al., 2022). During gas fermentation, the addition of nitrogen, minerals and vitamins is necessary (Woern & Grossmann, 2023).

1.8 Single cell protein production process

While a lot of research have been done on production of SCP, there are still technological bottlenecks that needs to be overcome to reach commercialization (Banks et al., 2022). Research in finding efficient methods of process optimization, and satisfy nutritional and food safety standards is still ongoing (Sagar et al., 2024). A traditional approach when doing process optimization is to change one parameter while keeping the other parameters involved constant and observing the effects, such as only varying carbon concentration through a series of experiments. However, this technique does not capture the interactive effect when varying several parameters. Therefore some studies are based on experimental designs utilizing statistical models, such as response surface methodology (RSM), where the effect of multiple variables and their interactions on the final results are determined (Reihani & Khosravi-Darani, 2019).

SCP is produced by using either submerged or solid-state fermentation. In submerged fermentation the process is carried out in a liquid state where all the nutrients are solubilized (Thiviya et al., 2022). Submerged fermentation is also used for fermentation processes such as production of yoghurt and alcoholic beverages. In solid-state fermentation the process is

carried out on the surface of a water-insoluble substrate and has traditionally been used for production of food like tempeh and sauerkraut (Voidarou et al., 2020). Submerged fermentation is a more lab-friendly process suited for a variety of bioreactors and is easier to scale up (Sekoai et al., 2024).

The most well-known SCP producers utilizes continuous fermentation, where nutrient concentrations can be controlled and maintained by continuously adding as much culture medium as is removed. A well-known example is the company Quorn growing the fungi *Fusarium venenatum* on glucose and ammonia in 150 m^3 bioreactors in which the nutrient concentrations are continuously monitored and controlled (Sobhi et al., 2023).

In addition to the substrate components already mentioned, process conditions also have a huge impact on the SCP production process, such as dissolved oxygen, pH, temperature and incubation period (Reihani & Khosravi-Darani, 2019). Using a bioreactor allows for better control of these parameters when compared to other systems such as shake flasks. Finding optimal values for the parameters to be controlled in the bioreactor is important, while also being aware of the interplay between these. Increasing stirring speed is often necessary to reach the desired dissolved oxygen concentration or for sufficient mixing of nutrients, but excess stirring can also induce shear stress on the cells, so a balance must be found (Doran, 2012).

The k_La (volumetric mass transfer coefficient) is a measurement of how efficient oxygen is transferred into the liquid medium from gas phase when being introduced into a bioreactor. This transfer will depend on medium composition, stirring speed and design of the impellers and aeration rate (Doran, 2012). The mass transfer coefficient, k_L, represents a sum of a series of resistances during this transfer, such as the resistance caused by the liquid film surrounding the air bubbles. Multiplying the k_L with the total area of the air bubbles in the media, “a”, results in the k_La (Larsson, 2020). During aerobic fermentation the efficiency of this oxygen transfer is of importance because the cells can only utilize the dissolved oxygen. Additionally, dissolved oxygen can often be the limiting substrate, and therefore determine the overall efficiency of the fermentation (Doran, 2012). The k_La is an important factor in process development and scaling-up. There are several different methods for calculating the k_La based on the equipment available (Aroniada et al., 2020).

1.9 Single cell protein market growth

The SCP market is predicted to reach 18.5 billion dollars by 2030, based on the increasing market growth for the past years (Koukoumaki et al., 2024). Within the feed market sector, SCP has the highest growth potential (Areniello et al., 2023).

Also, the number of publications related to SCP over the last decade has greatly increased; from 2011 to 2021 the number of annual publications tripled (Sobhi et al., 2023).

1.10 Yeast

Fungi is a highly diverse kingdom of heterotrophic eukaryotic microbes being used in a variety of biotechnological applications, such as production of drugs or enzymes (Li et al., 2023). Yeast is a unicellular fungi found naturally in a variety of habitats (Jach et al., 2022). Yeast dry weight biomass consists of 35%-60% protein, and has an almost optimal amino acid composition in terms of being used as a protein source for feed and food, limited only by the low contents of sulphur-containing amino acids methionine and cysteine (J. Ma et al., 2023). Yeasts also contain less nucleic acids than bacteria. Other advantages of using yeast to produce SCP is low contamination risk due to their rapid proliferation, and practical cell size and flocculation abilities which facilitates harvesting (Lapeña, Kosa, et al., 2020).

Cyberlindnera jadinii, previously known as *Candida utilis,* is a well-known yeast with a generally regarded-as-safe (GRAS) status and documented health-promoting effects (Sharma et al., 2018). *C. jadinii* is also recognized for its high protein content of more than 50% (Carranza-Méndez et al., 2022). There have been numerous studies exploring growing *C. jadinii* for SCP on different substrates (Jach et al., 2022), and its use in supplements or food additives has been marketed for more than 70 years.

Wickerhamomyces anomalus, previously known as *Hansenula anomala* and *Pichia anomala,* is a yeast able to grow in diverse environments under wide ranges of pH (2.0 – 12.4) and temperatures (3°C – 37°C) (Fredlund et al., 2002). *W. anomalus* is also well-known as a biocontrol agent, utilizing several antimicrobial mechanisms, such as producing compounds

like organic acids, ethanol and glycerol (Godana et al., 2024). Its protein content has been found to be similar to that of *C. jadinii* at around 50% (Lapeña, Kosa, et al., 2020).

Blastobotrys adeninivorans, also known as *Arxula adeninivorans*, is a yeast able to tolerant high temperatures and osmotic concentrations. *B. adeninivorans* has the ability to utilize a broad range of substrates, including aromatic compounds (Lapeña, Kosa, et al., 2020).

1.10.1 Yeast metabolism

The metabolic pathways in different types of yeast are highly similar. However, there are great differences in nutrient uptake and regulation of aerobic and anaerobic processes. Since yeast are heterotrophic, carbon acts as both a source for gaining ATP and for use in biosynthesis, and they are able to utilize a wide range of carbon sources, such as organic acids and alcohols, but have been found to prefer sugars (Rodrigues & Ludovico, 2006).

Different yeast species differ in how sugar is metabolized, and can be categorized as non-, facultative- or obligate-fermentative, meaning, respectively, that in the presence of glucose the yeast either do not exhibit, sometimes exhibit, or always exhibit alcohol fermentation. For the facultative-fermentative yeasts, sugar concentration and oxygen availability are factors influencing the regulation of aerobic respiration and alcohol fermentation. The Pasteur effect initiates alcohol fermentation when oxygen is unavailable, while the Crabtree effect initiates alcohol fermentation under aerobic conditions when glucose is in excess (Rodrigues & Ludovico, 2006).

Nutrients are taken up into the yeast cells by transport proteins in the cell membrane. These transporters are regulated based on the concentration of the specific nutrient. For the carbon source, such as glucose, the molecules are then used in a series of catabolic reactions through the pathways of glycolysis and the citric acid cycle. In these pathways, glucose is oxidized through a series of steps, generating all required compounds used for anabolic biosynthesis. The glucose is also used to generate energy, and for an aerobic process this is done by capturing the electrons from the oxidation steps and reducing the electron acceptors NAD^+/ $NADP^+$ to NADH/NADPH, which are then used to reduce oxygen leading to the formation of the energy molecule ATP. CO_2 is generated during the reduction of NAD^+/$NADP^+$ and is excreted as a waste product. For anaerobic processes, alternative electron acceptors other than

oxygen may be used. When not using oxygen, the amount of conserved energy is decreased leading to slower growth. Yeast utilizing anaerobic processes may therefore compensate by increasing the uptake and oxidization of the carbon source (Larsson, 2020).

The end product of glycolysis, in addition to ATP and NADH, is pyruvate, which is the starting point for the citric acid cycle where it is oxidized to CO_2. However, during anaerobic or high glucose concentration conditions the pyruvate may also be transformed to ethanol (Flores et al., 2000), at the cost of being less energetically favourable (Pronk et al., 1996). For SCP production, all formation of side-products (such as ethanol and acetate) means a loss in yield and should be minimized (Ugalde & Castrillo, 2002).

1.11 Aim of this study

The aim of this study was to investigate the performance of the yeasts *Blastobotrys adeninivorans, Cyberlindnera jadinii, Wickerhamomyces anomalus* in production of SCP in bioreactors. This included testing different media compositions and process parameters to optimize the production of yeast cells. Finally, based on these findings a continuous cultivation process was designed for SCP production for the best performing yeast.

2. Materials and method

2.1 Equipment

Table 1. Equipment and corresponding supplier used for this thesis.

Category	Equipment	Supplier
Appliances	Freezer, -20˚C	Bosch
	Freezer, -80˚C	Thermo Fisher Scientific
	Minitron incubation shaker	Infors HT
	Laminar Flow Workbench	Telstar
	Certoclav Sterilizer, A-4050	CertoClav
	Mixer table	Velp Scientifica
	Peristaltic pump	Cole-Parmer
	Freeze drier, DW 6-85	Heto-Holten A/S
	Measuring scale	Sartorius
Miscellaneous	Petri dishes, 9 cm	Heger
	Inoculation loop, 10 µL	Sarstedt
	Duetz-microtiter plate system	Enzyscreen
	Super Safe Sampler	Infors HT
	eve® bioprocess software	Infors HT
	96-welll microtiter filter plates	Merck Millipore
	STERITOP® filtration system, 0.2 µm	Merck Millipore
Instruments and columns	pH meter	inoLab
	EasyFerm Plus pH probe	Hamilton
	1.5 L stirred-tank Minifors 2 bioreactor	Infors HT
	2 L stirred-tank Minifors 1 bioreacto	Infors HT
	Ultimate 3000 HPLC	Thermo Fisher Scientific
	Rezex ROA-organic H^+, 300 x 7.8 mm column	Phenomenex
	RI-101 refractive index detector	Shodex
	OD600 meter	Amersham Biosciences
	VisiFerm DO probe	Hamilton
	BlueVary analyzer	Bluesens
	DUMAS	
	6890N Network Gas Chromatograph	Agilent Technologies
	7697A Headspace Sampler	Agilent Technologies
	CP-SIL 5CB column	Agilent Technologies
	Dionex ICS-6000	Thermo Fisher Scientific
	PA210-Fast-4 µm column	Thermo Fisher Scientific

2.2 Chemicals

Table 2. Chemicals and corresponding supplier used for this thesis.

Chemical	**Supplier**
Yeast extract peptone dextrose	Sigma
Agar powder	VWR
Yeast extract	Millipore
Glycerol ($C_3H_8O_3$)	Sigma
Glucose ($C_6H_{12}O_6$)	ITW Reagents
Meat peptone	Millipore
Bacterial peptone	Thermo Fisher Scientific
Yeast nitrogen base	Thermo Fisher Scientific
Ammonium sulphate ($(NH_4)_2SO_4$)	Sigma
Urea ($CO(NH_2)_2$)	Sigma
Monosodium glutamate ($C_5H_8NO_4Na$)	MP Biomedicals
Monosodium phoshpate (NaH_2PO_4)	Sigma
Xylose ($C_5H_{10}O_5$)	Sigma
Arabinose ($C_5H_{10}O_5$)	Sigma
Mannose ($C_6H_{12}O_6$)	Sigma
EDTA (Na) ($C_{10}H_{14}N_2Na_2O_8$-$2H_2O$)	Sigma
Calcium Chloride ($CaCl_2$-$2H_2O$)	Sigma
Zinc sulphate ($ZnSO_4$-$7H_2O$)	Sigma
Iron(II) sulphate ($FeSO_4$-$7H_2O$)	Sigma
Boric acid (H_3BO_3)	VWR
Manganese(II) chloride ($MnCl_2$-$4H_2O$)	Sigma
Sodium molybdate (Na_2MoO_4-$2H_2O$)	Sigma
Colbalt(II) chloride ($CoCl_2$-$6H_2O$)	Sigma
Copper(II) sulphate ($CuSO_4$)	VWR
Potassium iodide (KI)	VWR
Vitamin B10 ($C_7H_7O_2$)	Sigma
Vitamin B3 ($C_6H_5NO_2$)	Sigma
Vitamin B5 ($C_9H_{17}NO_5$)	Sigma

Vitamin B6 ($C_8H_{11}NO_3$)	Sigma
Vitamin B1 ($C_{12}H_{17}N_4OS$)	Sigma
Biotin ($C_{10}H_{16}N_2O_3S$)	Sigma
Histidine ($C_6H_9N_3O_2$)	Sigma
Leucine ($C_6H_{13}NO_2$)	Sigma
Lysine ($C_6H_{14}N_2O_2$)	Sigma
Methionine ($C_5H_{11}NO_2S$)	Sigma
Phenylalanine ($C_9H_{11}NO_2$)	Sigma
Serine ($C_3H_7NO_3$)	Sigma
Threonine ($C_4H_9NO_3$)	Sigma
Uracil ($C_4H_4N_2O_2$)	Sigma
Sulphuric acid (H2SO4)	Millipore
Sodium hydroxide (NaOH)	Sigma
Clerol	PMC Ouvrie
Glanapon	Busetti
Sodium chloride (NaCl)	Sigma

2.3 Microorganisms

2.3.1 Strain ID

The yeast strains used in this thesis were *Blastobotrys adeninivorans* (LS3, from Swedish University of Agricultural Sciences, Uppsala, Sweden)*, Cyberlindnera jadinii* (LYCC 7549, from Lallemand Yeast Culture Collection, Montreal, Canada)*, Wickerhamomyces anomalus* (CBS100487, from Swedish University of Agricultural Sciences, Uppsala, Sweden) and *Thermosacc® Dry* (from Lallemand Yeast Culture Collection, Montreal, Canada).

2.3.2 Yeast glycerol stocks

To make freezer stocks, pre-existing -80˚C stocks of *B. adeninivorans, C. jadinii, W. anomalus* and *Thermosacc® Dry* were streaked onto two YPD agar plates each. YPD agar

plates were made using 50 g/L YPD broth powder (Sigma) and 20 g/L agar powder (VWR). After autoclaving at 121˚C for 15 minutes, the medium was poured in 9 cm petri dishes in a laminar flow cabinet for ~20 minutes until the medium set. After streaking, the plates were incubated at 30˚C for 24 hours.

A single colony from each plate was picked with a 10 µL inoculation loop and used to inoculate sterile test tubes with 5 mL of YPD medium. YPD medium was prepared using 50 g/L YPD broth powder (Sigma) and then autoclaved at 121˚C for 15 minutes.

The test tubes were incubated at 30˚C for 24 hours with orbital shaking at 180 rpm.

~10 cryovials for each microbe were prepared using 375 µL of 80% glycerol (final concentration of 20% (v/v)) with 1.125 mL of overnight culture, and then kept in a -80˚C freezer.

2.4 Media and buffer preparation

2.4.1 Glucose solution

333 g glucose was mixed 1 L of milli-Q water. The solution was autoclaved for 15 minutes at 121°C.

2.4.2 YPD

50 g of YPD broth powder was mixed with 1 L of milli-Q water. The solution was autoclaved for 15 minutes at 121°C.

2.4.3 Yeast extract and meat peptone solution (YP)

To achieve varying C/N ratios (2, 3.5 or 6) when combining YP with a carbon source, different YP solutions were made. See Table 3 for media components.

Table 3. YP solutions with varying concentrations to achieve different C/N ratios.

C/N ratio (g_C/g_N) *	Yeast extract (g/L)	Meat peptone (g/L)	Milli-Q water (mL)
2.0	22.935	16.235	50
3.5	26.190	18.540	1000
6.0	7.683	5.438	50

pH was adjusted to 5.0. All solutions were autoclaved for 15 minutes at 121°C.

For the bioreactor experiments using only YP as nitrogen source, 52.38 g yeast extract and 37.08 g meat peptone were added directly into, and autoclaved with, the bioreactor.

2.4.4 Yeast extract and bacterial peptone solution (YBP)

13.095 g yeast extract and 9.21 g of bacterial peptone were mixed with 50 mL milli-Q water. The solution was autoclaved for 15 minutes at 121°C.

2.4.5 Yeast nitrogen base solution (YNB)

12 g yeast nitrogen base (without amino acids and ammonium sulphate) was mixed with 400 mL milli-Q water. The whole solution was poured directly into, and autoclaved with, the bioreactor.

2.4.6 Yeast nitrogen base and ammonium sulphate solution (YNBAS)

1.7 g of yeast nitrogen base (Thermo Fischer Scientific) and 26.93 g of ammonium sulphate (Sigma) were mixed with 1 L milli-Q water. pH was adjusted to 5.0. The solution was autoclaved for 15 minutes at 121°C.

2.4.7 Yeast nitrogen base and urea (YNBU)

1.7g of yeast nitrogen base (Thermo Fischer Scientific) and 12.25g of urea (Sigma) were mixed with 1 L milli-Q water. pH was adjusted to 5.0. The solution was autoclaved for 15 minutes at 121°C.

2.4.8 YP and ammonium sulphate solution (YPAS)

Two solutions were made: one solution of 75% YP with 25% ammonium sulphate, as well as a 50% YP with 50% ammonium sulphate solution.

26.24 g yeast extract, 18.57 g meat peptone and 26.93 g ammonium sulphate were mixed with 600 mL milli-Q water. The whole solution was poured directly into, and autoclaved with, the bioreactor.

39.36 g yeast extract, 27.86 g meat peptone and 13.49 g ammonium sulphate were mixed with 600 mL milli-Q water. The whole solution was poured directly into, and autoclaved with, the bioreactor.

2.4.9 Urea solution

61.24 g urea was mixed with 200 mL milli-Q water. The solution was sterile filtered through a STERITOP® filtration system with 0.2 µm pore size and 45 mm neck size.

2.4.10 Mono sodium glutamate solution (MSG)

4.5 g monosodium glutamate was mixed with 100 mL milli-Q water. The solution was autoclaved for 15 minutes at 121°C.

2.4.11 Ammonium sulphate and monosodium phosphate solution

0.1 g ammonium sulphate and 0.1 g monosodium phosphate were mixed with 100 mL milli-Q water. The solution was autoclaved for 15 minutes at 121°C.

2.4.12 Spruce model sugar solution

3.9 g glucose, 0.67 g xylose, 0.21 g arabinose and 1.58 g mannose were mixed with 100 mL milli-Q water. The solution was sterile filtered through a STERITOP® filtration system with 0.2 µm pore size and 45 mm neck size.

2.4.13 Birch model sugar solution

3.5 g glucose, 1.8 g xylose, 0.2 g arabinose and 0.278 g mannose were mixed with 100 mL milli-Q water. The solution was sterile filtered through a STERITOP® filtration system with 0.2 µm pore size and 45 mm neck size.

2.4.14 Trace elements solution

1.5 g EDTA (Na), 0.28 g $CaCl_2$-$2H_2O$, 0.45 g $ZnSO_4$-$7H_2O$, 0.549 g $FeSO_4$-$7H_2O$, 0.1 g H_3BO_3, 0.6 g $MnCl_2$-$4H_2O$, 0.035 g Na_2MoO_4-$2H_2O$, 0.04 g $CoCl_2$-$6H_2O$, 0.02 g $CuSO_4$ and 0.01 g KI were added to 500 mL of milli-Q water. The solution was sterile filtered through a STERITOP® filtration system with 0.2 µm pore size and 45 mm neck size.

2.4.15 Vitamin solution

0.02 g vitamin B10, 0.1 g vitamin B3, 0.1 g vitamin B5, 0.1 g vitamin B6, 0.1 g vitamin B1 and 0.005 g biotin was added to 100 mL of milli-Q water. The solution was sterile filtered through a STERITOP® filtration system with 0.2 µm pore size and 45 mm neck size.

2.4.16 Amino acid mix

0.525 g histidine, 1.65 g leucine, 1.8 g lysine, 0.6 g methionine, 0.75 g phenylalanine, 0.565 g serine, 0.3 g threonine and 0.6 g uracil were mixed with 500 mL milli-Q water. The solution was sterile filtered through a STERITOP® filtration system with 0.2 µm pore size and 45 mm neck size.

2.4.17 Calculating C/N ratios

C/N ratios were calculated based on the molecular weights of the carbon and nitrogen sources. This was done by first finding the molecular weight of the carbon source, then determining the percentage of carbon by using the molecular weight of carbon (12 g/mol). The carbon concentration of the carbon source was then determined. This carbon concentration was then divided by the desired C/N ratio, giving the nitrogen concentration needed from the nitrogen source.

For the undefined nitrogen sources, like yeast extract, the percentage of nitrogen was retrieved from their respective providers. For the other nitrogen sources, like ammonium sulphate, the molecular weight of the nitrogen source and the molecular weight of nitrogen (14 g/mol) was used to determine the percentage of nitrogen. The percentage of nitrogen from the nitrogen source and the nitrogen concentration needed was then used to determine the concentration of the nitrogen source needed.

To illustrate how the C/N ratio was calculated, two sample calculations for yeast extract and ammonium sulphate are outlined below (Equation 1).

Glucose contains six carbons with the molecular weight of carbon being 12 g/mol. The molecular weight of glucose is 180 g/mol.

$$\frac{(6 * 12\ g/mol)}{180\ g/mol} = 0{,}4 = 40\%$$

(1)

Using a glucose concentration of 100 g/L results in 40 g/L carbon. To achieve a C/N ratio of 3.5 the concentration of nitrogen needed can be calculated (Equation 2).

$$\frac{40\ g/L}{3.5} = 11.43\ g/L$$

(2)

11.43 g/L of nitrogen will result from a 50/50 mix of YP and ammonium sulphate, meaning 5.71 g/L nitrogen from YP and 5.71 g/L nitrogen from meat peptone. For YP 2.86 g/L nitrogen will come from yeast extract and 2.86 g/L nitrogen will come from meat peptone. Yeast extract and meat peptone contain 10.9% and 15.4% nitrogen, respectively. The concentrations of the compounds needed to reach the desired nitrogen concentrations can then be calculated (Equation 3 and 4).

$$\frac{10.9\%}{100\%} = \frac{2.86\ g/L}{x}$$

(3)

$$\frac{15.4\%}{100\%} = \frac{2.86\ g/L}{x}$$

(4)

This makes the concentrations of yeast extract and meat peptone needed at 26.24 g/L and 18.57 g/L, respectively.

Since ammonium sulphate is a defined chemical compound, its molecular weight (132.14 g/mol), its number of nitrogen atoms per molecule and the molecular weight of nitrogen (14 g/mol) is needed to calculate its nitrogen content (Equation 5).

$$\frac{2 * 14\ g/mol}{132.14\ g/mol} = 0.0212 = 21.2\%$$

(5)

The ammonium sulphate concentration needed (26.93 g/L) to reach the desired nitrogen concentration can then be calculated (Equation 6).

$$\frac{21.2\%}{100\%} = \frac{2.86\ g/L}{x}$$

(6)

2.5 Microtiter plate screening

All conditions, expect YPD, contained a carbon-to-nitrogen (C/N) ratio of 3.5, with a glucose concentration of 10 g/L. The C/N ratio of YPD was 4.8. Two of the carbon sources were prepared to simulate pretreated (by acid hydrolysis) spruce and birch substrates.

A total of 7 different media were prepared: yeast extract and meat peptone plus spruce model sugar (YP + S), yeast extract and meat peptone plus birch model sugar (YP + B), yeast nitrogen base and ammonium sulphate plus spruce model sugar (YNBAS + S), yeast nitrogen base and ammonium sulphate plus birch model sugar (YNBAS + B), yeast nitrogen base and urea plus spruce model sugar (YNBU + S), yeast nitrogen base and urea plus birch (YNBU + B) and YPD. See Table 4 for detailed media compositions.

200 µL of -80˚C freezer stock was added to 25 mL of YPD medium in 250 mL baffled shake flasks and incubated at 30˚C with orbital shaking at 220 rpm for ~16 hours.

The OD600 of the overnight culture was measured and used to obtain a starting OD600 of 0.5 in the wells by varying the amount of culture and milli-Q water added to the media.

The experiment was done in a Duetz-microtiter plate system (Duetz-MTPS) consisting of 24-square polypropylene deep well plates (11 mL well volume), sandwich covers and cover clamps, which were mounted in a shaker. The total volume for each well was 2.5 mL. Prior to addition of media and cultures, the microtiter plates were autoclaved at 121°C for 15 minutes.

Three wells were assigned to each type of media in order to have triplicate measurements. Each of the replicates had one well assigned to each sampling point, for a total of four wells for the four samples taken. This resulted in a total of 84 wells per strain. The full contents of the wells (2.5 mL) were taken as samples at 0, 3, 6, and 24 hours, and then centrifuged for 5 minutes at 10,000 g. The samples were used for measuring OD600, pH, and cell dry weight (CDW), and for high pressure liquid chromatography (HPLC) analysis as outlined in section 2.6.

These steps were repeated for each yeast, *B. adeninivorans*, *C. jadinii* and *W. anomalus.*

Table 4. Composition of the different media variants used in the microtiter plate experiment.

	YP + S	YNBAS + S	YNBU + S	YP + B	YNBAS + B	YNBU + B	YPD
C/N ratio (g_C/g_N)	3.5	3.5	3.5	3.5	3.5	3.5	4.8
Glucose (g/L)	10	10	10	10	10	10	10
Xylose (g/L)	1.71	1.71	1.71	5.14	5.14	5.14	
Arabinose (g/L)	0.54	0.54	0.54	0.57	0.57	0.57	
Mannose (g/L)	4.05	4.05	4.05	0.79	0.79	0.79	
Yeast extract (g/L)	5.24			5.24			5
Peptone (g/L)	3.71			3.71			10
YNB (g/L)		0.34	0.34		0.34	0.34	
Ammonium sulphate (g/L)		5.39			5.39		
Urea (g/L)			2.45			2.45	

2.6 Shake flask experiments

2.6.1 Investigating effects of C/N ratio, glucose concentration, peptone variants and antifoam in *C. jadinii* and *W. anomalus* cultivations

Different media compositions were tested for both *C. jadinii* and *W. anomalus*, all of which contained 100% YP as the nitrogen source and glucose as the carbon source. C/N ratio (2, 3.5 and 6), bacterial peptone compared to meat peptone and initial glucose concentration (50 and 100 g/L) were tested for both microbes. Additionally, the effect of antifoam on the *W. anomalus* cultivations in shake flasks were investigated by comparing three conditions: Clerol antifoam, Glanapon antifoam and no antifoam. Working volume was 50 mL using 250 mL shake flasks autoclaved at 121˚C for 15 minutes. See Table 5 and Table 6 for a detailed media description.

The shake flasks were incubated for 24 hours at 30˚C at 200 rpm in a shaker.

Samples were taken at 0, 2, 4, 6, 8, 10, 12, and 24 hours. The samples were used for measuring CDW and for HPLC analysis.

The experiment was repeated with the nitrogen source from the 3.5 C/N condition being changed to 75% YP and 25% ammonium sulphate. A washing step was included. Samples were taken at 0, 2, 4, 6, 12, 14, and 16 hours.

Table 5. Composition of the media used for *C. jadinii* shake flask fermentations. YP = 50% yeast extract + 50% meat peptone, YBP = 50% yeast extract + 50% bacterial peptone.

C. jadinii	**C/N (g_C/g_N)**	**Glc (g/L)**	**N source**
1	3.5	100	YP
2	2.0	100	YP
3	6.0	100	YP
4	3.5	100	YBP
5	3.5	50	YP

Table 6. Composition of the media used for W. anomalus shake flask fermentations. YP = 50% yeast extract + 50% meat peptone, YBP = 50% yeast extract + 50% bacterial peptone.

W. anomalus	**C/N**	**Glc (g/L)**	**N source**	**Antifoam, mL**
1	3.5	100	YP	1:10 Clerol, 0.5
2	2.0	100	YP	1:10 Clerol, 0.5
3	6.0	100	YP	1:10 Clerol, 0.5
4	3.5	100	YBP	1:10 Clerol, 0.5
5	3.5	50	YP	1:10 Clerol, 0.5
6	3.5	100	YP	1:10 Glanapon, 0.5
7	3.5	100	YP	No antifoam

2.6.2 Freeze stock experiment

250 mL baffled empty shake flasks were autoclaved at 121°C for 15 minutes. 250 µL of reference *C. jadinii* and *W. anomalus* freeze stock culture was added to 2 shake flasks each with a working volume of 50 mL containing a glucose concentration of 50 g/L and a C/N ratio of 3.5, and to 2 shake flasks with a working volume of 50 mL containing YPD media with a glucose concentration of 50 g/L. For comparison, 250 µL of *C. jadinii* and *W. anomalus* freeze stock prepared by the author were added to 2 250 mL baffled shake flasks with 50 mL YPD media.

The experiment was performed in two rounds with samples taken at 0, 4, 6, 8, and 24 hours for the first set of experiments and at 0, 12, 14, 16, 18 hours for the second experiment. The samples were used for measuring OD600, CDW and for HPLC analysis as outlined in section 2.6.

2.7 Cultivation in 1.5 L bioreactors

2.7.1 Inoculum preparation

The inoculums for the bioreactors were prepared from -80°C glycerol stocks. 500 µL of thawed stock culture was added directly to 500 mL baffled shake flasks containing 100 mL

YPD media. The microbes were incubated at 30°C with orbital shaking at 220 rpm for ~16 hours.

2.7.2 Bioreactor

For the bioreactor experiments, 1.5 L stirred-tank Minifors 2 bioreactors were used. A bioreactor provides a closed and controlled environment with automatic monitoring of parameters and the ability to regulate these parameters. These parameters commonly include pH, temperature, and dissolved oxygen (DO) and are monitored automatically by sensors.

The pH was automatically regulated to the set value by additions of 5 M sulfuric acid and 5 M sodium hydroxide and was monitored by an EasyFerm Plus pH probe.

The temperature was regulated to the set value using an electric heating block with built-in cooling spiral and was monitored using a Pt100 sensor.

The DO was regulated to the set value by the flow rate of air into the bioreactor (aeration rate) and the stirring speed of the impellers. The DO was monitored by a VisiFerm DO probe.

To regulate the DO, a cascade feature was used, increasing the stirring speed up to a set maximum rpm to maintain the set DO value. The bioreactors were equipped with two 6-bladed Rushton impellers. In some experiments the aeration rate was included in the cascade feature, increasing to a set maximum (vessel volume per minute, vvm).

To avoid foaming, a sensor was used at a set height inside the bioreactor. If foam would reach the sensor, the system automatically pumps in 1:10 Clerol antifoam to the medium.

To monitor the carbon dioxide produced from the fermentation a BlueVary analyzer was used, equipped with infrared sensors measuring the percentage of carbon dioxide in the off-gas leaving the bioreactor. The infrared light is reflected by the gas in the analyzer, and the weakened light is measured by a detector. Before entering the detector, the off-gas was filtered through a 0.2 µm PTFE filter attached to a condenser.

Aseptic sampling from the bioreactor was done using a Super Safe Sampler, equipped with integrated non-return valves avoiding contamination of the bioreactor medium. The sample is

extracted directly from the bioreactor using a syringe, and the remaining liquid in the tube can be pushed back into the bioreactor by sterile filtrated air.

For the monitoring and collection of fermentation data from the bioreactors the bioprocess platform software eve® was used.

2.7.2.1 Set-up of bioreactor and selected parameters

The set-up of the bioreactors after autoclaving before each fermentation included adding 5M sulphuric acid and 5M sodium hydroxide to dedicated glass bottles and connecting the tubing via peristaltic pumps to ports in the bioreactor headplate. The tubes with acid, base and antifoam were then purged, filling the tube completely with liquid. All sensors were then connected to the bioreactor. Stirring speed, temperature, aeration rate, pH and antifoam control were turned on and let run for ~30 minutes before the pO_2 probe was calibrated. The DO and cascade function of the stirring speed and/or aeration rate was turned on right after inoculation.

Unless otherwise stated, the bioreactor parameters used for the fermentations were 30°C, pH of 5, aeration rate of 1 vvm and DO=30%. Stirring speed was controlled with a cascade function between 300 – 1250 rpm. Working volume was 1 L.

2.7.3 Batch cultivations

2.7.3.1 Cultivation of *B. adeninivorans, C. jadinii* and *W. anomalus* using 100 g/L glucose and 50% organic nitrogen source

The media was made to have a C/N ratio of 3.5, where the nitrogen source consisted of 50% nitrogen from YP (50% nitrogen from yeast extract, and 50% nitrogen from meat peptone), and 50% nitrogen from ammonium sulphate.

Inoculum was prepared as previously described in Section 2.7.1 using media containing the same concentrations as the media used during the bioreactor cultivations.

Four bioreactors were set up with the parameters as previously described in Section 2.7.2.1. Each microbe was assigned to one bioreactor, with *C. jadinii* being run in parallel in two bioreactors.

Samples were taken at 0, 2, 4, 6, 10, and 24 hours, with an extra sampling point for the *B. adeninivorans* fermentation at 48 hours. After removal of the supernatant, the cells were washed twice with 0.9% NaCl. The samples were used for measuring CDW and for HPLC analysis.

2.7.3.2 Cultivation of *C. jadinii* and *W. anomalus* using 100 g/L glucose and 100%/75% organic nitrogen source

To investigate the importance of organic nitrogen sources, another experiment was set up where, instead of 50% organic nitrogen, 100% and 75% organic nitrogen was used (here *B. adeninivorans* was not included).

The media had a C/N ratio of 3.5, where the nitrogen source consisted of 100% or 75% nitrogen from YP (50% nitrogen from yeast extract, and 50% nitrogen from meat peptone), and 0% or 25% nitrogen from ammonium sulphate. The initial glucose concentration was set to 100 g/L.

Inoculum was prepared as previously described in Section 2.7.1 using media containing the same concentrations as the media used during the fermentation.

Four bioreactors were set up with the parameters as previously described in Section 2.7.2.1 but with the aeration rate set up in a cascade of 1.0-1.5 vvm. The bioreactors were set up with the following conditions: *C. jadinii* (100% YP), *W. anomalus* (100% YP), *C. jadinii* (75% YP) and *W. anomalus* (75% YP).

Samples were taken at 0, 2, 4, 6, 10, 12, 14, 21, 24 and 48 hours. After removal of the supernatant, the cells were washed twice with 0,9% NaCl. The samples were used for measuring CDW and for HPLC analysis.

2.7.3.3 Cultivation of *C. jadinii* and *W. anomalus* using 100 g/L and 50 g/L glucose

To investigate possible effects on ethanol production (i.e. Crabtree-effect), the starting glucose concentration was lowered.

The media had a C/N ratio of 3.5, where the nitrogen source consisted of 100% nitrogen from YP (50% nitrogen from yeast extract, and 50% nitrogen from meat peptone). The glucose concentration was set to 100 g/L or 50 g/L.

Inoculum was prepared as previously described in Section 2.7.1 but with an incubation time of 11 and 13 hours for *W. anomalus* and *C. jadinii*, respectively.

Four bioreactors were set up with the parameters as previously described in Section 2.7.2.1 but with the aeration rate set up in a cascade of 1.0-2.0 vvm. The bioreactors were set up with the following conditions: *C. jadinii* (100 g/L glucose), *W. anomalus* (100 g/L glucose), *C. jadinii* (50 g/L glucose) and *W. anomalus* (50 g/L glucose).

Samples were taken at 0, 2, 4, 6, 8, 10, 12, 22, 24 and 26 hours. After removal of the supernatant, the cells were washed twice with 0.9% NaCl. The samples were used for measuring CDW and for HPLC analysis.

2.7.3.4 Cultivation of *W. anomalus* using 100 g/L glucose and 100%/50% organic nitrogen source, with lower agitation rates

The media had a C/N ratio of 3.5, where the nitrogen source consisted of 100% or 50% nitrogen from YP (50% nitrogen from yeast extract, and 50% nitrogen from meat peptone), and 0% or 50% nitrogen from ammonium sulphate. The glucose concentration was set to 100 g/L.

Inoculum was prepared as previously described in Section 2.7.1 but with an incubation time of 11 hours.

Two bioreactors were set up with the parameters as previously described in Section 2.7.2.1 but with the aeration rate set up in a cascade of 1.0-2.0 vvm and the maximum rpm set at 900.

The bioreactors were set up with the following conditions: *W. anomalus* (100% YP) and *W. anomalus* (50% YP).

Samples were taken at 0, 24, 26, 28, 30, 32, 48, 72 and 96 hours. After removal of the supernatant, the cells were washed twice with 0.9% NaCl. The samples were used for measuring CDW and for HPLC and high-performance anion-exchange chromatography analysis (HPAEC).

2.7.3.5 Cultivation of *W. anomalus* at 100 g/L and 50 g/L glucose using urea as nitrogen source

The media had a C/N ratio of 3.5, where the nitrogen source consisted of 100% or 50% of nitrogen from YP (50% nitrogen from yeast extract, and 50% nitrogen from meat peptone), and 0% or 50% nitrogen from urea. The glucose concentration was set to 100 g/L or 50 g/L glucose.

Inoculum was prepared as previously described in Section 2.7.1 but with an incubation time of 11 hours.

Two bioreactors were set up with the parameters as previously described in Section 2.7.2.1 but with the aeration rate set up in a cascade of 1.0-2.0 vvm and the maximum rpm set at 900. The bioreactors were set up with the following conditions: *W. anomalus* (100 g/L glucose with 50% YP and 50% urea) and *W. anomalus* (50 g/L glucose with 100% YP).

Samples were taken at 0, 2, 4, 6, 8, 12, 24, 31,5 and 48 hours. After removal of the supernatant, the cells were washed twice with 0.9% NaCl. The samples were used for measuring CDW and for HPLC analysis.

2.7.3.6 Cultivation of *W. anomalus* using vitamin and trace solution, YP adjustments and defined media

In this experiment the effects of adding a vitamin and trace solution, increasing the amount of meat peptone in relation to the yeast extract and using a defined media was investigated. The defined media, a high cell density media, was made in accordance to (Roberts et al., 2020).

The first media recipe contained 100 g/L glucose and a C/N ratio of 3.5, with the nitrogen source being YP (50% nitrogen from yeast extract, and 50% nitrogen from meat peptone). In addition, a vitamin and trace solution were added.

The concentrations of the trace elements were 0.0300 g/L EDTA (Na), 0.0056 g/L $CaCl_2$-$2H_2O$, 0.0090 g/L $ZnSO_4$-$7H_2O$, 0.0109 g/L $FeSO_4$-$7H_2O$, 0.0020 g/L H_3BO_3, 0.0012 g/L $MnCl_2$-$4H_2O$, 0.0007 g/L Na_2MoO_4-$2H_2O$, 0.0008 g/L $CoCl_2$-$6H_2O$, 0.0004 g/L $CuSO_4$ and 0.0002 g/L KI, and the concentrations of the vitamins were 0.0002 g/L vitamin B10, 0.001 g/L vitamin B3, 0.001 g/L vitamin B5, 0.001 g/L vitamin B6 and 0.001 g/L vitamin B1.

The second media recipe contained 100 g/L glucose, with the nitrogen source being YP with a different yeast extract/meat peptone ratio (40 g/L yeast extract, and 60 g/L meat peptone).

The third media was the defined media consisting of 60 g/L glucose, 12 g/L YNB, 4.5 g/L MSG, amino acid mix, 1 g/L ammonium sulphate and 1 g/L monosodium phosphate.

The concentrations of the amino acids were 0.21 g/L histidine, 0.66 g/L leucine, 0.72 g/L lysine, 0.24 g/L methionine, 0.3 g/L phenylalanine, 0.225 serine, 0.12 g/L threonine and 0.24 g/L uracil.

Inoculum was prepared as previously described in Section 2.7.1 but with an incubation time of 12 hours.

Three bioreactors were set up with the parameters as previously described in Section 2.7.2.1 but with the aeration rate set up in a cascade of 1.0-2.0 vvm and the maximum rpm set at 900.

Samples were taken at 0, 2, 4, 6, 8, 12, 22, 24, 48 and 94 hours. After removal of the supernatant, the cells were washed twice with 0.9% NaCl. The samples were used for measuring CDW and for HPLC analysis.

The experiment was repeated for all conditions except for the defined media. For this repeated experiment, the maximum agitation rate was increased to 1250 rpm, and the vitamin/trace solution was now included for both conditions.

2.7.4 Continuous cultivation in bioreactors

A continuous fermentation experiment was conducted, using two Minifors 2 bioreactors with media containing 100 g/L glucose and a C/N ratio of 3.5, with the nitrogen source being YP (50% of nitrogen from yeast extract, and 50% of nitrogen from meat peptone), and including vitamins and trace elements.

The concentrations of the trace elements were 0.0300 g/L EDTA (Na), 0.0056 g/L $CaCl_2$-$2H_2O$, 0.0090 g/L $ZnSO_4$-$7H_2O$, 0.0109 g/L $FeSO_4$-$7H_2O$, 0.0020 g/L H_3BO_3, 0.0012 g/L $MnCl_2$-$4H_2O$, 0.0007 g/L Na_2MoO_4-$2H_2O$, 0.0008 g/L $CoCl_2$-$6H_2O$, 0.0004 g/L $CuSO_4$ and 0.0002 g/L KI, and the concentrations of the vitamins were 0.0002 g/L vitamin B10, 0.001 g/L vitamin B3, 0.001 g/L vitamin B5, 0.001 g/L vitamin B6 and 0.001 g/L vitamin B1.

Inoculum was prepared as previously described in Section 2.7.1 but with an incubation time of 12 hours.

Two bioreactors were set up with the parameters as previously described in 2.4.2.1 but with the aeration rate set up in a cascade of 1.0-2.0, and 2.5 M sulfuric acid and 2.5 M sodium hydroxide used as acid and base.

After 50 hours of batch fermentation, a continuous fermentation process was initiated as outlined below.

Two 2 L bottles with tubing (Figure 1A) were autoclaved at 121°C for 15 minutes. The bottles were filled with 2 L of feed media with a C/N ratio of 3.5, containing 200 g/L glucose, 104.85 g/L yeast extract and 74.21 g/L meat peptone and including the same vitamin and

trace elements with the same concentrations as in the bioreactor medium. The tubing from the bottles were connected to an integrated peristaltic pump (Figure 1B) mounted on each bioreactor and from there onto an addition port on the headplate of the bioreactor (Figure 1C). Another pair of tubes were connected to an outlet leading from a dip tube in the bioreactor and from there to another pair of peristaltic pumps (Figure 1D) leading into two waste bottles (Figure 1E).

Figure 1. Set-up for the continuous fermentation, with feed bottles (A), peristaltic pumps with tubing leading into the bioreactor (B), bioreactors (C), peristaltic pumps with tubing leading from the bioreactors (D) and waste bottles (E).

Pumping of the feed was set to start towards the end of the batch fermentation process, but before complete glucose depletion, with an aim to maintain a glucose concentration in the bioreactor of ~10 g/L. The pumping rate of the feed media into the bioreactor was decided by first looking at the glucose consumption rate from the previous batch fermentation using the same media. The pumping of the feed was initiated after 50 hours.

To calculate the feed pumping rate, the glucose concentration of the feed media (200 g/L), the working volume in the bioreactor (1 L) and the glucose consumption rate (1.875 g glucose per hour) was cross multiplied to give a feed pump rate of 9.375 mL per hour.

After 48 hours after the continuous process was initiated the pump rate for one bioreactor was increased to 14.062 mL per hour. After 72 hours, the pump rates for both bioreactors were increased to 19.062 mL per hour, to investigate the maximum capacity of the system before oxygen would become the limiting factor.

Samples were taken at 0, 4, 12, 29, 50, 72, 96, 117, 144 and 166 hours. After removal of the supernatant, the dry cells were washed twice with 0.9% NaCl. The samples were used for measuring CDW and for HPLC analysis.

2.8 Assessment of k_La

To assess the efficiency of the oxygen transfer from gas to liquid phase in the bioreactor systems used for the cultivation experiments a k_La assessment experiment was set up with three Minifors 2 bioreactors with 1 L milli-Q water. Additionally, three Minifors 1 bioreactors with 1.5 L working volume were also used as to compare the two bioreactor systems.

Each of the three bioreactors were set at different aeration rates of 0.5, 1.0 and 1.5 vvm with an initial agitation rate of 300 rpm. First, air flow into the bioreactors was stopped and nitrogen gas was used to flush out the oxygen. When the pO_2 value reached 0%, air flow was returned until it reached 100%. The pO_2-probe was then calibrated, and the process was repeated for each of the following agitation rates: 400, 500, 600, 700, 800, 900 and 1000 rpm.

For calculating the k_La values the time (s) and pO_2 (%) data from when the pO_2 went from ~10% to ~90% was collected. An example of a data set from one of the cycles is shown in Table 7.

Table 7. Example of raw data collected from one process cycle of the k_La assessment.

time (s)	pO_2 (%)
6126	9.00
6128	13.46
6131	19.01
6134	24.96
6136	29.48
6139	36.16

6141	42.15
6144	48.17
6146	51.02
6149	52.68
6151	54.72
6154	57.14
6157	59.28
6160	61.70
6162	63.62
6165	65.25
6167	66.50
6170	67.58
6172	69.20
6175	72.51
6178	75.96
6180	79.08
6183	81.70
6185	83.86
6188	85.58
6191	86.96
6194	88.05
6196	90.30

Two equations were used for each of these parameters (Equation 7 and 8).

$$\ln\left(\frac{C^* - C1}{C^* - C2}\right) \quad (7)$$

$$t2 - t1 \quad (8)$$

Where, C* is assumed constant and is the saturated dissolved oxygen concentration, which in this case is 100%. C1 is the pO_2 value at a given time point (t1), and C2 is the pO_2 value at another given time point (t2). For equation 7, using the data from Table 7 as an example, the first calculation was done using C2 = 90.3 and C1 = 9, the second calculation was done using C2 = 88.05 and C1 = 13.46, and so on with C2 moving one data point up the column and with

C1 moving one data point down the column. The same was done with the time data using equation 8. These values were put together in Table 8.

Table 8. Example of calculations done for assessing the k_La value of one process cycle.

(t2-t1)	ln((C*-C1)/(C*-C2))
70	2,238733621
66	1,979875456
60	1,826304134
54	1,649405173
49	1,474595695
44	1,249478893
39	1,017147814
34	0,768250023
29	0,577589749
23	0,429418349
19	0,334089918
13	0,246393551
8	0,1585398
2	0,051430723

The values from equation 8 were then plotted against the values from equation 7 to make a dot diagram (Figure 2).

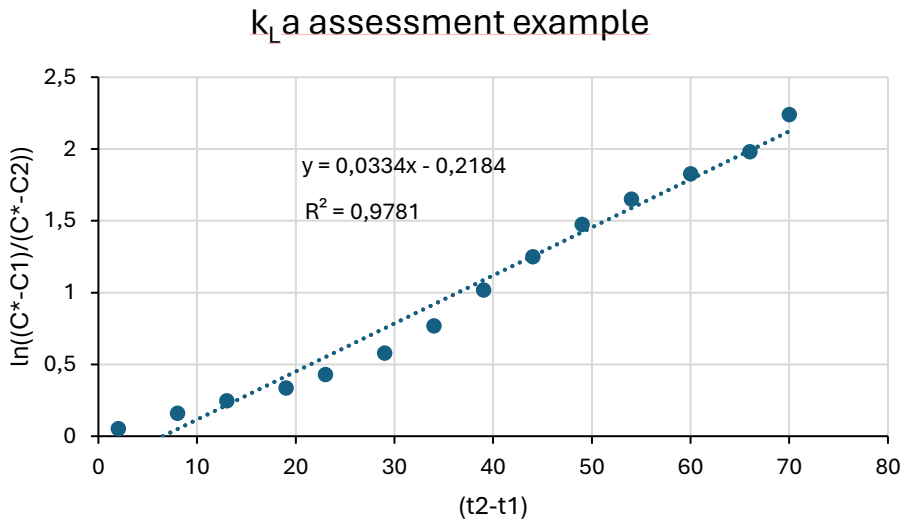

Figure 2. Example of a plot diagram used to assess the k_La value.

A linear trendline was then used to approximate the data. The slope of this curve is equal to the k_La value, which for the example in Figure 2 is 0.0334.

The repeatability of this kLa assessment was investigated by repeating a select few of the process cycles three times. The repeated process cycles were done at an agitation rate of 1000 rpm using an aeration rate of 0.5, 1.0, 1.5 vvm for the Minifors 1, and at an agitation rate of 1000 rpm using an aeration rate of 1.5 vvm for the Minifors 2.

The oxygen transfer rate (OTR) is defined by the k_La value and the difference between C* and C at a given time point as shown Equation 9.

$$OTR = kLa \times (C^* - C) \quad (9)$$

Oxygen concentrations in mmol/L was obtained by first converting the pO_2 (%) values to partial pressure (atm) by dividing by 100. This value was then multiplied by Henry's Law constant, which for oxygen is 0.00130.

2.8 Analytical methods

2.8.1 High pressure liquid chromatography (HPLC)

HPLC was used for analysis of glucose, ethanol, formic acid, acetic acid, propionic acid, and succinic acid on an Ultimate 3000 HPLC with an autoinjector using a Rezex ROA-organic H^+, 300 x 7.8 mm column.

The HPLC was done in reverse phase, using a polar eluent (5 mM H_2SO_4), separating molecules based on polarity so that the most polar molecules exit the column first. A refractive index (RI) detector was used with refractive light being sent through the eluent and analyte, and then compared to the light being sent only through the eluent in a reference cell.

The analytes have distinct retention times, and the height of each peak signal reveals the concentrations based on the standards used.

For each analyte, five standards were prepared with the follow concentrations: 10, 5, 2.5, 1 and 0.5 g/L.

The samples taken for HPLC analysis consisted of 1 mL of supernatant from centrifuged fermentation samples. Dilution of the samples was done only if the glucose concentration was suspected to exceed the highest standard concentration of 10 g/L. 200 µL of sample was then transferred to and filtered through a 96 well microtiter filter plate (0.45 µm pore size). 100 µL of the filtered sample was then transferred to an HPLC vial compatible with the HPLC system.

2.8.2 Cell dry weight (CDW)

The remaining pellets of the fermentation samples after supernatant removal were freeze dried in a Heto-Holten A/S Model DW 6-85 freeze-drier. The samples were frozen to -80°C prior to freeze-drying. The samples were then put in glass jars connected to the freeze-drier. Temperatures in the freeze-drier were kept at -80°C at low pressure (<0.4 mbar) in a vacuum. This makes the ice transition directly into water vapor without going through the liquid phase.

The freeze-dried pellets were then measured on a scale. Using this measurement together with the weight of the empty sample tube and the weight of the total liquid sample before centrifugation, CDW could be calculated using the follow formula:

$$CDW\ \left(\frac{g}{L}\right) = \frac{(Weight\ of\ dried\ pellet\ and\ tube_g - Weight\ of\ empty\ tube_g)}{(Weight\ of\ liquid\ and\ tube_g - Weight\ of\ empty\ tube_g)} \times 1000 \frac{g}{L}$$

(10)

2.8.3 Dumas

Dumas analysis was used to determine the carbon, nitrogen, and sulphur contents of the cells after drying and weighing. The method is based on combusting a sample of known mass at high temperatures (~900˚C) in the presence of oxygen. This releases gases containing carbon, nitrogen and sulphur which are then sent through and separated in columns and then quantified by detectors. To assess the protein content of the cells, the nitrogen content was multiplied by 6.25.

All Dumas analysis was carried out by LabTek, NMBU.

2.8.4 Headspace gas chromatography (HSGC)

HSGC was used for the analysis of ethyl acetate using an Agilent Technologies 7679A automatic headspace sampler and a 6890 series GC system with a flame ionisation detector, where the analytes are ionised by combustion in a hydrogen flame, and then detected by electrodes. 10 g of liquid from the supernatant of each of the centrifuged fermentation samples were transferred to HSGC vials. The HSGC instrument vaporizes the liquid sample by using a temperature program which gradually increases the temperature reaching up to 130˚C. Helium was used as a carrier gas transferring the analytes in gas form through a CP-SIL 5CB column.

2.8.5 High-performance anion-exchange chromatography (HPAEC)

HPAEC was used for the analysis of mannitol, fucose, arabinose, galactose, glucose, xylose, and mannose using an Dionex ICS-6000 system based on the anion exchange principle with a PA210-Fast-4 µm column.

For each analyte, five standards were prepared with the follow concentrations: 0.01, 0.007, 0.005, 0.003 and 0.001 g/L.

High pH is used to promote deprotonation of the analytes. This gives the analytes one or several negative charges which will then interact with the positively charged stationary phase of the columns. The number of negative charges of the molecule will influence the retention time, but also the alignment/spatial configuration of its hydroxyl groups (which is where the protonation occurs).

The samples were prepared and collected in the same manner as the HPLC samples (Section 2.6.1).

3. Results and discussion

3.1 Microtiter plate experiment

To gain some initial insights into growth performance of the three yeasts *B. adeninivorans, C. jadinii* and *W. anomalus* on different carbon and nitrogen sources, a screening experiment in microtiter plates was performed. Microtiter plate-based systems have the advantage of using small volumes and many separate wells making it possible to include a range of different variables in one experiment without it being overly labour-intensive and costly in terms of media. However, as opposed to using bioreactors, pH and oxygen levels are uncontrolled. Uncontrolled pH values may be problematic as the pH range when using yeast for producing SCP should be at 4.5 – 5.5. This acidic pH range reduces bacterial contamination (Lapeña, 2019). For aerobic cultivations, dissolved oxygen can be the limiting factor for cell growth. As the oxygen is depleted as cell biomass accumulate the yeast may switch its metabolism to be anaerobic, which is less efficient in terms of cell growth and may lead to side products such as ethanol being formed (Anderlei et al., 2007).

The CDW results from the microtiter plate experiment (Figure 3) showed the highest growth for all three microbes when grown on YPD media. The YPD condition was included as a control, as YPD is known to be a reliable medium for yeast cultivation (Kurcz et al., 2018). The nitrogen in YPD comes from yeast extract and peptone, which are complex sources that are more expensive compared to simple inorganic nitrogen sources like ammonium sulphate or urea. It is therefore industrially relevant to investigate using non-organic nitrogen sources.

The highest CDW achieved on an inorganic nitrogen source was 6.5 g/L achieved by *W. anomalus* grown on spruce model sugar (S) + yeast nitrogen base and urea (YNBU). This was very similar to *W. anomalus* grown on YPD which reached 6.7 g/L. Even though *B. adeninivorans* and *C. jadinii* showed higher CDW on YPD medium at 6.6 g/L and 8.2 g/L respectively, they showed less versatility than *W. anomalus* as the CDW values of the non-YPD medium was significantly lower than that of the YPD medium.

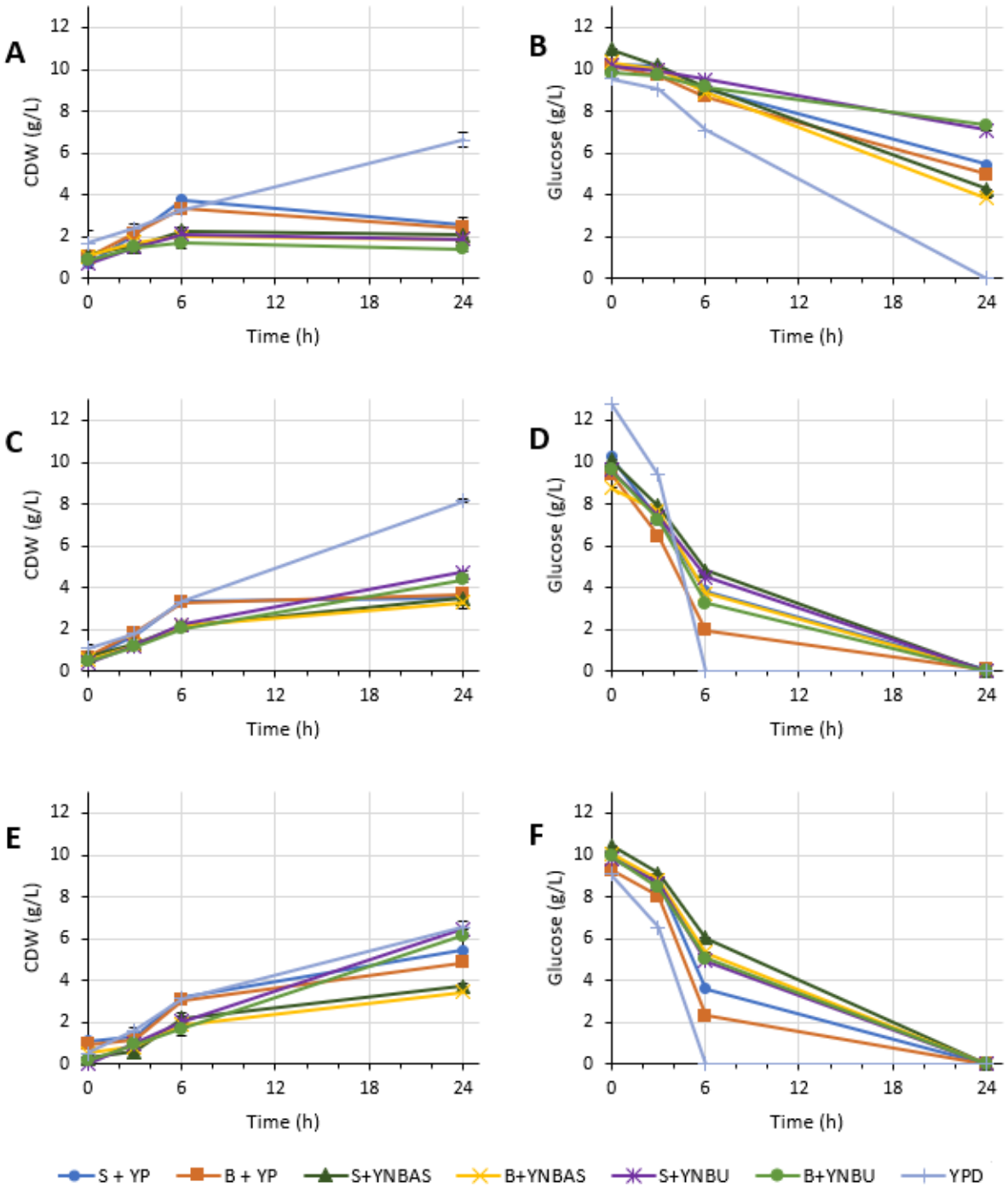

Figure 3. CDW and glucose concentrations from yeasts grown on different media in microtiter plates. A/B = *B. adeninivorans,* C/D = *C. jadinii*, E/F = *W. anomalus*. Abbreviations; S = spruce model sugar; B = birch model sugar; YP = yeast extract and meat peptone; YNBAS = yeast nitrogen base and ammonium sulphate; YNBU = yeast nitrogen base.

Acidification was observed to varying degrees during the first six hours of fermentation in all cultivations (Figure 4). The experiments with ammonium as a nitrogen source showed the most acidification and may be due to the production of protons when ammonium is

consumed. When carbohydrates are metabolized organic acids may be produced, decreasing the pH. Cultivation of all three yeasts on YPD led to an increase in pH from 6 to 24 hours, which was also observed for *C. jadinii* and *W. anomalus* grown on YNBU on both sugar sources. This can be explained by possible consumption of the produced organic acids (Munro, 1970).

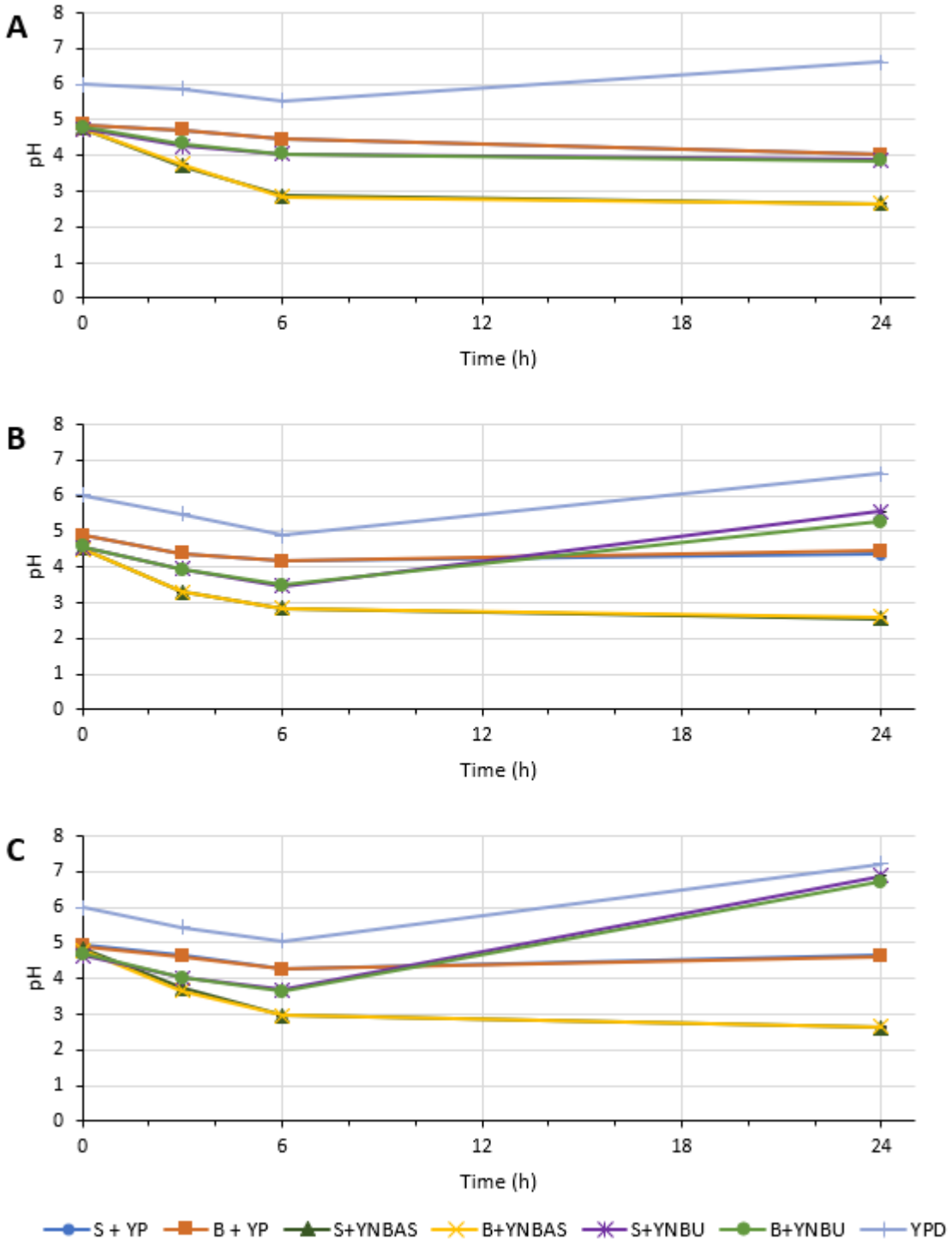

Figure 4. pH values from yeasts grown on different media in microtiter plates. A = *B. adeninivorans*, B = *C. jadinii*, C = *W. anomalus*. Abbreviations; S = spruce model sugar; B = birch model sugar; YP = yeast extract and meat peptone; YNBAS = yeast nitrogen base and ammonium sulphate; YNBU = yeast nitrogen base.

The concentration of glucose was measured at each sample point (Figure 3) to follow glucose consumption by the yeasts. The amount and rate of glucose consumption provide insight into

the growth rate of the yeast and may vary between different yeasts and growth media. For *B. adeninivorans,* the glucose was only fully consumed using the YPD medium. Also, a slower glucose consumption was shown compared to the other two yeasts after three and six hours even though *B. adeninivorans* had comparable or even higher CDW at these times. This may indicate unfavourable conditions causing the yeast to utilize other carbon sources in the media, such as xylose, arabinose, mannose or even from the smaller amounts of carbon found in YP/YNB.

For *C. jadinii* and *W. anomalus* all glucose was consumed in all media conditions after twenty-four hours, with all glucose being consumed after only six hours in YPD cultivations. Generally, *C. jadinii* consumed glucose more rapidly than *W. anomalus,* however the yield of CDW per g/L of glucose was higher for all media variants for *W. anomalus* indicating that *C. jadinii* may produce more side products (Figure 5, Figure 6, Figure 7).

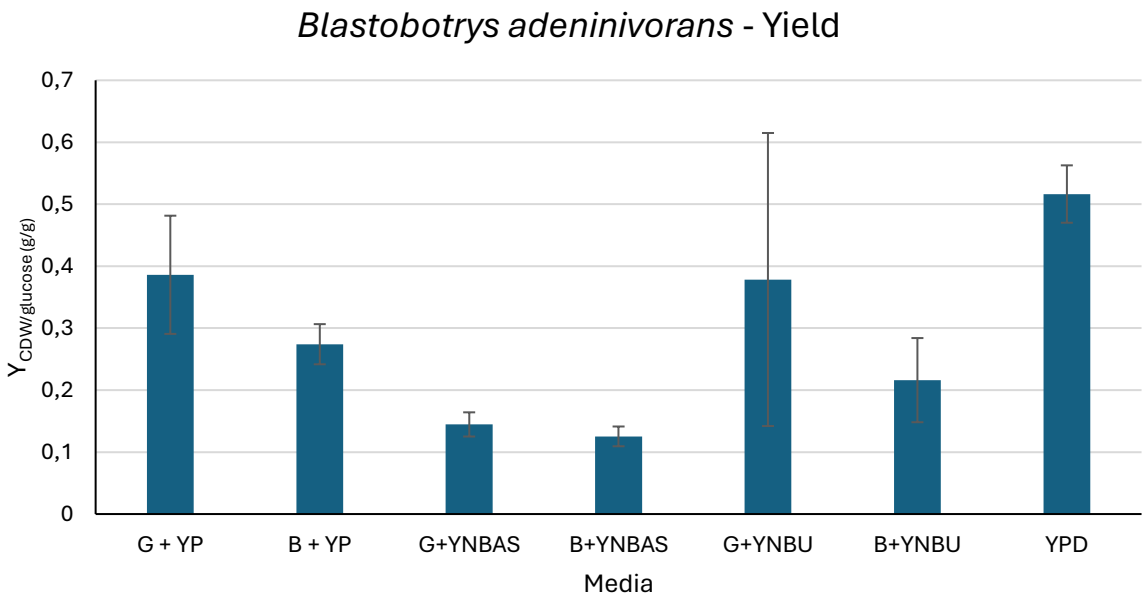

Figure 5. Yield, g dry yeast per g consumed glucose by *B. adeninivorans* grown on different media in microtiter plates. Abbreviations; S = spruce model sugar; B = birch model sugar; YP = yeast extract and meat peptone; YNBAS = yeast nitrogen base and ammonium sulphate; YNBU = yeast nitrogen base.

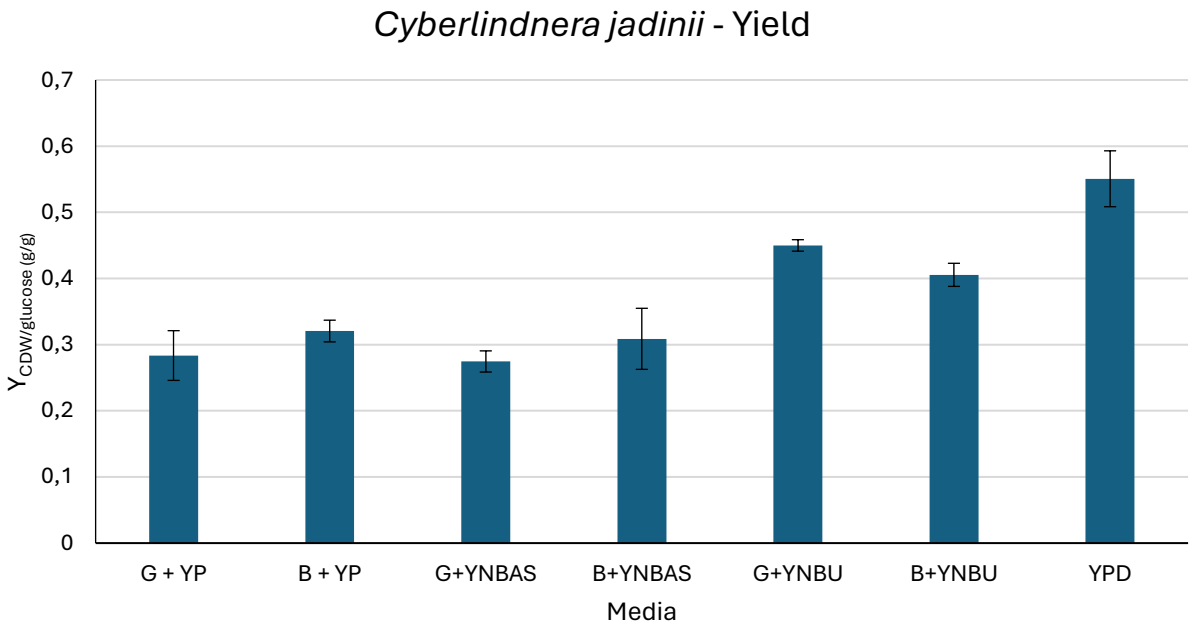

Figure 6. Yield, g dry yeast per g consumed glucose by *C. jadinii* grown on different media in microtiter plates. Abbreviations; S = spruce model sugar; B = birch model sugar; YP = yeast extract and meat peptone; YNBAS = yeast nitrogen base and ammonium sulphate; YNBU = yeast nitrogen base.

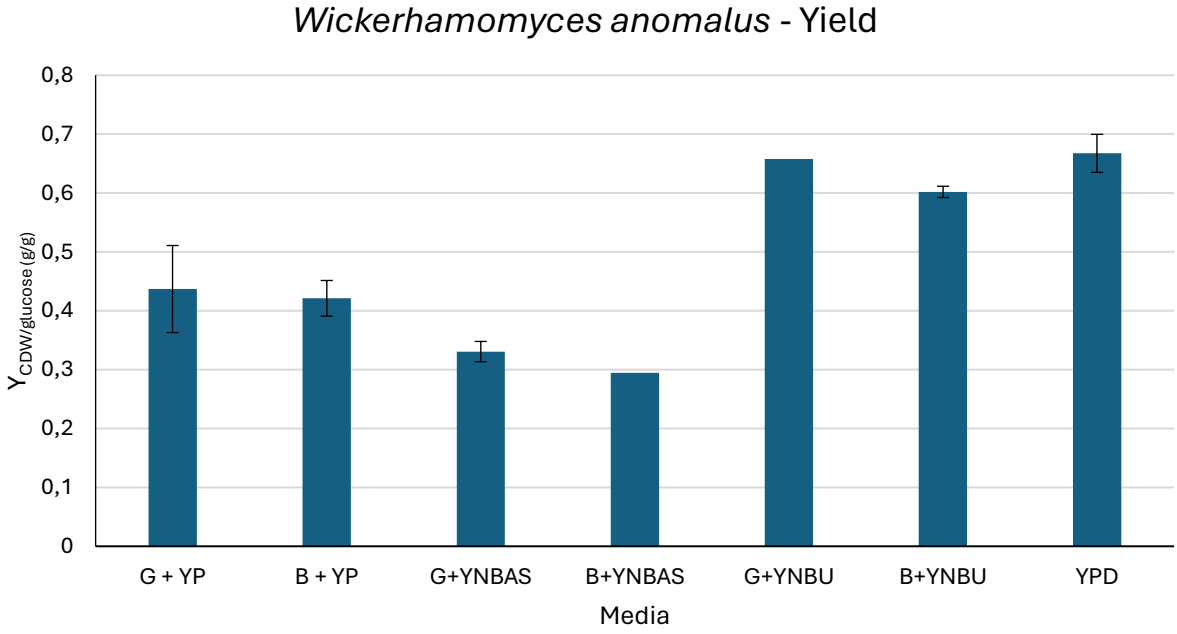

Figure 7. Yield, g dry yeast per g consumed glucose by *W. anomalus* grown on different media in microtiter plates. Abbreviations; S = spruce model sugar; B = birch model sugar; YP = yeast extract and meat peptone; YNBAS = yeast nitrogen base and ammonium sulphate; YNBU = yeast nitrogen base.

The results from the microtiter experiment revealed *W. anomalus* as an interesting candidate for future experiments because of its versatility in metabolizing multiple nitrogen sources and

its efficient use of the carbon source. *C. jadinii* also proved an interesting candidate, showing the highest total CDW overall. *B. adeninivorans* generally achieved lower CDW and yield than the two other yeasts.

3.2 Cultivations in 1.5 L bioreactors

3.2.1 Batch cultivations

3.2.1.1 Cultivation of *B. adeninivorans*, *C. jadinii* and *W. anomalus* using 100 g/L glucose and 50% organic nitrogen source

To gain further insights into the growth capabilities and behaviour, such as side product formation and lag phase, of *B. adeninivorans, C. jadinii* and *W. anomalus* were cultivated in bioreactors with 100 g/L glucose and 50% organic nitrogen source. For a culture not limited by oxygen, typically a cell yield of 50% can be expected (Ugalde & Castrillo, 2002), which for this fermentation experiment would theoretically result in 50 g/L CDW.

Continuous automatic CO_2 measurements give a good indication of the growth performance of the yeasts during fermentation as CO_2 is a natural byproduct of the respiratory pathway, and the concentration will increase with an increasing number of cells consuming the glucose in the media (Ugalde & Castrillo, 2002). Comparing the growth performance based on CO_2 production between the three yeasts showed interesting similarities and differences (Figure 8). The growth cycles of the two *C. jadinii* replicates were highly similar which shows good reproducibility of the cultivations in the bioreactor system.

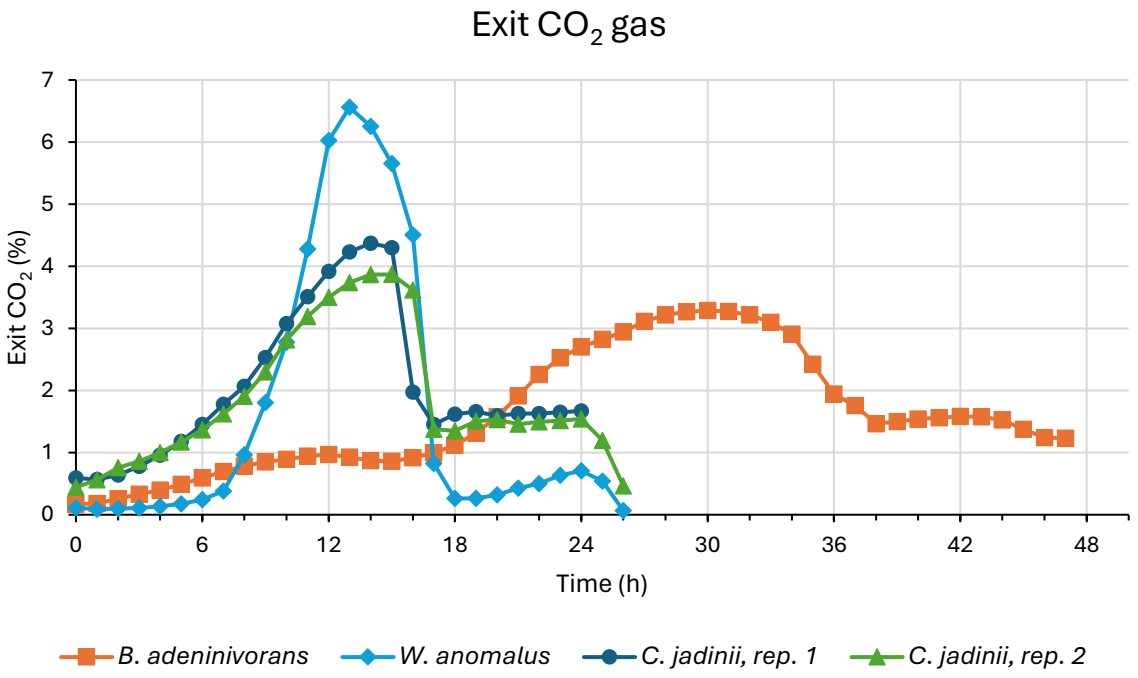

Figure 8. Production of CO_2 by *B. adeninivorans, W. anomalus* and *C. jadinii* (x2) grown on 100 g/L glucose and 50% organic nitrogen source media.

Figure 8 shows that the peak level of CO_2 in the *C. jadinii* and *W. anomalus* cultivations was reached at similar times (after ~13-14 hours). *W. anomalus* released higher amounts of CO_2 at this peak compared to *C. jadinii*, which may indicate either more cells in the bioreactor, or that *C. jadinii* transformed more of the pyruvate into ethanol instead of oxidizing it into CO_2. The latter explanation seems the most plausible as the CDW of *W. anomalus* was generally lower than that of *C. jadinii* (Figure 9), and the HPLC results showed *C. jadinii* produced high amounts of ethanol (~9 g/L) after 10 hours while *W. anomalus* produced significantly smaller amounts (~0.8 g/L after 24 hours) (Figure 10). *B. adeninivorans* had a much longer lag phase and reached its CO_2 peak after ~30 hours and this fermentation was therefore run for 48 hours as compared to 24 hours. *B. adeninivorans* produced ethanol, which reached 5.3 g/L after 24 hours (although may have continued to produce ethanol after this sample point) which was subsequently consumed by the end of the fermentation.

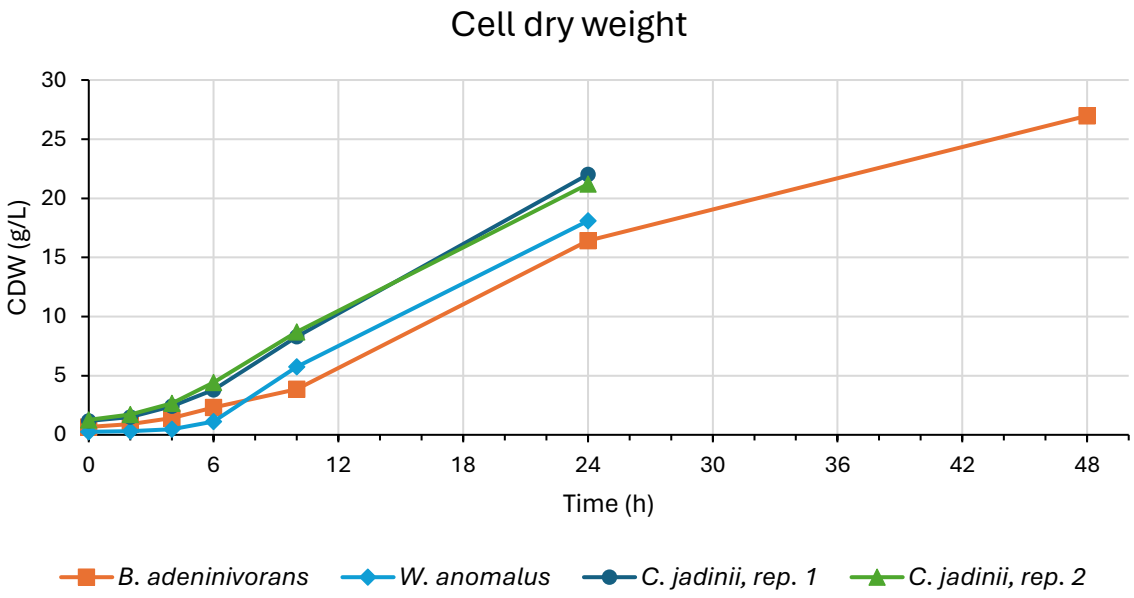

Figure 9. Cell dry weight of *B. adeninivorans, W. anomalus* and *C. jadinii* (x2) grown on 100 g/L glucose and 50% organic nitrogen source media.

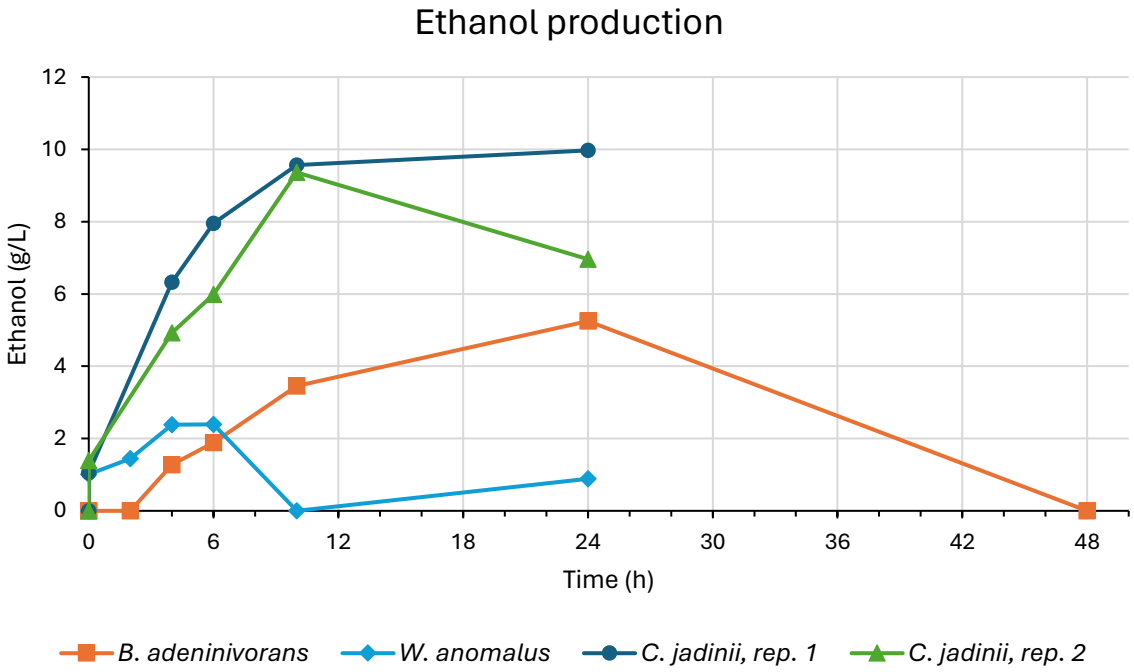

Figure 10. Cell dry weight of *B. adeninivorans, W. anomalus* and *C. jadinii* (x2) grown on 100 g/L glucose and 50% organic nitrogen source media.

Interestingly ethanol production is often a sign of anaerobic conditions, however *W. anomalus* was the only yeast being exposed to pO_2 lower than 30% (at the lowest ~10%) as a

result of the bioreactor system not being able to keep up with the high oxygen demand (Figure 11). This high oxygen demand is also demonstrated by the fact that the stirring speed of the bioreactor reached its maximum of 1250 rpm from 10-16 hours, whereas for the *C. jadinii* the stirring speed did not reach the maximum once during the fermentation. For *B. adeninivorans,* the stirring speed just about reached the maximum for a short period after ~28 hours. This may show that *W. anomalus* has a naturally higher oxygen demand compared to *C. jadinii*, as this can differ between different yeasts (Hagman et al., 2014).

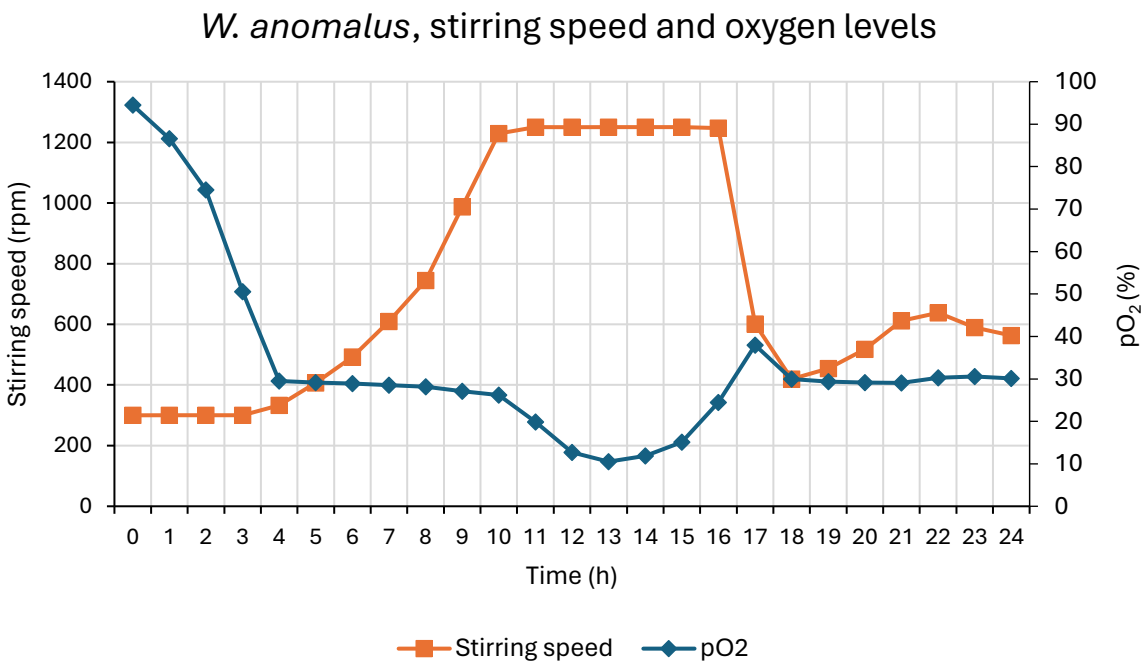

Figure 11. Stirring speed and oxygen levels during cultivation of *W. anomalus* grown on 100 g/L glucose and 50% organic nitrogen source media.

As for the *C. jadinii* and *W. anomalus* fermentations, the CO_2 concentration starts to drop rapidly right after the CO_2 peak is reached. For *B. adeninivorans,* the CO_2 concentration is at its peak for a longer period of time (~8 hours) and then drops. This drop indicates that the yeast has run out of a necessary nutrient, such as glucose or nitrogen compounds, or is suffering from other conditions which cause stress and stops cell growth, such as shear stress from high stirring speeds. In this case, based on the glucose measurements, it is uncertain at what time point glucose was exhausted. It is however possible that the CO_2 drop is caused by glucose exhaustion because the glucose measurements indicates that glucose was exhausted

somewhere between 10 and 24 hours for *C. jadinii* and *W. anomalus,* and between 24 and 48 hours for *B. adeninivorans*, which corresponds with the drop in CO_2. A smaller second CO_2 drop happens for all the yeasts towards the end of the fermentation, indicating that the yeasts may have utilized small amounts of carbon present in the complex nitrogen sources until then.

HPLC analysis of the inoculum (Figure 12) may explain the lag phase of *B. adeninivorans* and the high ethanol production by *C. jadinii*. *B. adeninivorans* consumed considerably less glucose than the two other yeasts and 63 g/L glucose still remained in the inoculum media at the time of inoculation, whereas *C. jadinii* and *W. anomalus* had ~10 g/L to ~2 g/L glucose left respectively. The inoculum of *C. jadinii* and *W. anomalus* contained very high amounts of ethanol at around ~40 g/L, which may be because a shake flask environment provides no form of oxygen control. The ethanol production from *C. jadinii* continued for 10 hours right after inoculation indicating that the cells were still utilizing metabolic pathways triggered by the anaerobic conditions from the shake flasks. However, *W. anomalus* seemed to have a metabolic pathway shift to respiration, when *W. anomalus* was inoculated, as *W. anomalus* produced only small amounts of ethanol during the fermentation.

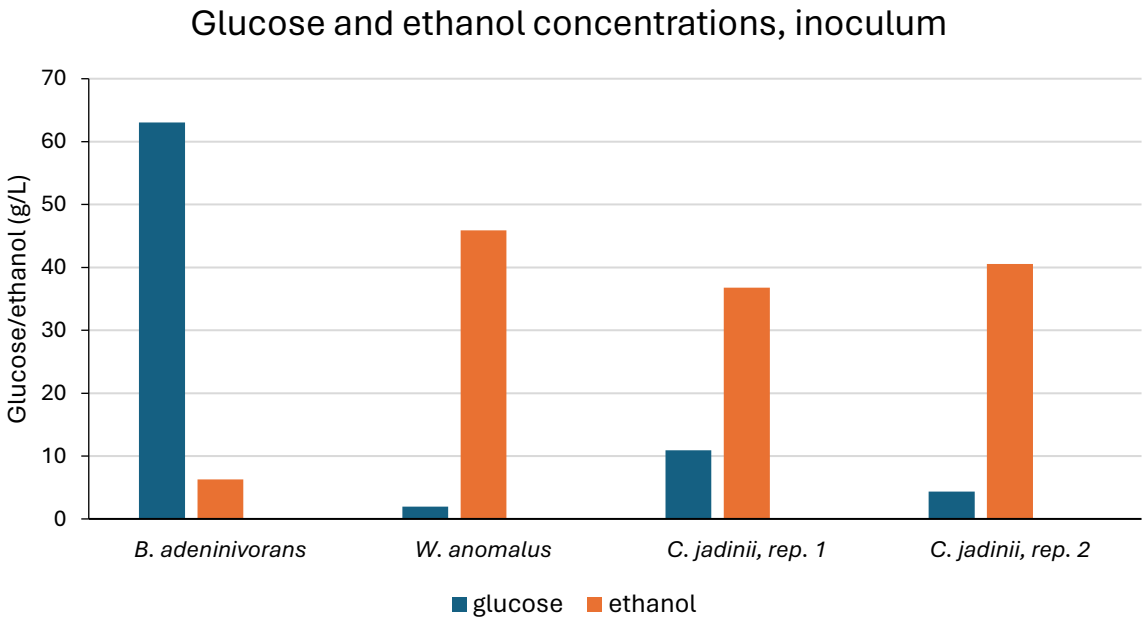

Figure 12. Glucose and ethanol concentrations in the inoculum of *B. adeninivorans, W. anomalus* and *C. jadinii* (x2) used for cultivation using 100 g/L glucose and 50% organic nitrogen source media.

An unknown compound was detected on HPLC after 10 hours in the *W. anomalus* cultivations, coinciding with a drop in pO_2. On the Rezex column, this compound had the same retention time as formic acid, however no evidence for formic acid production by *W. anomalus* has been described in previous literature. *W. anomalus* is known to produce esters such as ethyl acetate during anaerobic conditions (Padilla et al., 2018), however this standard was not available in the laboratory.

The highest CDW was achieved for *B. adeninivorans* at 27.0 g/L. *C. jadinii* achieved the second highest CDW at 21.2/22.0 g/L. For one of the *C. jadinii* replicates it may seem that the yeasts had begun consuming some of the ethanol produced towards the end of the fermentation and may therefore have been able to achieve higher CDW if the fermentation time had been extended. *W. anomalus* achieved the lowest CDW at 18.1 g/L. *C. jadinii* and *W. anomalus* had consumed all of the glucose after 24 hours, while *B. adeninivorans* still had 51.3 g/L left, however all was consumed after 48 hours (Figure 13).

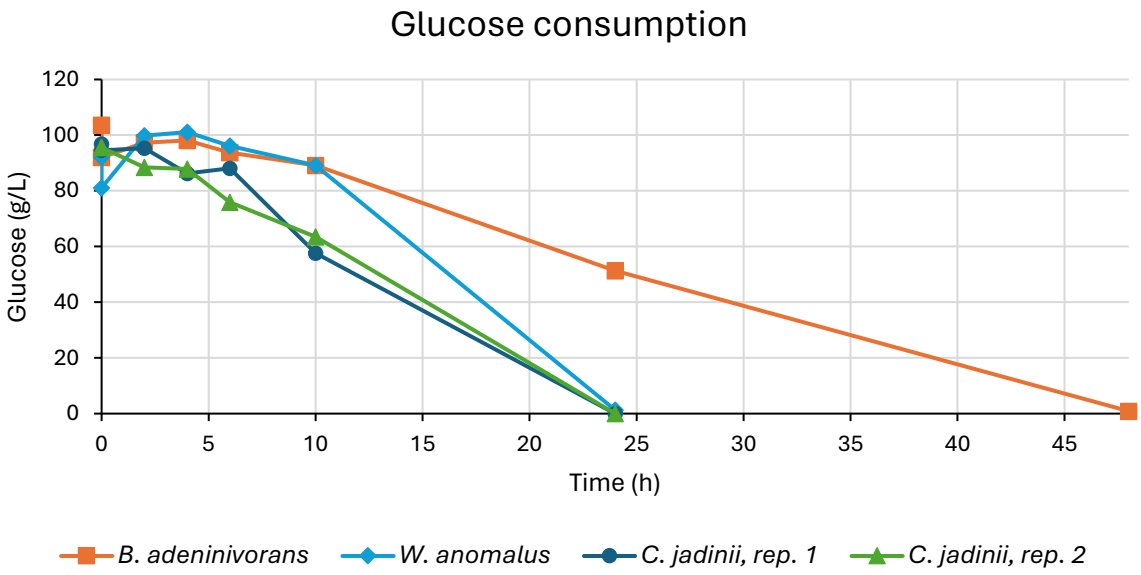

Figure 13. Glucose consumption by *B. adeninivorans, W. anomalus* and *C. jadinii* (x2) grown on 100 g/L glucose and 50% organic nitrogen source media.

When the goal is obtaining maximum cell yields this experiment highlighted a challenge since the cells were utilizing a less efficient anaerobic pathway to produce cells and side products which decreases the cell yield. Due to its long lag phase and relatively slow growth, *B. adeninivorans* was excluded from further investigation.

3.2.1.2 Cultivation of *C. jadinii* and *W. anomalus* using 100 g/L glucose and 100%/75% organic nitrogen source

In order to assess cell growth, an experiment with a higher ratio of complex nitrogen sources to simpler nitrogen was carried out.

The CO_2 measurements for *C. jadinii* and *W. anomalus* (Figure 14) show that the lag phase is ~5 hours shorter when using a higher inclusion of organic nitrogen sources (section 1.2.1.1.). This may be because of the higher quantity of essential nutrients found in these nitrogen sources, as the HPLC revealed the inoculum (Figure 20) to be similar with high amounts of ethanol at ~40 g/L. The maximum CO_2 production was highly similar to the previous cultivations of the same strains. Also, both these experiments showed a rapid drop after the peak, indicating glucose exhaustion which was confirmed by the glucose results (Figure 15).

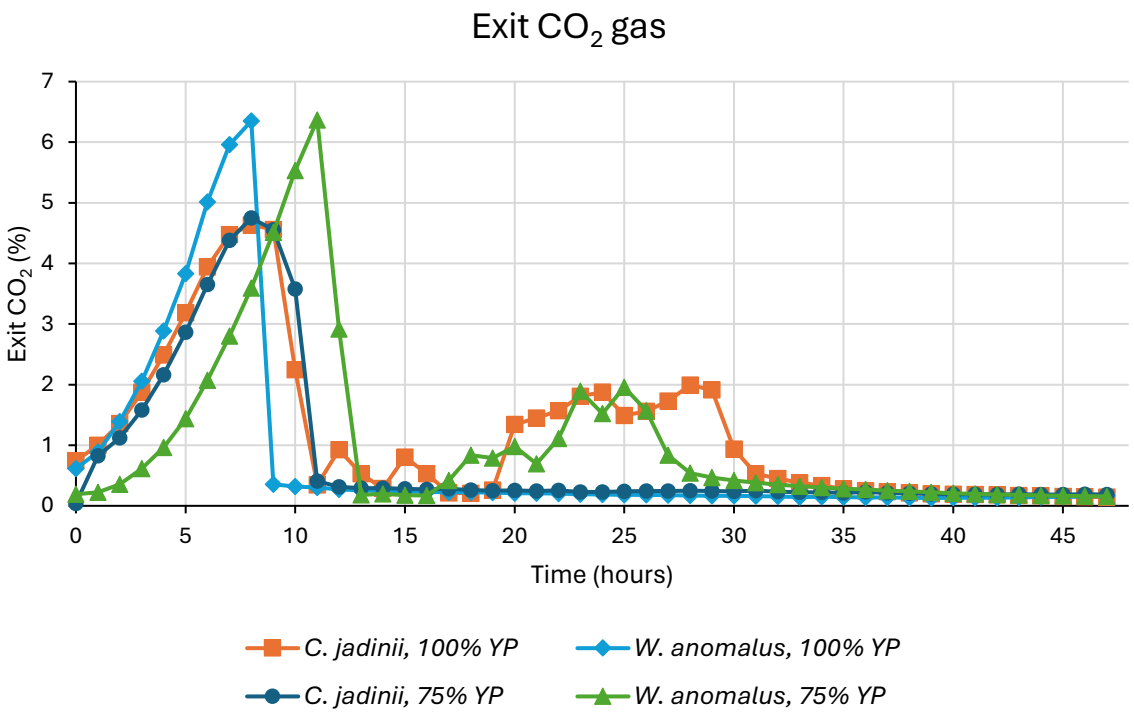

Figure 14. Production of CO_2 by *C. jadinii* and *W. anomalus* grown on 100 g/L glucose and 100/75% organic nitrogen source media.

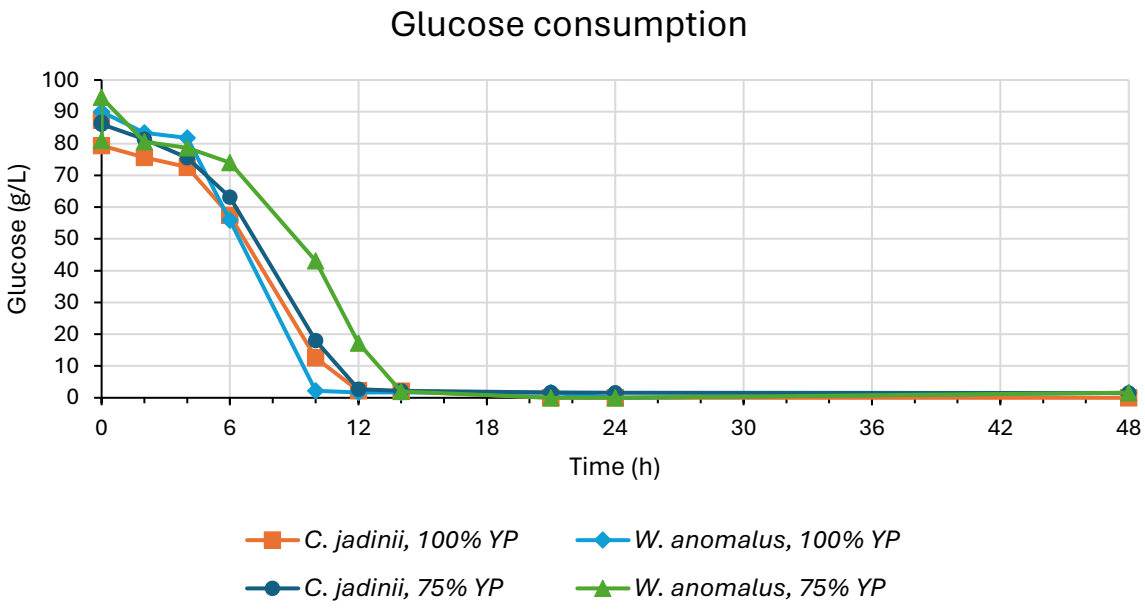

Figure 15. Glucose consumption by *C. jadinii* and *W. anomalus* grown on 100 g/L glucose and 100/75% organic nitrogen source media.

Two of the four fermentations had a second growth phase after glucose exhaustion, while the two others had very low CO_2 production for the rest of the fermentation indicating no further growth. Interestingly, the two fermentations where a second growth phase occurred were that of *C. jadinii* using 100% YP and *W. anomalus* using 75% YP: the two most different experimental set-ups of the four. These two fermentations were the only ones out of the four where the pO_2 went below 30% (Figure 16, Figure 17). This pO_2 drop occurred either right before or during the increase in CO_2 during the second growth phase.

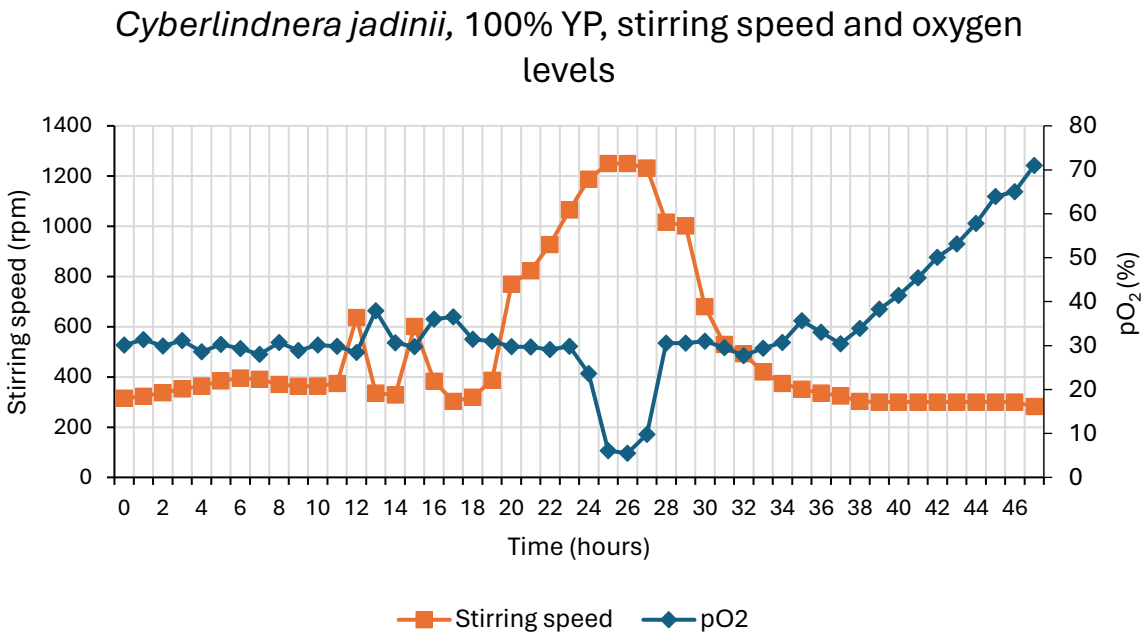

Figure 16. Stirring speed and oxygen levels during cultivation of *C. jadinii* using 100 g/L glucose and 100% organic nitrogen source media.

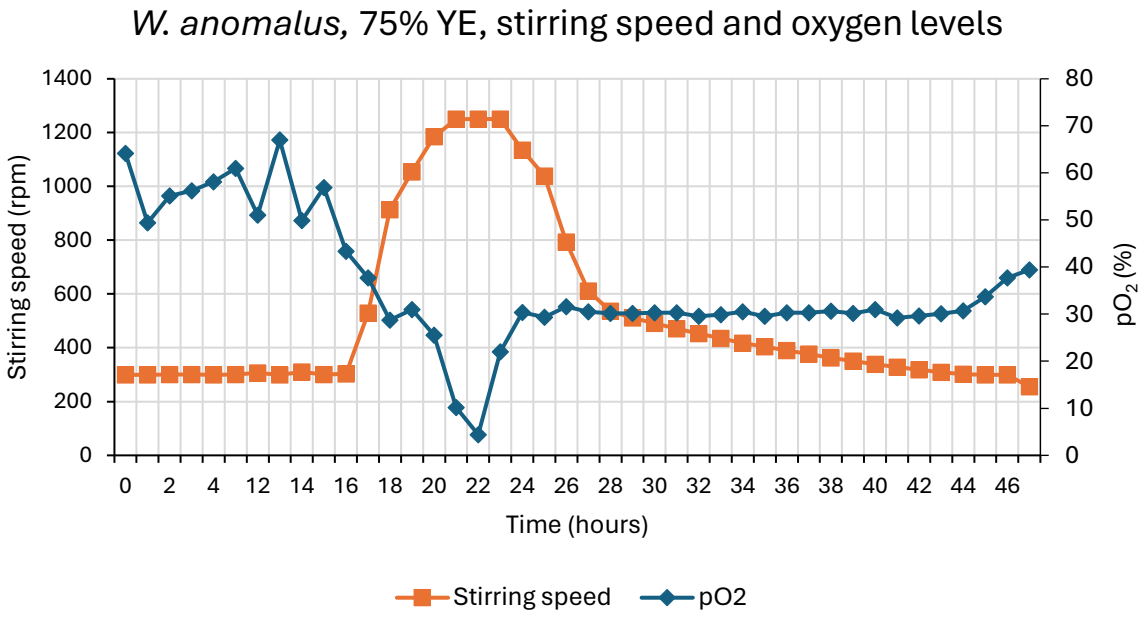

Figure 17. Stirring speed and oxygen levels during cultivation of *W. anomalus* using 100 g/L glucose and 75% organic nitrogen source media.

During the first growth phase where the glucose was consumed, the stirring speed of the system for the cultivations using *C. jadinii* grown on 100% YP and *W. anomalus* grown on 75% YP was between 300-400 rpm (Figure 16, Figure 17), showing relatively low use of

oxygen which may indicate that these yeasts were growing anaerobically. This is further corroborated by the high ethanol production during this time (Figure 18).

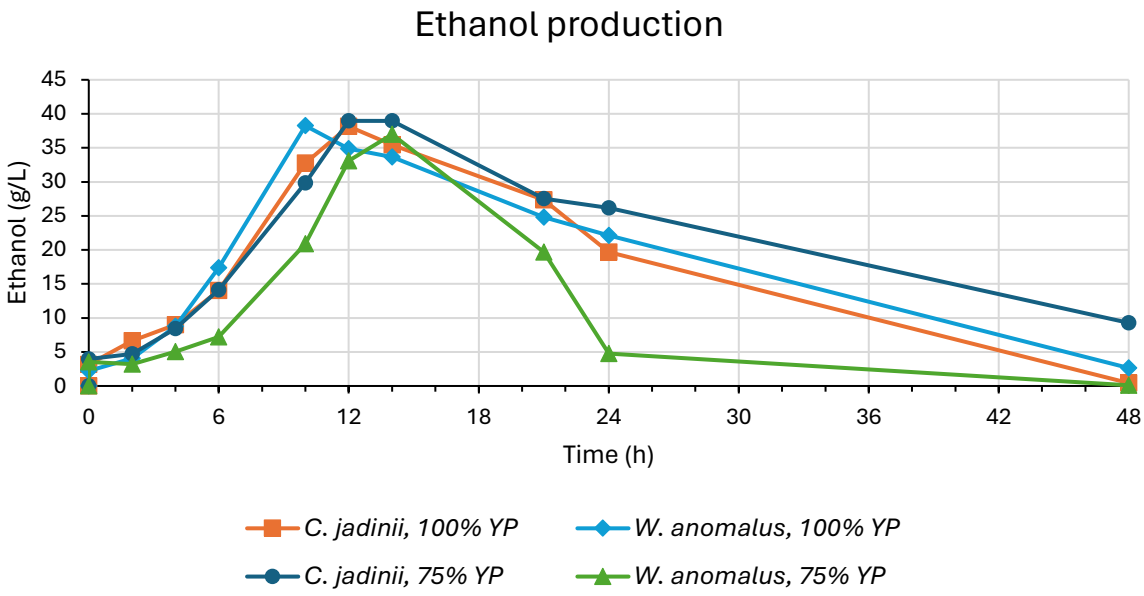

Figure 18. Ethanol production by *C. jadinii* and *W. anomalus* grown on 100 g/L glucose and 100/75% organic nitrogen source media.

Whereas the stirring speed of the fermentations with *W. anomalus* using 100% YP and *C. jadinii* using 75% YP stayed at lower values near 300 rpm, the two other fermentations had a subsequent increase in stirring speed values eventually reaching maximum rpm right after the first growth phase, indicating a switch to aerobic growth. However, as the glucose was exhausted at this time, the high amounts of ethanol produced from all fermentations was consumed as an alternative carbon source somewhere after 10 hours. This time frame matched the second increase in CO_2 and a second increase in CDW (Figure 19) after the first growth phase, indicating that the fermentations with *C. jadinii* using 100% YP and *W. anomalus* using 75% YP was then utilizing ethanol as a carbon source.

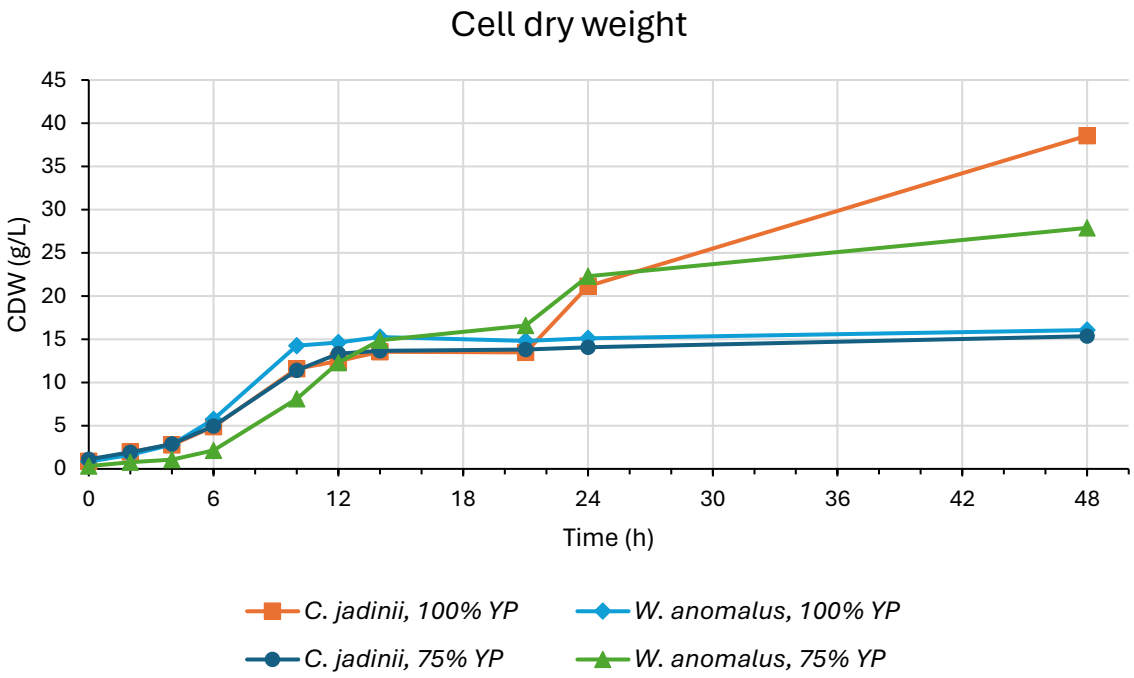

Figure 19. Cell dry weight of *C. jadinii* and *W. anomalus* grown on 100 g/L glucose and 100/75% organic nitrogen source media.

The consumption of ethanol during the two fermentations where no second growth phase occurred may mean that these yeasts used the ethanol for other cellular maintenance activities, however it is unknown why this occurred. Interestingly, the rate of ethanol consumption looks to be higher for the fermentations where a second growth phase occurred, further corroborating that there were different purposes for the consumption of ethanol. As with a previous fermentation (see section 1.2.1.1), the inoculum used for these fermentations all contained high amounts of ethanol (Figure 20).

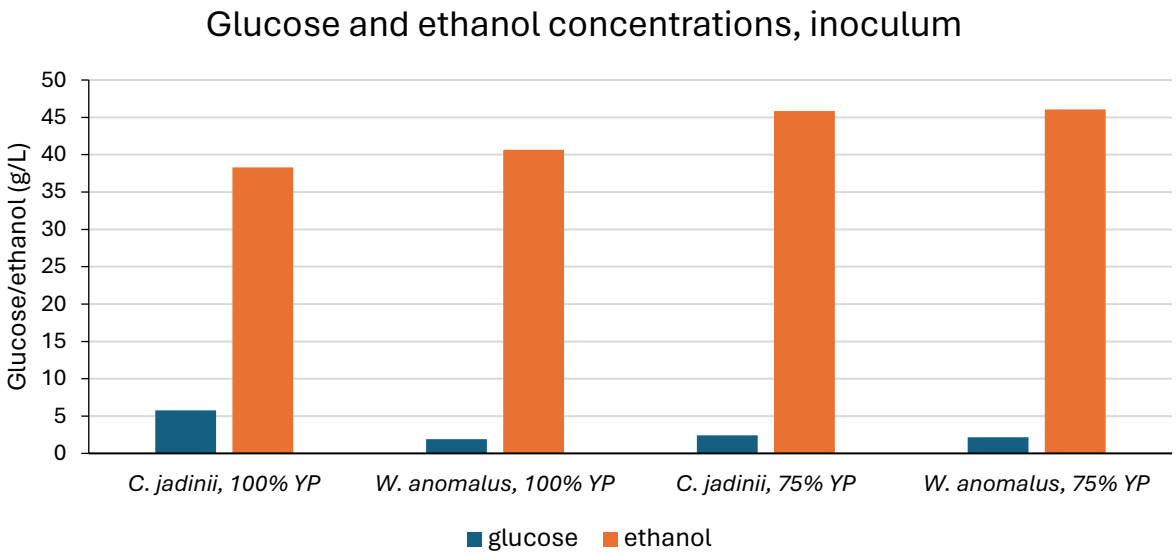

Figure 20. Glucose and ethanol concentrations in the inoculum of *W. anomalus* and *C. jadinii* used for cultivation using 100 g/L glucose and 100/75% organic nitrogen source media.

W. anomalus grown on 100% YP showed the highest CDW after the first growth phase (~10 hours) at 14.3 g/L, but as with *C. jadinii* using 75% YP the cell growth stopped after the first growth phase. For the two fermentations where a second growth phase occurred *C. jadinii* grown on 100% YP reached the highest CDW at 38.6 g/L.

3.2.1.3 Cultivation of *C. jadinii* and *W. anomalus* using 100 g/L and 50 g/L glucose

No evidence of *C. jadinii* and/or *W. anomalus* being Crabtree-positive has previously been described in literature, however Crabtree-like effects have been observed (Ding et al., 2023; López-Enríquez et al., 2023). To investigate the potential role of this, a lower glucose concentration was tested along with a shorter incubation time for the inoculum to prevent the yeasts from potentially switching to anaerobic growth. The incubation time was shortened to ~11 and ~13 hours, for the *W. anomalus* and *C. jadinii* inoculum respectively, based on results from the shake flask experiment (see section 1.1.2), where *C. jadinii* was observed to have a slightly longer lag phase than *W. anomalus*.

The CO_2 measurements (Figure 21) showed an expected difference between the fermentations using different glucose concentrations, where the fermentations with lower glucose

concentrations showed a rapid CO_2 drop before the ones with higher glucose concentrations, indicating glucose exhaustion. The fermentations using *W. anomalus* had a shorter lag phase compared to previous experiments (see section 1.2.1.1 and 1.2.1.2). This may be explained by the shortened inoculum time causing the inoculum to contain cells earlier in their growth phase where growth is exponential.

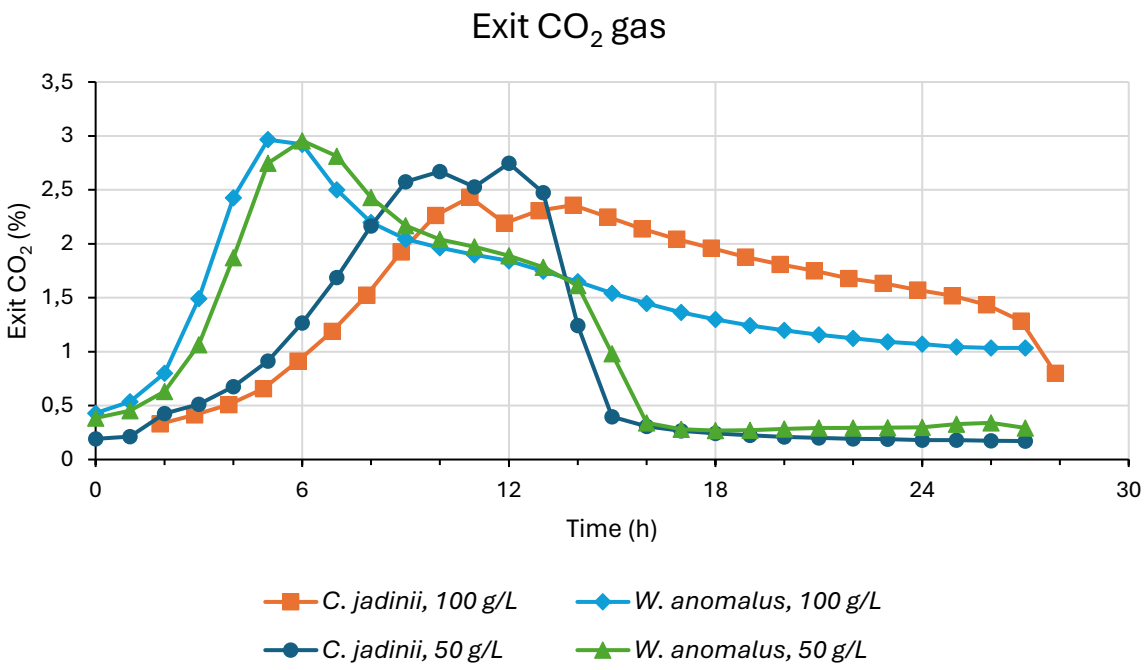

Figure 21. Production of CO_2 by *C. jadinii* and *W. anomalus* grown on 100 g/L and 50 g/L glucose media.

The height of the CO_2 peaks for both yeasts decreased when compared with previous experiments (see section 1.2.1.1 and 1.2.1.2). For *W. anomalus* this was shown to not correlate with lower rate of cell growth as the CDW (Figure 22) obtained was comparable to previous experiments, and for the fermentation using 50 g/L glucose the conversion of glucose to CDW reached 38%, the highest percentage so far when compared to the experiments in section 1.2.1.1 and 1.2.1.2. For *C. jadinii* the CDW was generally lower than previous experiments. Also, when using a higher glucose concentration, the CDW should in theory be higher when compared to using a lower glucose concentration, however in this experiment the CDW between the two 100 g/L and 50 g/L glucose conditions was highly similar for each yeast, meaning something else other than glucose to be limiting growth.

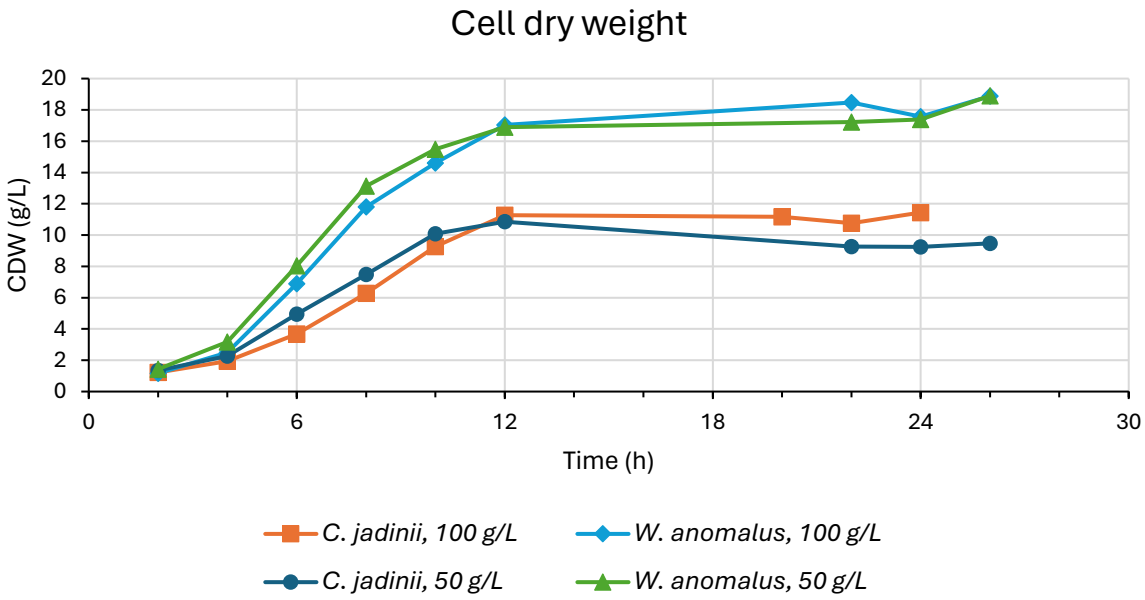

Figure 22. Cell dry weight by *C. jadinii* and *W. anomalus* grown on 100 g/L and 50 g/L glucose media.

For the fermentations using 100 g/L glucose the cell growth stopped before the glucose was fully consumed (Figure 23), and at the end of the fermentation *W. anomalus* still had 22 g/L glucose left. This is an indication of either some essential nutrient missing other than carbon, such as nitrogen or vitamins, or unfavourable fermentation conditions.

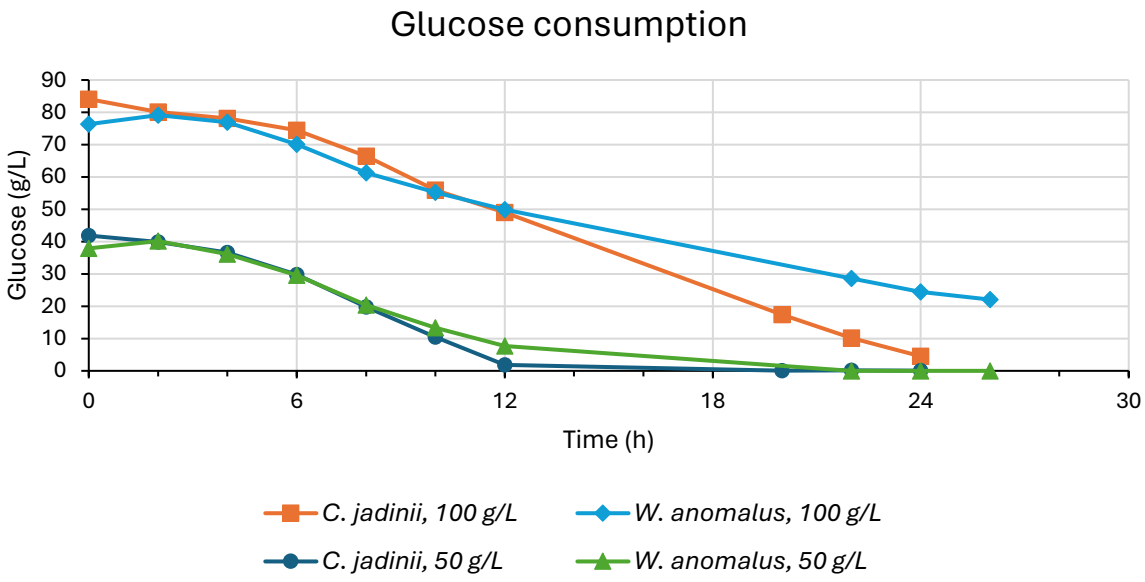

Figure 23. Glucose consumption by *C. jadinii* and *W. anomalus* grown on 100 g/L and 50 g/L glucose media.

Shortening the inoculum times for *W. anomalus* proved to stop the excessive ethanol production (Figure 24) during the fermentations as only low amounts of ethanol was observed for both the 100 g/L and 50 g/L condition. This reveals no apparent Crabtree-effect for *W. anomalus* when using 100 g/L glucose. Also, the unknown compound previously observed in section 1.2.1.1 was not detected for *W. anomalus*. For *C. jadinii,* excessive ethanol was still being produced and ethanol consumption was only observed for the 50 g/L glucose fermentation, likely because of glucose exhaustion, however no increase in CDW was observed as a result of this. Ethanol was still present in the inoculum of both yeasts, however at much lower concentrations (Figure 25) when compared to previous experiments (see section 1.2.1.1 and 1.2.1.2).

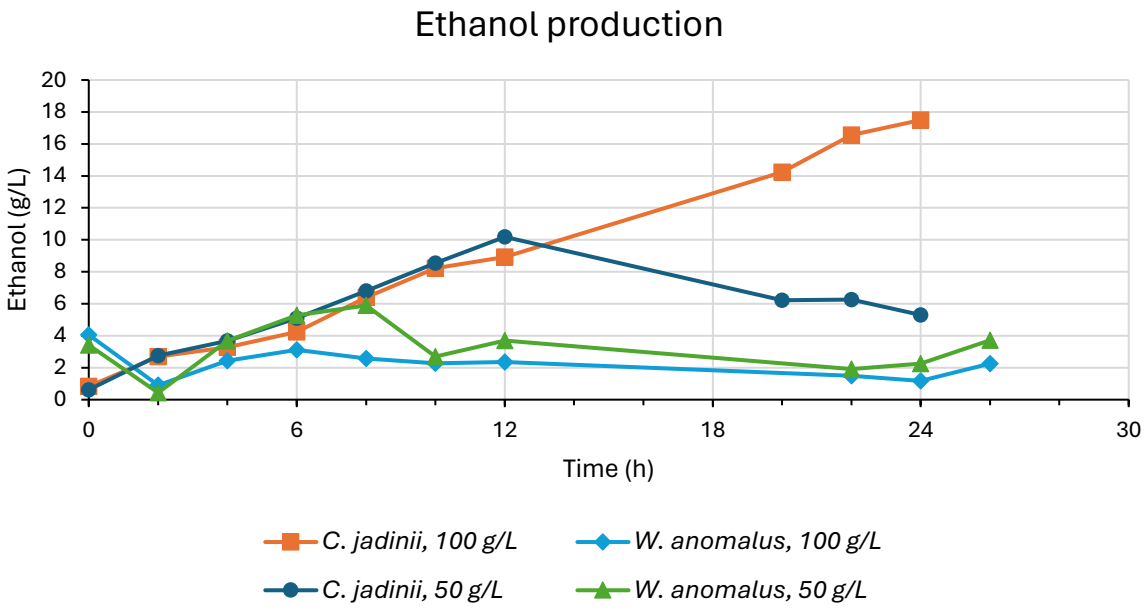

Figure 24. Ethanol production by *C. jadinii* and *W. anomalus* grown on 100 g/L and 50 g/L glucose media.

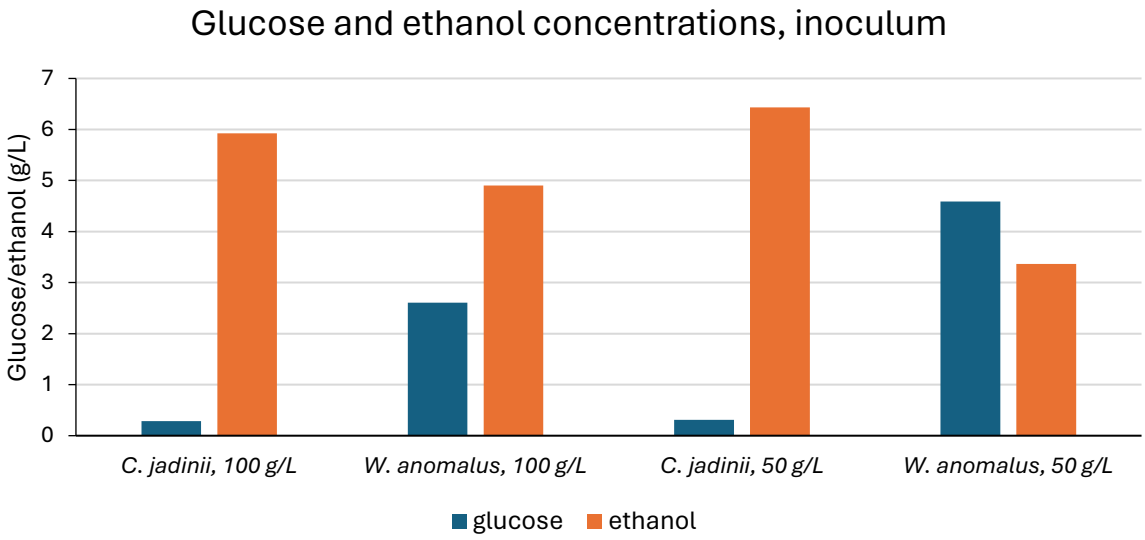

Figure 25. Glucose and ethanol concentrations in the inoculum of *C. jadinii* and *W. anomalus* used for cultivation using 100 g/L and 50 g/L glucose media.

For the *W. anomalus* fermentations high oxygen demand, indicated by agitation rates reaching over 1000 rpm, was observed (Figure 26).

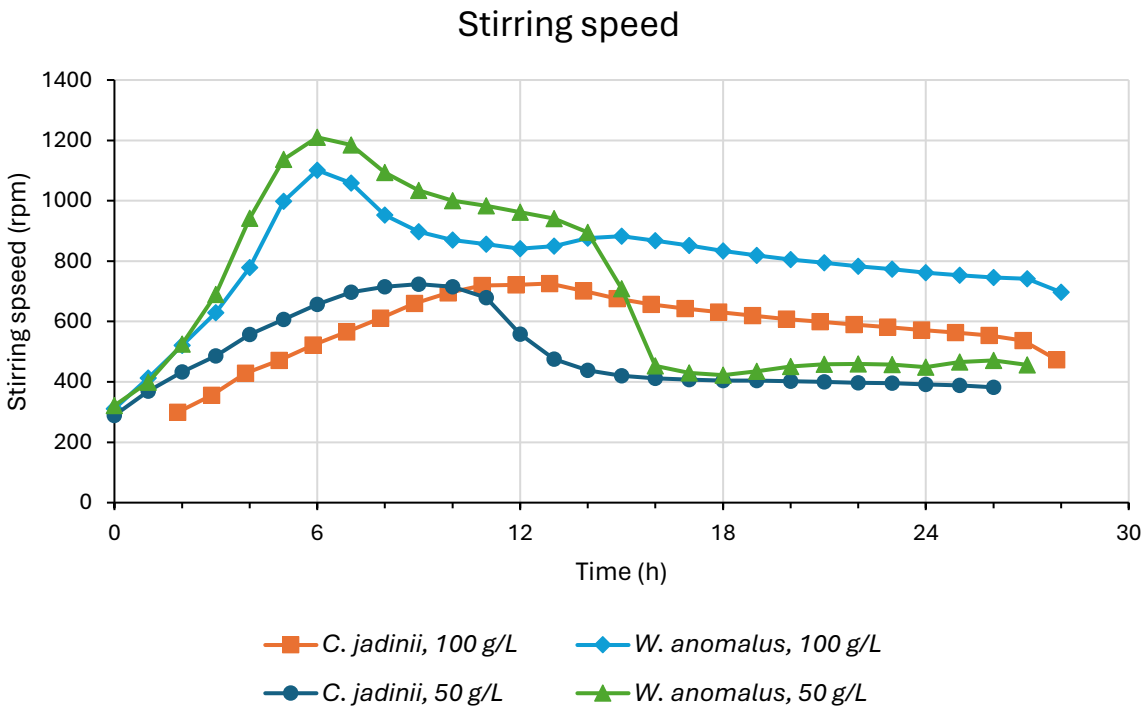

Figure 26. Stirring speeds during cultivation of *C. jadinii* and *W. anomalus* using 100 g/L and 50 g/L glucose media.

3.2.1.4 Cultivation of *W. anomalus* using 100 g/L glucose and 100%/50% organic nitrogen source, with lower agitation rates

In the previous experiments cell growth stagnated even when glucose was still present in the media, indicating that accessible carbon was not the limiting factor. This trend was occurring even with more glucose left in the media indicating that the carbon source was not the limiting factor. Therefore, an experiment was set up to investigate if the reason for the drop in growth was due to shear stress caused by high agitation rates.

When lowering the maximum agitation rate, the DO levels in the media might be compromised. It was therefore decided that using an agitation rate of 900 rpm as a maximum would be a suitable middle ground between maintaining a DO of 30% and avoiding high shear stress. Also, a secondary cascade function with increasing aeration rate from 1.0-2.0 vvm was employed to help with the upkeep of DO. To investigate the possible effects of shear stress it was decided to use media consisting of 100 g/L glucose and 100%/50% YP as it then could be easier compared to previous experiments.

As using an inoculum time of ~11 hours had proved satisfactory in terms of keeping ethanol production low (see section 1.2.1.3), it was decided to maintain this going forward. However, for this experiment, almost no glucose was consumed in the inoculum and therefore minimal cell growth occurred (less than 1 g/L CDW). This made the lag phase for the fermentation in the bioreactors somewhat longer, but surprisingly only by a few hours when compared to the experiment seen in section 1.2.1.1.

The growth cycles (Figure 27, Figure 28) between the 100% and 50% YP conditions revealed a highly similar lag phase. However, it is apparent that the glucose is consumed at a much higher rate during the 100% YP fermentation by looking at the sudden drop in CO_2 to ~0.5% due to glucose exhaustion at ~48 and at ~80 hours for the 100% YP and the 50% YP fermentations, respectively. This is also backed by the glucose analysis results (Figure 29). A previous experiment (see section 1.2.1.2) using 100% and 75% YP also showed somewhat higher glucose consumption rate for the fermentations with higher amounts of complex nitrogen sources.

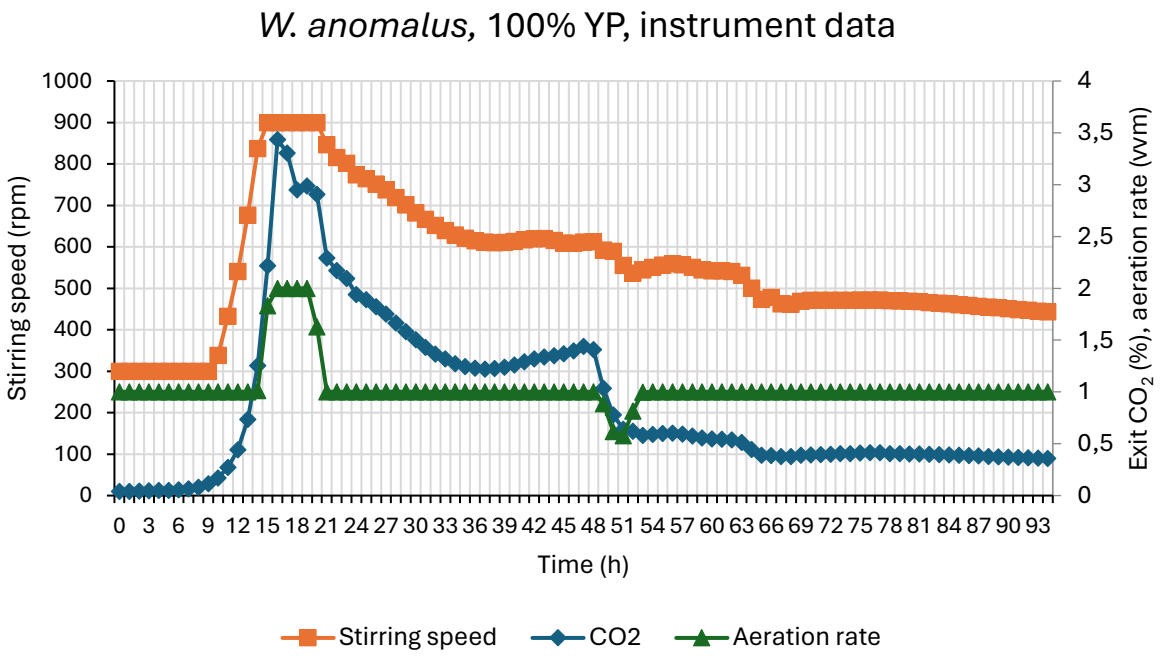

Figure 27. Stirring speeds, CO_2 production and aeration rates during cultivation of *W. anomalus* grown on 100 g/L glucose and 100% YP media with a lower maximum stirring speed.

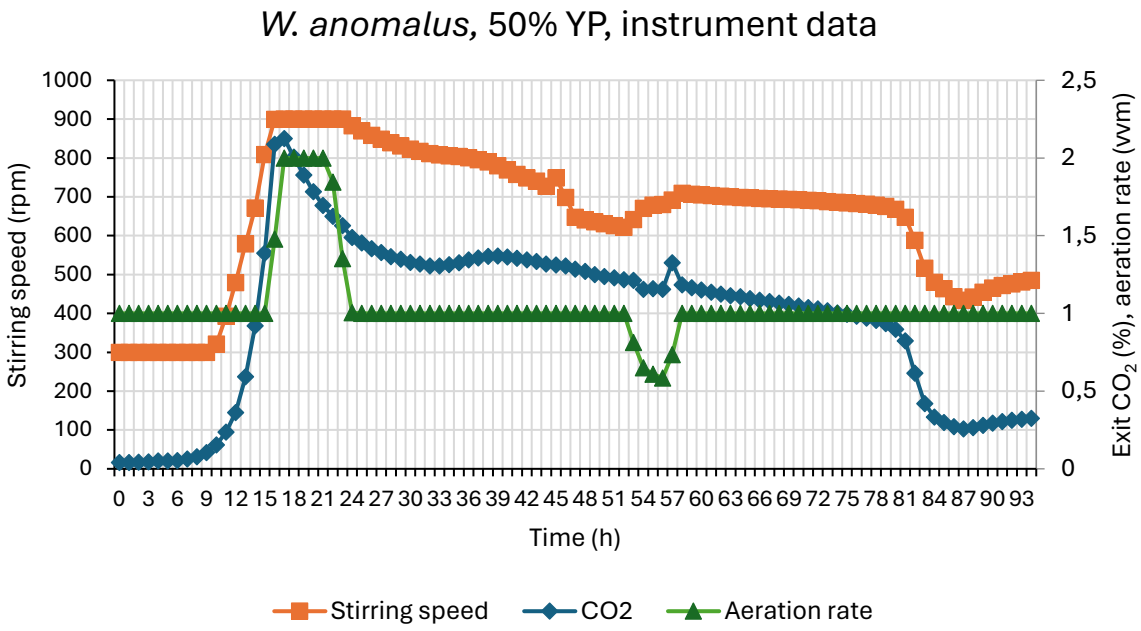

Figure 28. Stirring speeds, CO_2 production and aeration rates during cultivation of *W. anomalus* grown on 100 g/L glucose and 50% YP media with a lower maximum stirring speed.

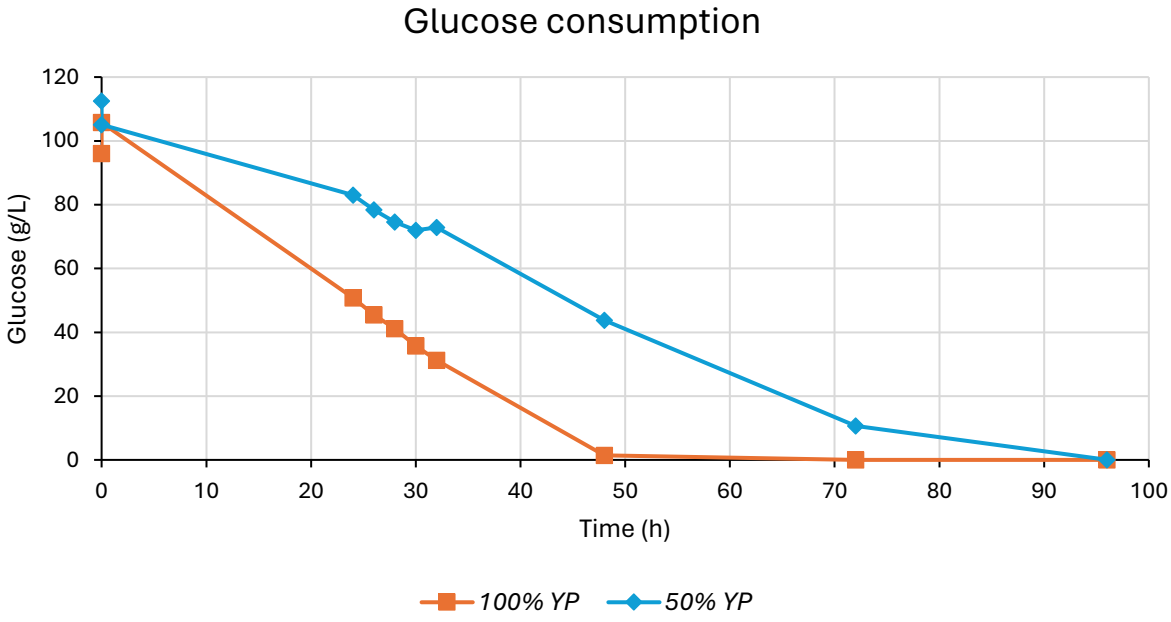

Figure 29. Glucose consumption by *W. anomalus* grown on 100 g/L glucose and 100/50% YP media with a lower maximum stirring speed.

With maximum stirring speed of 900 rpm, he DO could not be maintained at 30% and a DO drop happened after ~16 hours for both yeasts, with the DO stabilizing again at 30% after

~21-23 hours (data not shown). At 24 hours, the yeasts in the 50% YP fermentation started producing the unknown compound previously described in section 1.2.1.1, where the production also seemed to be triggered by a drop in DO. The unknown compound might be ethyl acetate, as *W. anomalus* is reported producing ethyl acetate from glucose under oxygen-limiting conditions (Hoffmann et al., 2021). However, this unknown compound was not detected for the 100% YP condition where there also was a drop in DO. The excessive production of the unknown compound in this experiment and in section 1.2.1.1 were both under conditions containing 50% YP, which may mean it is triggered by a lack of certain compounds from the complex nitrogen sources. Interestingly, ethyl acetate production by *W. anomalus* is known to occur when there is a lack of iron (Hoffmann et al., 2021).

The CDW of the fermentation using 100% YP was the highest achieved compared to previous experiments (see section 1.2.1.1, 1.2.1.2, 1.2.1.3) when using 100 g/L glucose, reaching 25.8 g/L (Figure 30). Growth continued steadily 48 hours after glucose exhaustion occurred (around 48 h), however at a low rate. This indicates that the yeasts were utilizing an alternative carbon source, perhaps from the YP, as no ethanol was detected. For the 50% YP condition, relatively little cell growth occurred leading to a final CDW of 12.0 g/L after 96 hours.

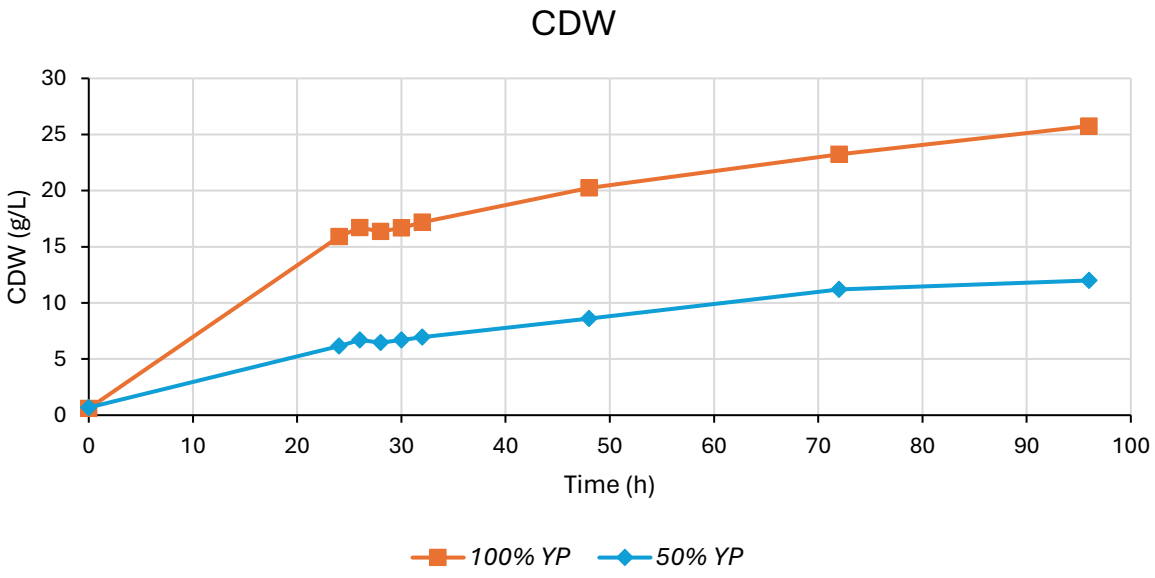

Figure 30. Cell dry weight of *W. anomalus* grown on 100 g/L glucose and 100/50% YP media with a lower maximum stirring speed.

Optimally, the lower stirring speed would keep the yeasts in a higher cell growth state until glucose was exhausted, however a decrease in CO_2 and cell growth rate was still observed before glucose exhaustion after 22 hours.

3.2.1.5 Cultivation of *W. anomalus* using 100 g/L and 50 g/L glucose using urea as non-organic nitrogen source

As the experiment using 900 rpm as a maximum agitation rate yielded the highest CDW for *W. anomalus* using 100 g/L glucose (see section 1.2.1.6) it was decided to continue with this setup for the following experiment. Using urea as a nitrogen source for *W. anomalus* during the microtiter plates experiment (see section 1.0) had yielded the highest CDW of the simple nitrogen sources and it was therefore decided to investigate this in a bioreactor fermentation using 50% YP and 50% urea. In addition, using 50 g/L glucose for *W. anomalus* had previously yielded the highest conversion of glucose to CDW (see section 1.2.1.3) so this was investigated with the new maximum agitation rate setting in a separate bioreactor.

The inoculum had very little growth (less than 1 g/L CDW) and no detected ethanol similar to what was previously observed (see section 1.2.1.6).

For the fermentation using urea there was a very long lag phase (~17 hours) and the CO_2 did not reach its peak until after 26 hours (Figure 31). There was little oxygen demand from the yeasts as the agitation rate maxed out at ~540 rpm. For the fermentation using 50 g/L glucose the lag phase was similar to previous experiments (Figure 32), reaching a peak in CO_2 after 10 hours and the agitation rate did not reach the set maximum of 900 rpm but peaked at ~800 rpm.

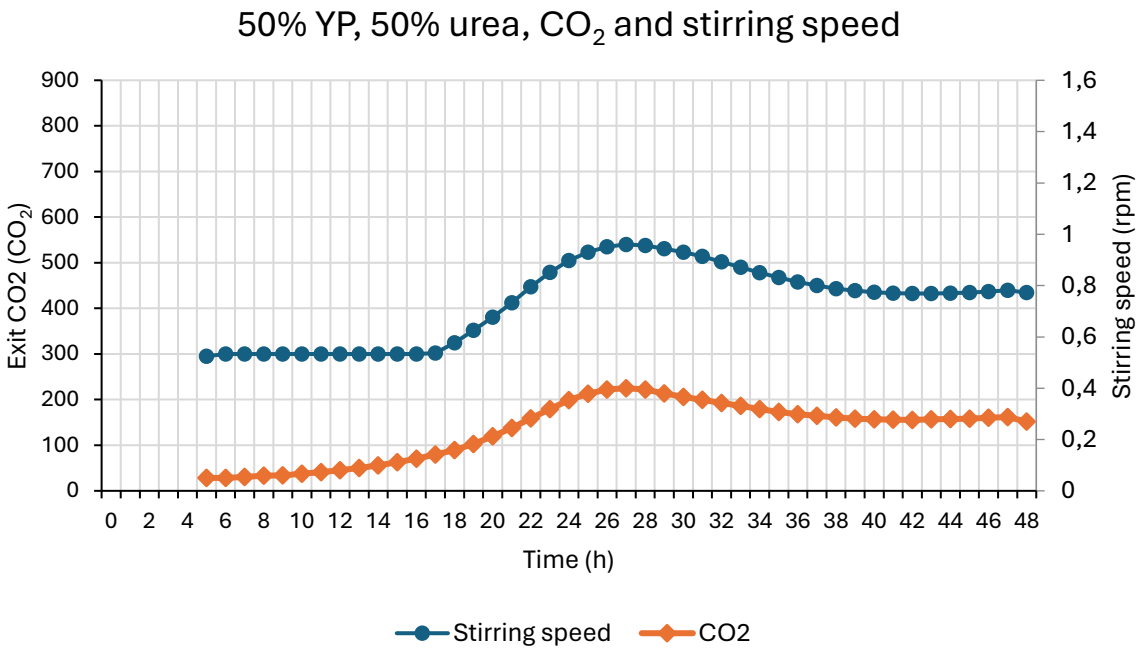

Figure 31. Stirring speeds and CO_2 production by *W. anomalus* grown on 100 g/L glucose and 50% urea nitrogen source media.

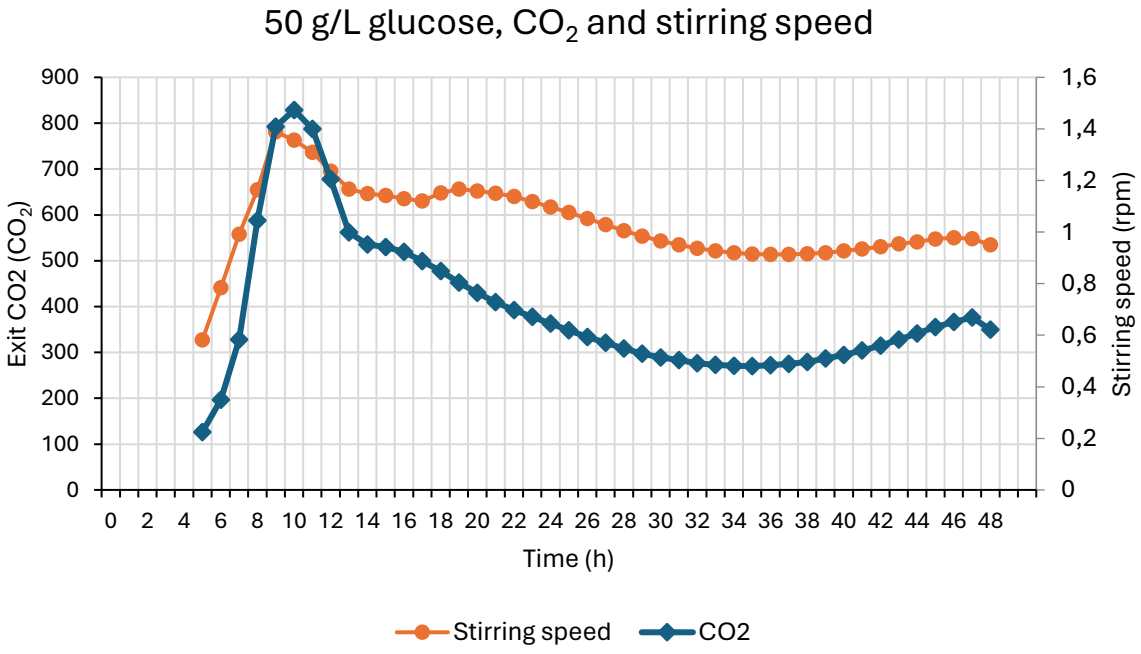

Figure 32. Stirring speeds and CO_2 production by *W. anomalus* grown on 50 g/L glucose and 100% organic nitrogen source media.

There was very little growth for the fermentation using urea and not even 10 g/L glucose was consumed (Figure 33). However, it seems that all the glucose consumed was utilized for cell growth as the conversion of glucose to CDW reached more than theoretical maximum of 50% (Figure 34). It seemed that the yeasts prioritized some other carbon than glucose, possibly from the complex nitrogen source, as most of the cell growth happened without any glucose being consumed. Even though the nitrogen source consisted of 50% YP it seems that the addition of 50% urea had a negative effect on the ability of the yeasts to consume glucose.

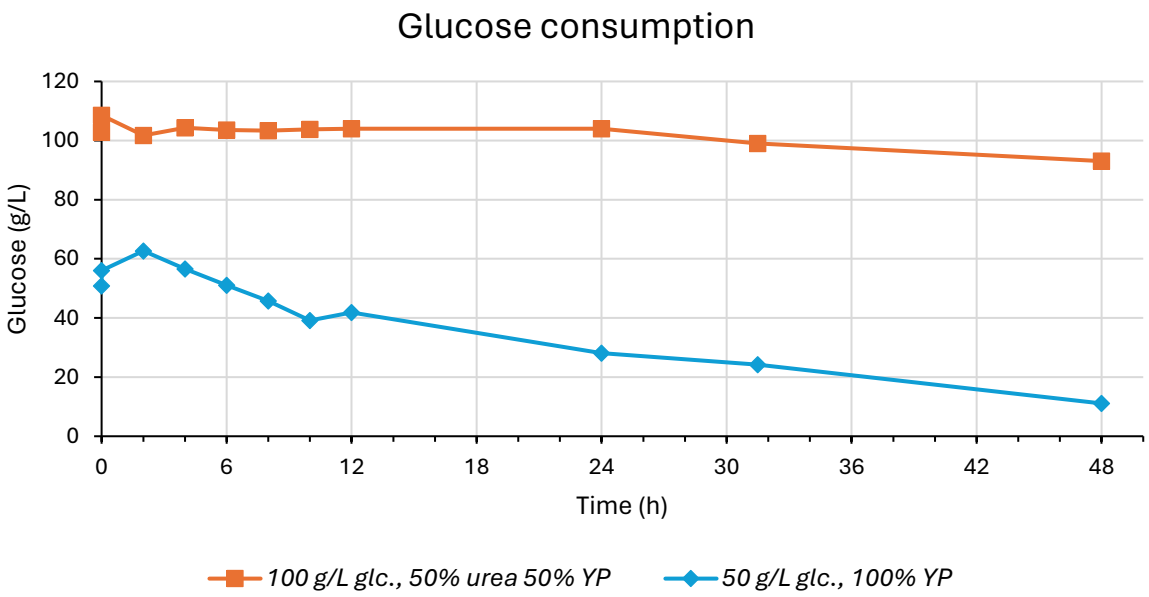

Figure 33. Glucose consumption by *W. anomalus* grown on 100/50 g/L glucose and 100%/50% organic nitrogen source media.

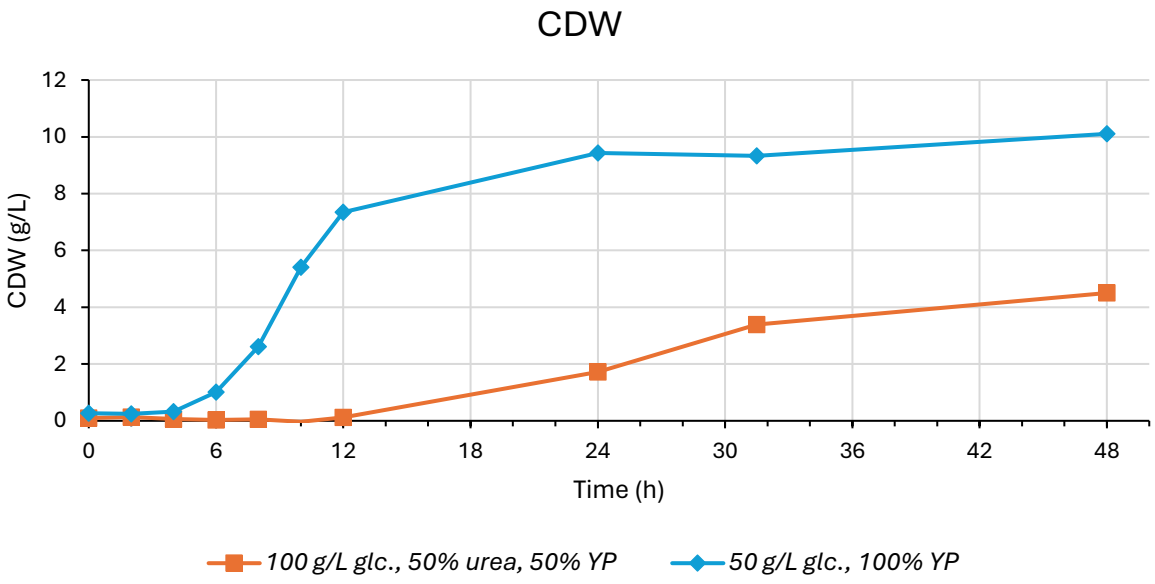

Figure 34. Cell dry weight of *W. anomalus* grown on 100/50 g/L glucose and 100%/50% organic nitrogen source media.

For the fermentation using 50 g/L glucose there was still ~11 g/L glucose left in the media after 48 hours, and the CDW reached only ~10 g/L. Essentially, this fermentation had the same conditions as a previous experiment using 50 g/L glucose (see section 1.2.1.3) but had much lower growth. Even though the inoculum times were the same between these the two experiments, the inoculum for this experiment may have had little growth making the cultivation less efficient in terms of cell growth.

3.2.1.6 Cultivation of *W. anomalus* using vitamin and trace solution, YP adjustments and defined media

Yeast extract contains high amounts of vitamins and trace elements (Tao et al., 2023), however due to its complexity its composition may vary (J. Zhang et al., 2003). The addition of a vitamin and trace element solution to the fermentation media was investigated to see if this improved growth. Additionally, another bioreactor was set up with the ratio of yeast extract/meat peptone adjusted. For all previous experiments YP was made so as to contain 50% nitrogen from yeast extract and 50% nitrogen from meat peptone. This ratio was adjusted in accordance with a previous study using *W. anomalus* for SCP production (Lapeña, Kosa, et al., 2020). A bioreactor with a defined high cell density media as described by

(Roberts et al., 2020) was also set up to compare growth with the fermentations using undefined media for all other experiments.

For the fermentations using the vitamin and trace solution, after the lag phase, there were ~7 hours of high oxygen demand with the stirring speed and aeration rate going at maximum capacity (Figure 35). During this period the DO reached below 30% for ~8 hours (Figure 36).

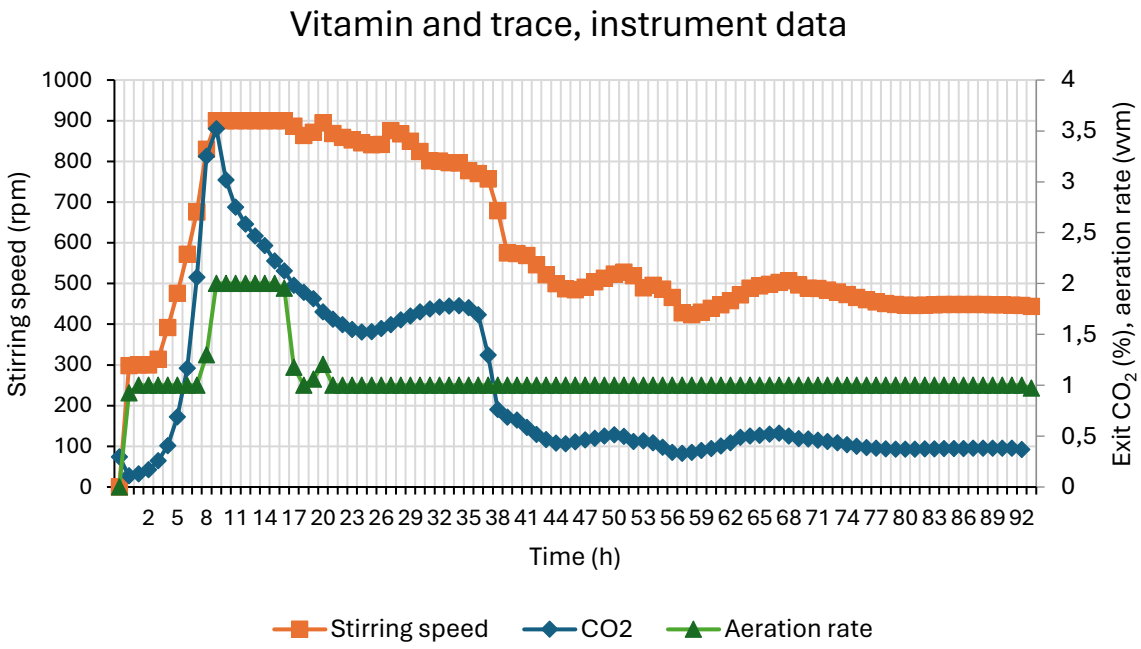

Figure 35. Stirring speeds, CO_2 production av aeration rates during cultivation of *W. anomalus* grown on media with the addition of a vitamin and trace solution.

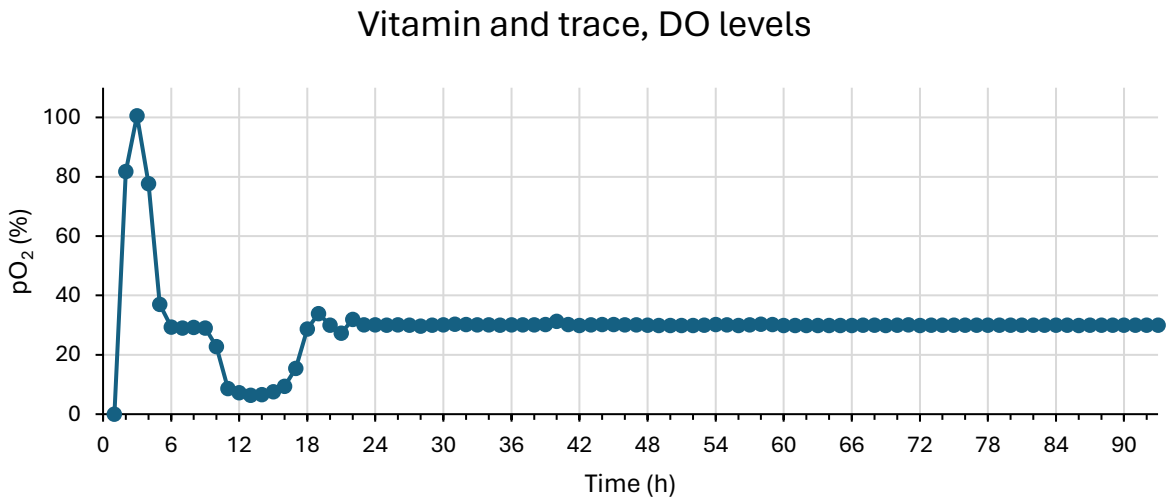

Figure 36. Oxygen levels during cultivation of *W. anomalus* grown on media with the addition of a vitamin and trace solution.

Production of the unknown compound started after ~10 hours right as the DO levels started to drop and was subsequently consumed somewhere between 24 and 48 hours when the DO had stabilized again, further indicating that the production of this compound is associated with an oxygen-limiting environment. Also, after 12 hours the rate of glucose consumption and the growth rate decreased indicating a metabolic switch possibly triggered by the limited oxygen.

No glucose was consumed for the first 8 hours (Figure 37), even though the agitation speed and CO_2 were gradually starting to increase before this, and the CDW had by then reached 3.7 g/L (Figure 38), indicating that the yeasts used a different carbon source than glucose either from the nitrogen source or from the vitamins. The CDW reached 31.4 g/L, making it the highest achieved for *W. anomalus* using 100 g/L glucose compared to previous experiments (see sections 1.2.1.1-1.2.1.5). This may show that the addition of a vitamin and trace solution is necessary for higher growth even when using yeast extract. However, the low levels of DO over several hours during this fermentation may have been limiting the cell growth.

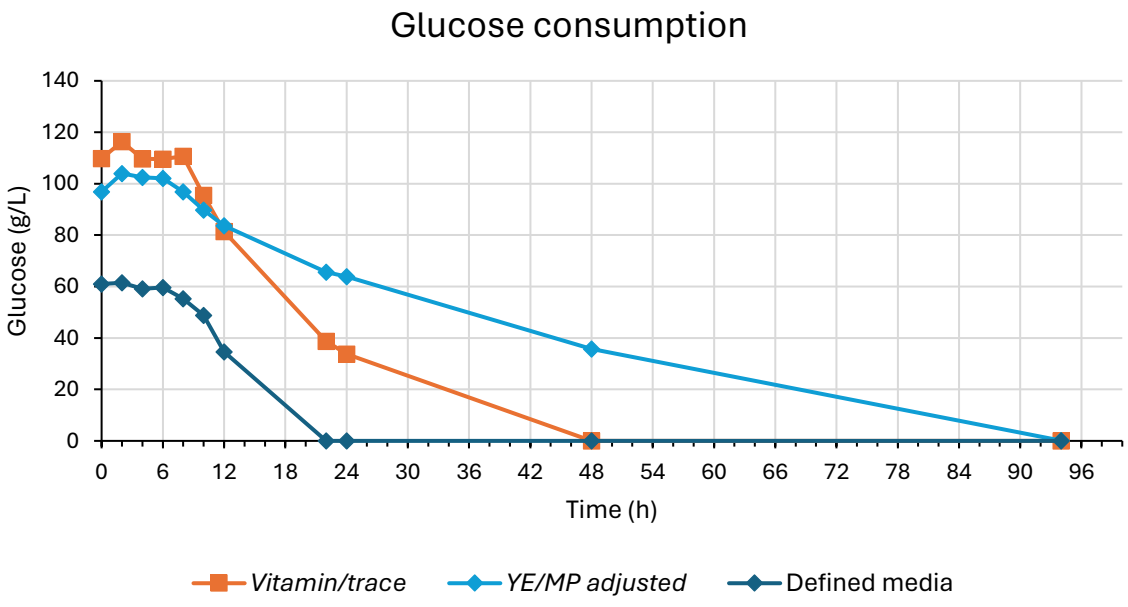

Figure 37. Glucose consumption by *W. anomalus* grown on media with the addition of a vitamin and trace solution.

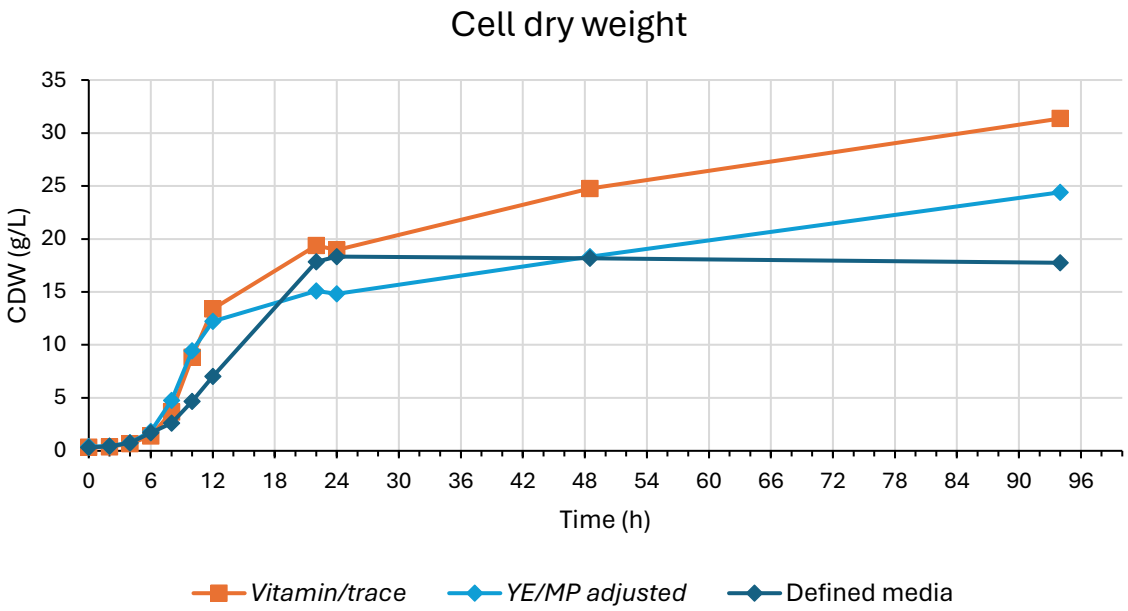

Figure 38. Cell dry weight of *W. anomalus* grown on media with the addition of a vitamin and trace solution.

For the fermentations using the adjusted yeast extract/meat peptone ratio, the stirring speed and aeration rate increased similarly the first few hours to the vitamin/trace fermentation described above, and also reached its maximum capacity indicating a high oxygen demand (Figure 39). However, the time at which the CO_2 was at its peak and the stirring speed and aeration rate were at maximum was significantly shorter than during the vitamin/trace fermentation. When looking at the CDW measurements between the two fermentations the initial exponential growth for the first 12 hours were highly similar. It seems there is an advantage in having more vitamins and trace elements in the media as this leads to higher growth rate after the initial exponential growth. After 10 hours the glucose consumption rate is also significantly lower than that of the vitamin/trace fermentation. Glucose consumption rate may therefore be correlated with the amount of vitamin and trace elements in the media, as seen in a previous fermentation with a higher glucose consumption rate when compared to a media with less yeast extract (see section 1.2.1.4).

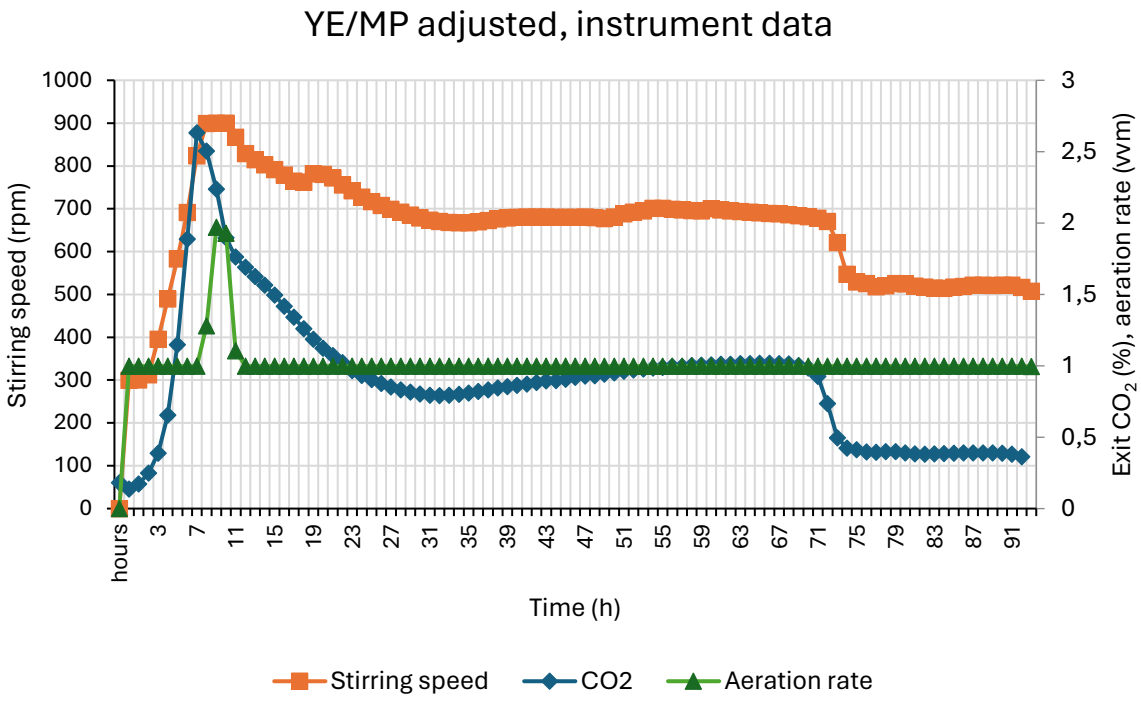

Figure 39. Stirring speeds, CO_2 production and aeration rates during cultivation of *W. anomalus* grown on media with an adjusted YE/MP ratio.

In the fermentations with defined media, the growth completely stopped when the glucose was exhausted (Figure 40), which was not the case for the two other fermentations. This possibly shows that components from either yeast extract or meat peptone are utilized as a carbon source at the same time as glucose, or after glucose exhaustion, as yeast extract and meat peptone were not a part of the defined media.

The media containing a vitamin and trace element solution and the media with an adjusted yeast extract/meat peptone ratio was of the most interest as these fermentations had oxygen-limiting conditions, but still relatively good growth. The oxygen-limiting conditions may be avoided by increasing the maximum stirring speed. Therefore, the experiment was repeated for the two medias of interest, but with an increased maximum stirring speed of 1250 rpm. Also, a vitamin and trace element solution were added to the media with the adjusted yeast extract/meat peptone ratio.

As a result of increasing the maximum stirring speed neither of the fermentations had the DO drop below 30%. Also, the unknown compound was not detected. The fermentation using the non-adjusted yeast extract/meat peptone ratio consumed glucose at a much lower rate (Figure 40) compared to the fermentation with a lower maximum stirring speed, and according to the

CO_2 curve glucose exhaustion happened after ~56 hours (Figure 41). This may be explained by a metabolic switch triggered by oxygen limitation where the yeast has to consume more glucose to achieve the same amount of ATP. The CDW was the highest achieved for the batch fermentations ending up at 40.2 g/L (Figure 42).

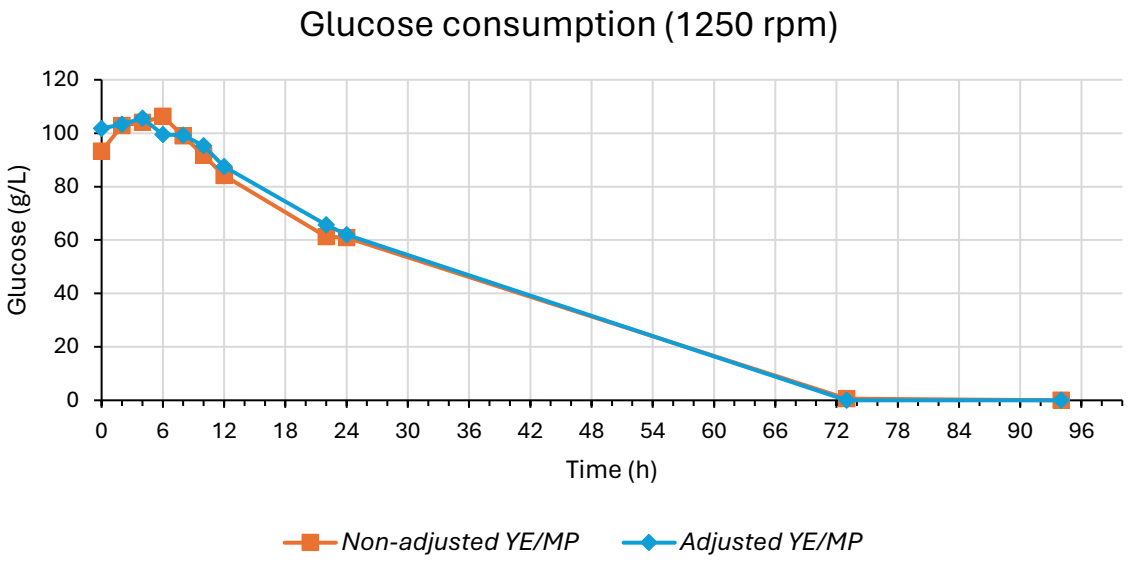

Figure 40. Glucose concentrations during cultivation of *W. anomalus* grown on media with an adjusted and non-adjusted YE/MP ratio.

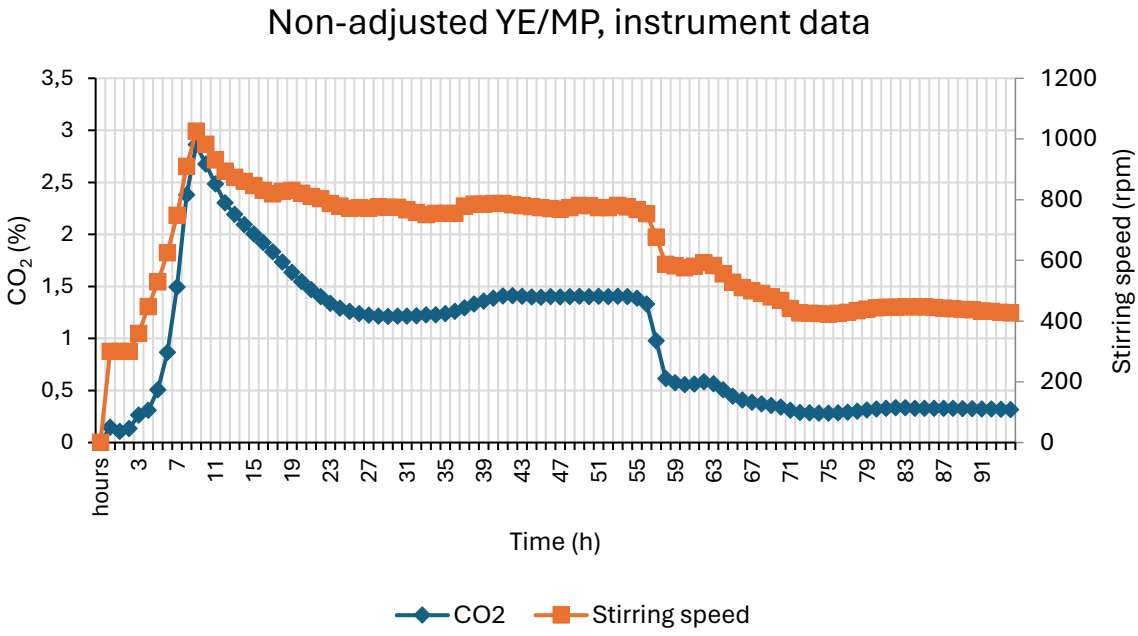

Figure 41. Stirring speed and CO_2 production during cultivation of *W. anomalus* grown on media with a non-adjusted YE/MP ratio.

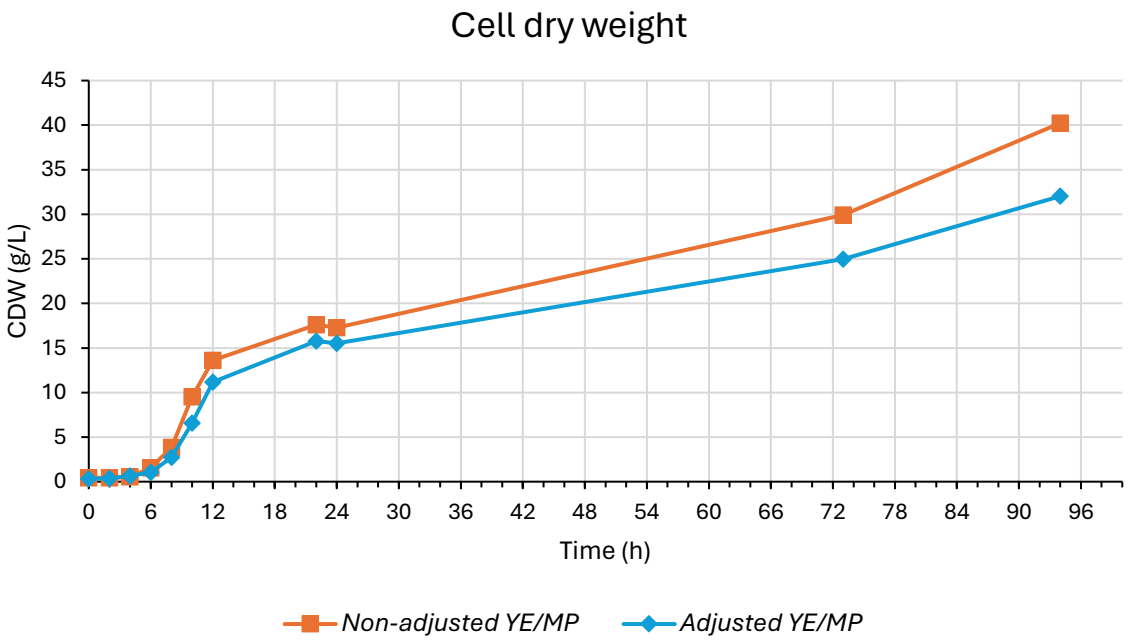

Figure 42. CDW of *W. anomalus* grown on media with an adjusted and non-adjusted YE/MP ratio.

The fermentation using the adjusted yeast extract/meat peptone ratio consumed glucose at a higher rate when the maximum stirring speed was increasing, and no oxygen limitation occurred. This does not match the notion that more glucose is consumed during oxygen limitation but may have something to do with the increased vitamins and trace elements making the yeast able to utilize more of the glucose, as mentioned earlier. According to the CO_2 curve, the glucose was exhausted 10 hours later than the fermentation with the non-adjusted yeast extract/meat peptone ratio (Figure 43). As the media with the adjusted yeast extract/meat peptone ratio contains less yeast extract and more meat peptone than the non-adjusted media, either having less yeast extract or having more meat peptone in the media seems to make glucose consumption slower. As both of these components are complex nutrient sources it is difficult to assess exactly for what reason this happened; both contain alternative carbon sources which may be consumed at the same time as glucose, and nutrients which are required for cell growth and may make the yeast utilize glucose more efficiently.

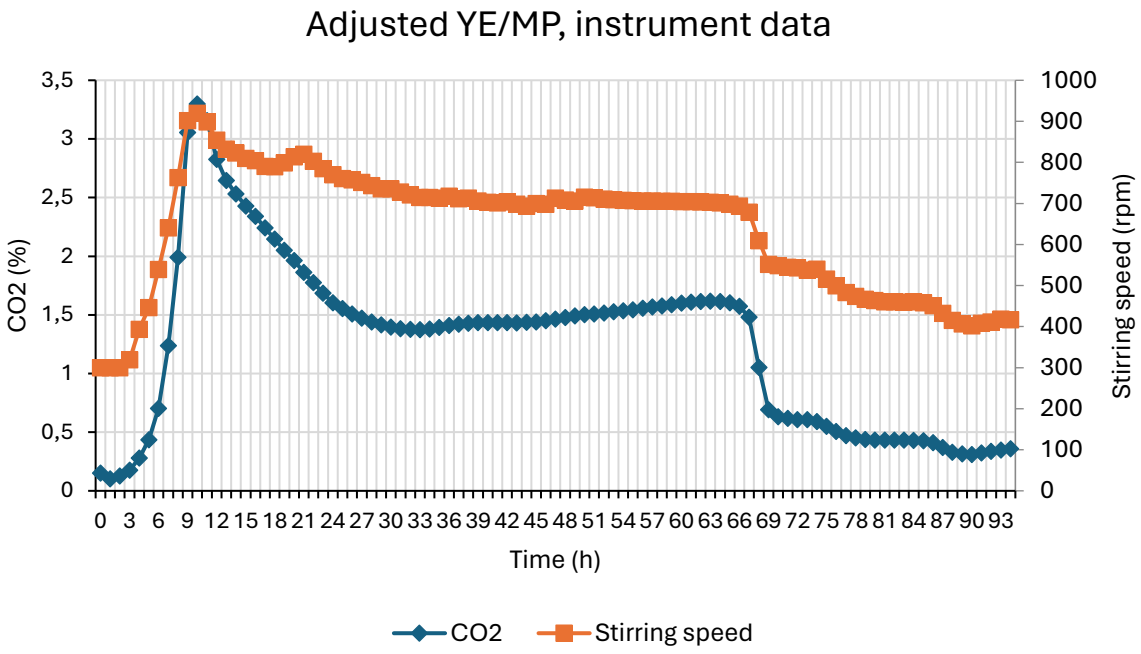

Figure 43. Stirring speed and CO_2 production during cultivation of *W. anomalus* grown on media with an adjusted YE/MP ratio.

DUMAS analysis revealed the protein content of the yeasts to be ~40% after 95 hours.

Since the non-adjusted YE/MP media provided the highest growth, it was decided to use this media for a continuous cultivation experiment.

3.2.2 Continuous cultivation in 1,5 L bioreactors

As continuous production processes are highly industrially relevant for SCP production (Ritala et al., 2017) a continuous cultivation experiment was carried out using the media which achieved the highest growth in a previous batch cultivation (see section 1.2.1.6). Based on off-gas CO_2 data from this cultivation, it could be concluded that glucose depletion happened after ~56 hours, indicated by a sudden and permanent drop in CO_2. The previous HPLC sample analysis before this time point, at 24 hours, showed a glucose concentration of ~60 g/L. Based on this, the average glucose consumption rate was calculated to be 1.875 glucose/L/h within this timeframe (32 hours). This rate of glucose consumption was subsequently used to estimate when to initiate the continuous process and which initial feed pump rate to employ.

In order to avoid the glucose concentration getting too low, the feed rate was adjusted throughout the cultivation as needed. 94 hours after inoculation, a sample was taken from each bioreactor to monitor if the glucose concentration had gone below 10 g/L. Bioreactor 6 (rep. 2) had only 1.2 g/L glucose left, while bioreactor 5 (rep. 1) had sufficient glucose levels at 19.7 g/L (Figure 44). Therefore, only the feed rate of bioreactor 6 was increased at this time.

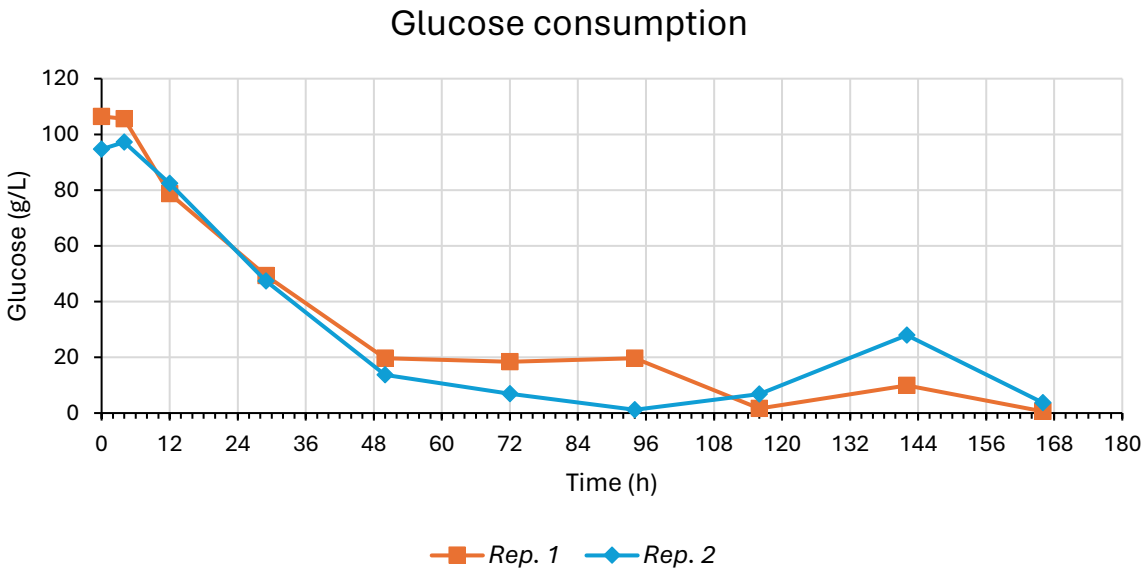

Figure 44. Glucose concentration rates during continuous cultivation of *W. anomalus*.

When the feed pumping was initiated, and for each time the feed rate was increased, the production of CO_2 and the stirring speed subsequently increased (Figure 45, Figure 46). This indicates that the substrate, most likely glucose, was the limiting factor for growth. This is the basis for a continuous process; being able to control the rate of growth by feeding substrate at a restricted rate into the bioreactor (Larsson, 2020).

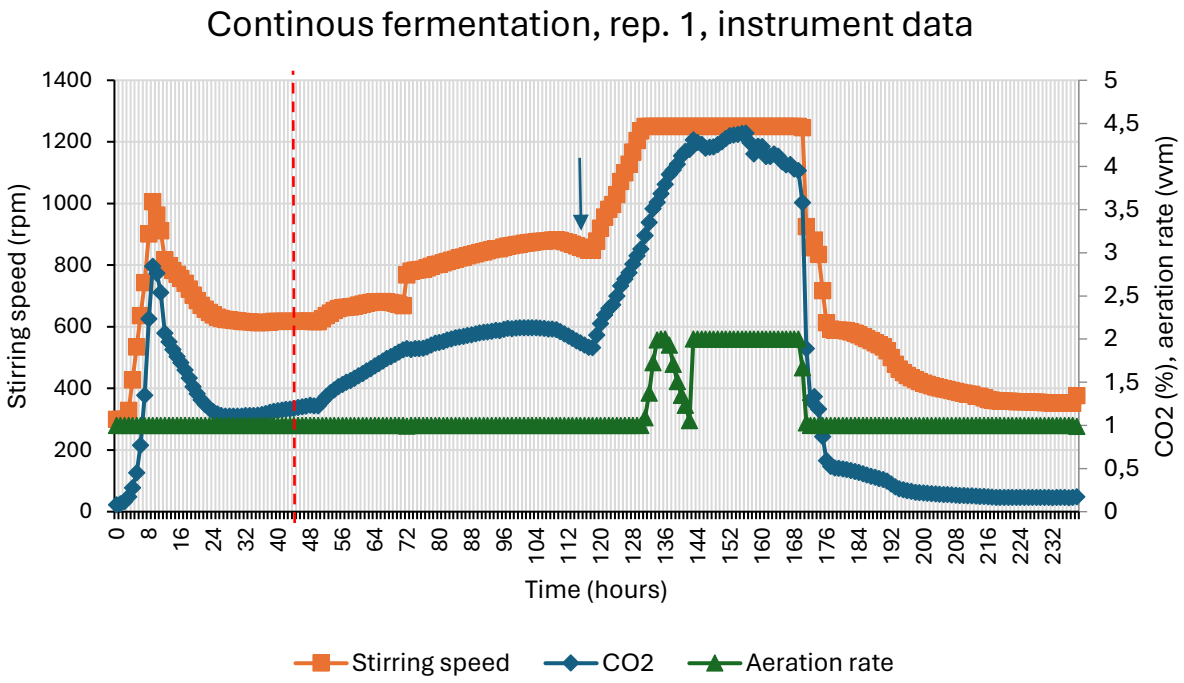

Figure 45. Stirring speeds, CO_2 production and aeration rates during continuous cultivation of *W. anomalus*, first duplicate. Red dotted line indicates the start of the continuous process initiated by the pumping of feed substrate into the bioreactor. Blue arrow indicates the increase in feed rate from 9.375 mL per hour to 19.062 mL per hour.

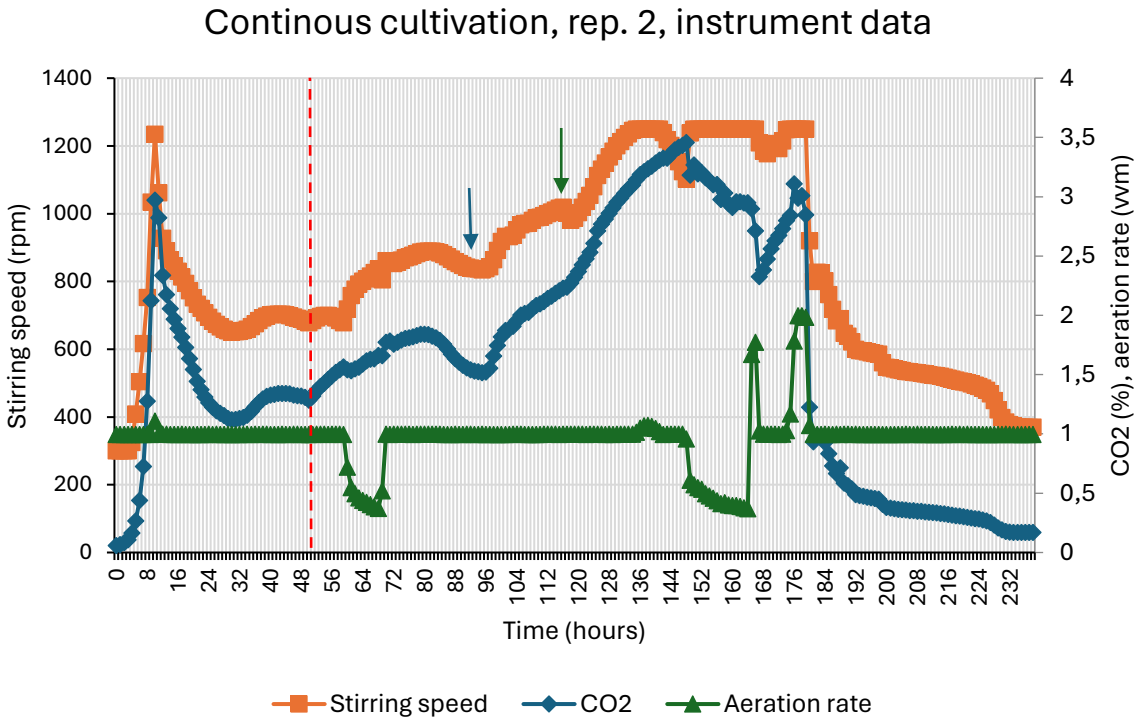

Figure 46. Stirring speeds, CO_2 production and aeration rates during continuous cultivation of *W. anomalus*, second duplicate. Red dotted line indicates the start of the continuous process initiated by the pumping of feed substrate at 9.375 mL per hour into the bioreactor. Blue arrow indicates the increase in feed rate to 14.062 mL per hour. Green arrow indicates the increase in feed rate from 14.062 mL per hour to 19.062 mL per hour.

After the final increase of the feed rate at 116 hours the stirring speeds of both bioreactors increased until reaching the maximum setpoint of 1250 rpm after ~130 hours. After 140-150 hours the DO levels for both bioreactors dropped below 30% pO_2 (Figure 47) for ~20 hours until the feed bottles with the media were completely empty, at which point the production of CO_2 and the stirring speed of both bioreactors rapidly dropped. The oxygen limitation is unwanted and avoiding this can be one of the advantages of utilizing a continuous cultivation process by keeping the number of cells and concentrations of the substrate relatively low in the bioreactor. Glucose consumption rates can increase with an increased feed rate and subsequently increase the oxygen demand in the bioreactor.

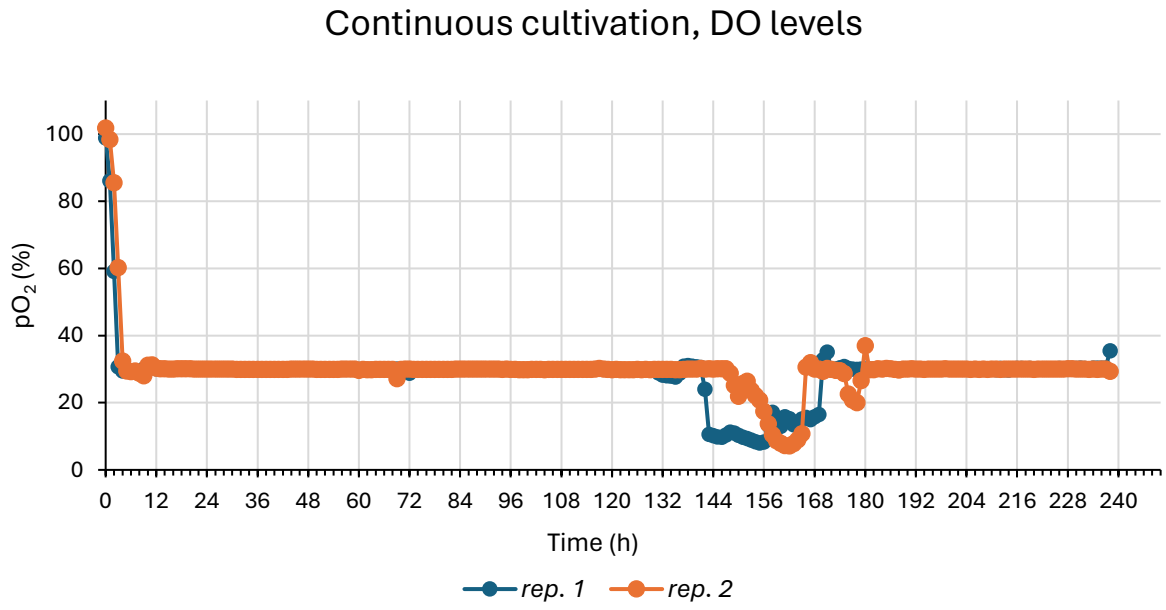

Figure 47. Oxygen levels during continuous cultivation of *W. anomalus*.

Optimally, for a continuous cultivation the process will eventually reach a "steady-state" where there is a balance between the flow of liquid into the bioreactor and the cell growth, so that the specific growth rate equals the dilution rate. During a steady-state process the CDW would remain constant (Larsson, 2020). The average CDW production rate from 12 to 166 hours for rep. 1 was 0.5 g/L/h. The CDW was steadily increasing during the first 120 hours of the cultivation. After 116 hours, at a dilution rate of 0.019062 L/h, the CDW seemed to stabilize at around ~30-35 g/L possibly reaching or getting near to a steady-state (Figure 48).

The specific growth rate for rep. 1 and rep. 2 from 116 to 166 hours was at 0.0211 h^{-1} and 0.0187 h^{-1}, respectively.

A steady-state where the concentration of cells is unchanged may be achieved at a range of feed rates up until a point where an unsteady-state is reached because the cells are being washed out of the bioreactor. Finding the optimal feed rate with good growth and a maintainable oxygen demand for a specific cultivation process requires research with multiple assessments of oxygen demand and cell and substrate concentrations in the bioreactor at different feed rates (Larsson, 2020).

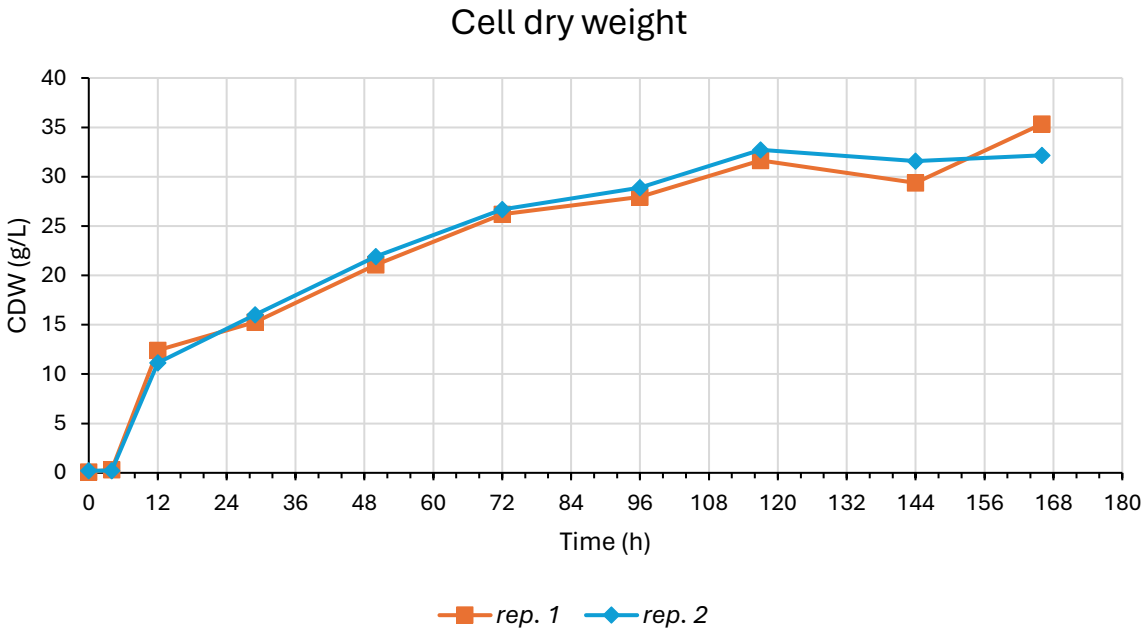

Figure 48. CDW of *W. anomalus* during continuous cultivation.

A carbon mass balance calculation was done for rep. 1 between 50 to 116 hours. Total carbon exiting the bioreactor as CO_2 was 37.9 g, while total carbon in the cells exiting and the cells accumulating inside the bioreactor was 12.0 g, yielding 49.9 g total carbon being utilized by the yeast metabolism. Total carbon from glucose being fed into the bioreactor in this period was 49.5 g. More carbon was being utilized than what was fed into the bioreactor from glucose and may possibly be explained by the glucose already in the bioreactor or alternative carbon sources from for example YP.

To evaluate the accessibility of oxygen in the system, the oxygen transfer rate (OTR) was calculated for different stirring speeds used during the continuous cultivation (Table 9). The k_La values was obtained from the k_La assessment experiment described in Section 1.3.

Table 9. OTR calculated from k_La values at different stirring speeds. All OTR measurements were done at timepoints where the DO = 30%.

Aeration rate (vvm)	Stirring speed (rpm)	k_La (s^{-1})	OTR (mmol/L/h)
1	700	0.0290	226.8
1	800	0.0316	248.4
1	900	0.0343	266.4
1	1000	0.0357	277.2

According to the OTR calculations, an increase in the stirring speed is less efficient at higher stirring speeds in terms of maintaining oxygen levels as the difference in OTR between 900 and 1000 rpm is less than between the lower stirring speeds. Which is also clear when looking at just the k_La values.

To estimate the respiration rate for rep. 1 at 116 hours, it was first made an estimation from Table 9 that the OTR at 850 rpm and 1 vvm would be 257.4 mmol/L/h, based on the known OTR values at 800 and 900 rpm. Using the CDW at 116 hours of 31.6 g/L the respiration rate was estimated to be 130 mg oxygen/g CDW. Since the DO was maintained constant at 30% it was assumed that all the O_2 transferred to the liquid was taken up by the cells.

In terms of protein, DUMAS analysis revealed that the yeasts from both rep. 1 and rep. 2 had a protein content of ~40% from 29 to 116 hours. After 116 hours, when the highest feed rate was initiated, the protein content increased to 44.1% and 43.2% for rep. 1 and rep. 2, respectively.

3.3 Shake flask experiments

3.3.1 Investigating effects of C/N ratio, glucose concentration, peptone variants and antifoam in *C. jadinii* and *W. anomalus* cultivations

As a result of possible restricting, or not optimal, conditions during fermentations which may be caused by inoculums being in an anaerobic state with high ethanol concentrations, a shake flask experiment was set up to optimize the inoculum conditions. Shake flask experiments can provide the same advantages as a microtiter plate-based experiment in being a high-throughput screening method, although less so than a microtiter plate-based system but more so than a bioreactor system. Shake flasks also has the same disadvantages as microtiter plates with the lack of control of pH and oxygen levels. For this experiment, a shake flask system was used to resemble inoculum conditions.

The aim of this experiment was to find an optimized method of preparing an inoculum for use in future single-cell protein production fermentations.

The high amount of ethanol previously observed in the inoculum may imply that the yeast has switched to anaerobic respiration due to lack of oxygen, which is unwanted in terms of optimizing biomass production (Fredlund et al., 2004). Therefore, samples were taken every two hours for 16 hours (except for at 8 and 10 hours), to mimic previous inoculum preparation and to investigate what time the ethanol production started.

Different C/N ratios and an alternative peptone source were investigated to see what effect this could have on the inoculum. To investigate if a Crabtree effect was the cause of high ethanol levels, a set of shake flasks with lower glucose concentration was included.

High amounts of foam were previously observed in *W. anomalus* inoculum bottles; however, this foam was not present in this experiment. Strangely, the shake flask containing zero antifoam showed some foam compared to the other shake flasks containing antifoam, but not anywhere near the amounts previously seen. This means that the high amounts of foam previously seen was at least partly caused by something other than missing antifoam.

Overall, *W. anomalus* produced more ethanol than *C. jadinii* (Figure 49). However, *W. anomalus* generally consumed more of the glucose than *C. jadinii* (Figure 50), indicating that

W. anomalus is not necessarily more prone to produce ethanol, but that *W. anomalus* was able to utilize more carbon. When comparing the 50 g/L glucose condition, where both yeasts consumed all the glucose, the ethanol levels are similar with *W. anomalus* producing 9.9 g/L ethanol and *C. jadinii* producing 11.2 g/L ethanol.

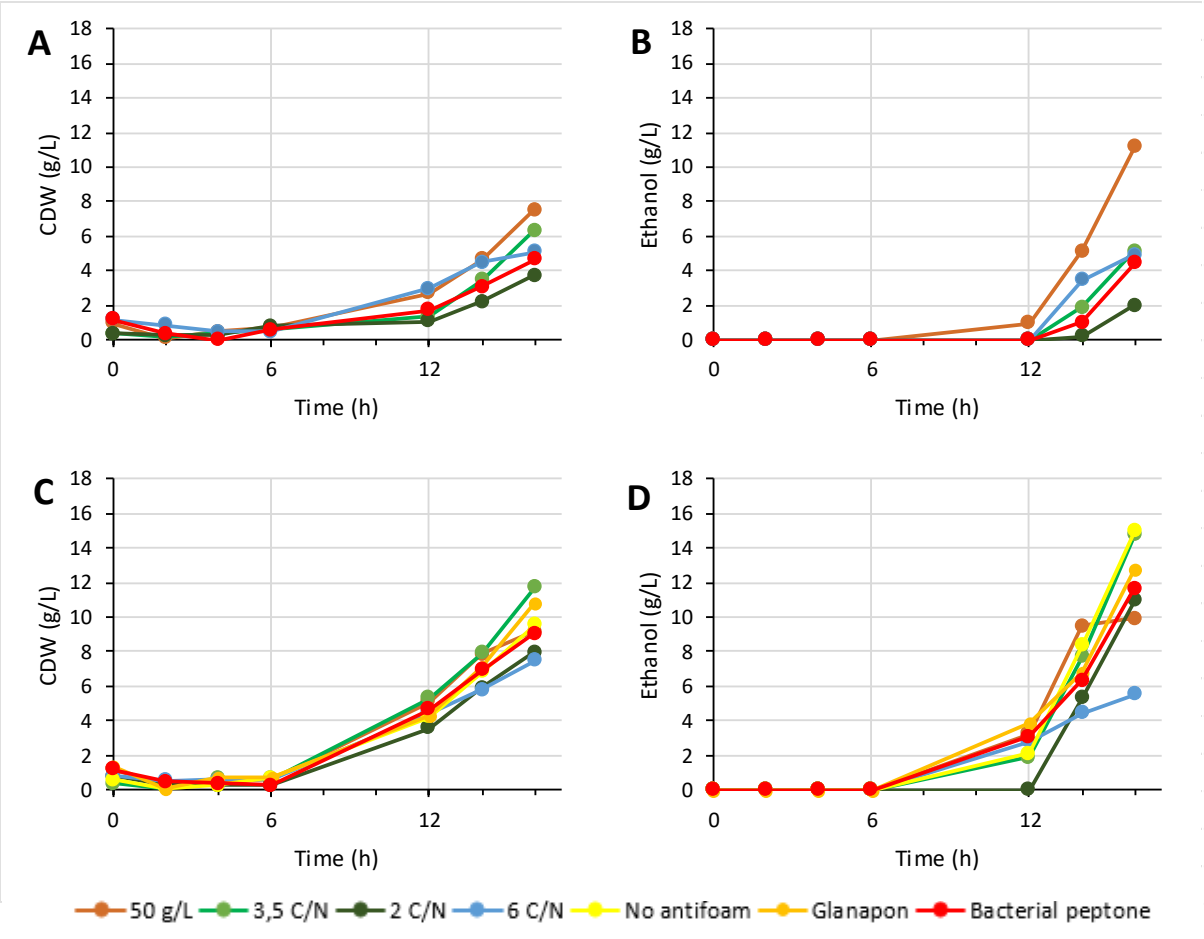

Figure 49. CDW and ethanol concentrations of yeasts grown on different media in shake flasks. A/B = *C. jadinii*, C/D = *W. anomalus*. All media used for *W. anomalus* contained Clerol antifoam, unless stated otherwise. '50 g/L' = media with 50 g/L glucose with a C/N ratio of 3.5, '3.5 C/N' = media with 100 g/L glucose with a C/N ratio of 3.5, '2 C/N' = media with 100 g/L with a C/N ratio of 2, '6 C/N' = media with 100 g/L glucose with a C/N ratio of 6, 'No antifoam' = media with 100 g/L glucose with a C/N ratio of 3.5 without antifoam, 'Glanapon' = media with 100 g/L glucose with a C/N ratio of 3.5 with Glanapon antifoam, 'Bacterial peptone' = media with 100 g/L glucose with a C/N ratio of 3.5 using bacterial peptone instead of meat peptone.

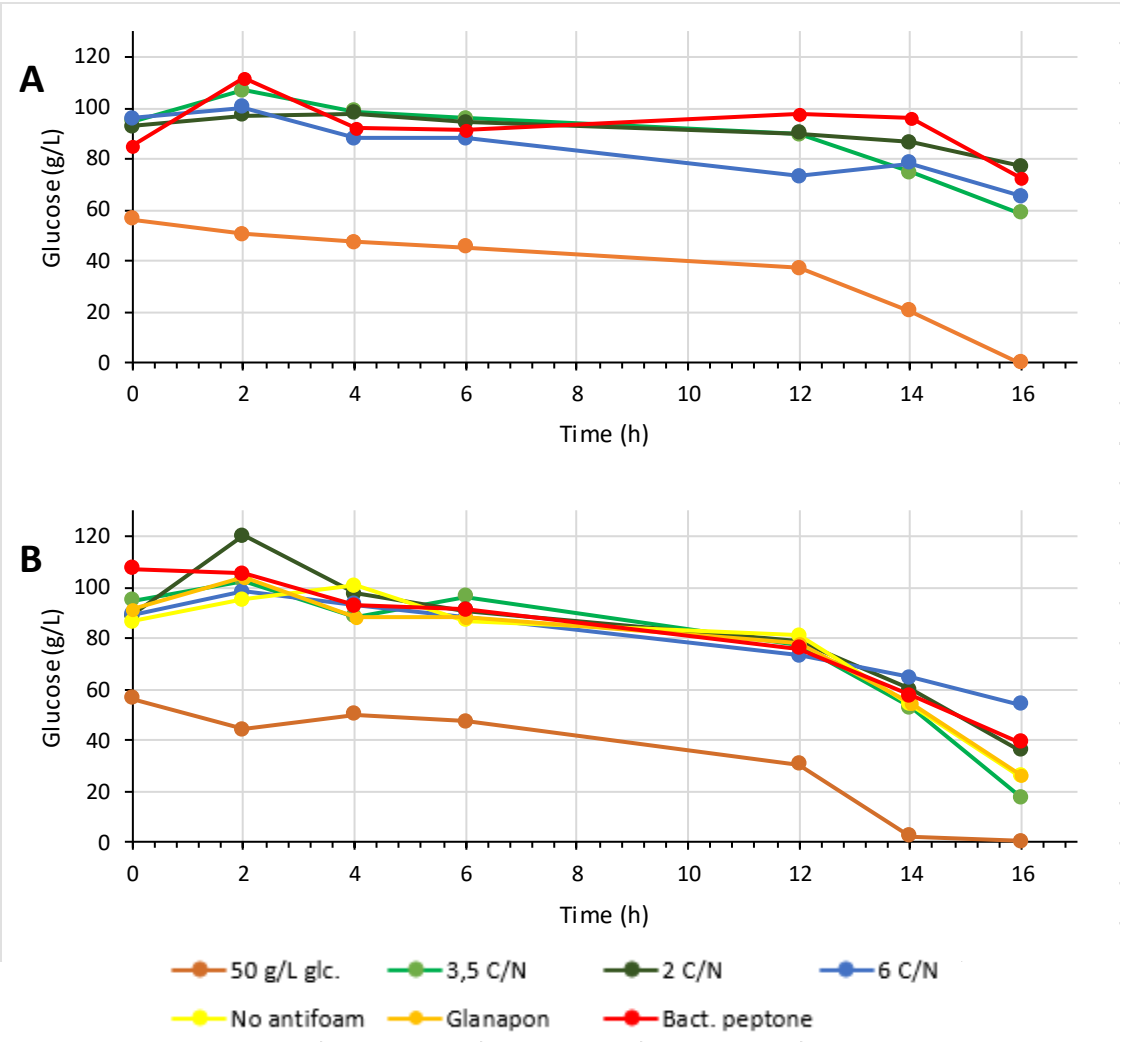

Figure 50. Glucose concentrations of yeasts grown on different media in shake flasks. A = *C. jadinii*, B = *W. anomalus*. All media used for *W. anomalus* contained Clerol antifoam, unless stated otherwise. '50 g/L' = media with 50 g/L glucose with a C/N ratio of 3.5, '3.5 C/N' = media with 100 g/L glucose with a C/N ratio of 3.5, '2 C/N' = media with 100 g/L with a C/N ratio of 2, '6 C/N' = media with 100 g/L glucose with a C/N ratio of 6, 'No antifoam' = media with 100 g/L glucose with a C/N ratio of 3.5 without antifoam, 'Glanapon' = media with 100 g/L glucose with a C/N ratio of 3.5 with Glanapon antifoam, 'Bacterial peptone' = media with 100 g/L glucose with a C/N ratio of 3.5 using bacterial peptone instead of meat peptone.

The CDW of *W. anomalus* was higher than that of *C. jadinii* (Figure 49), which corresponds with higher glucose consumption. The carbon taken up by the strains was almost evenly utilized for ethanol and biomass production in several of the conditions. The concentrations in terms of carbon content are relatively comparable as ethanol contains ~52% carbon and the yeast cells contain ~42% carbon (carbon content of the cells was assessed by DUMAS analysis). However, at the twelve-hour sample point the ethanol concentration was lower than that of the CDW, which may indicate that the production of ethanol starts later than the cell

growth, followed by an exponential production of ethanol eventually exceeding or reaching similar concentrations of the CDW.

Based on these results, the inoculation time was shortened to ~11 hours for *W. anomalus* and ~13 hours for *C. jadinii*, to let the inoculum reach a phase where cell growth had started while at the same time minimizing the amount of ethanol production.

3.2.2 Freeze stock experiment

As *C. jadinii* and *W. anomalus* are not commonly associated with high ethanol production, an experiment was set up comparing the freeze stocks to a reference freeze stock with the same yeast used for similar experiments, to exclude contamination and wrongly labelled freeze stocks tubes.

Glucose consumption, ethanol levels and CDW, using YPD as media, compared between the two freeze stocks are shown in Figure 51.

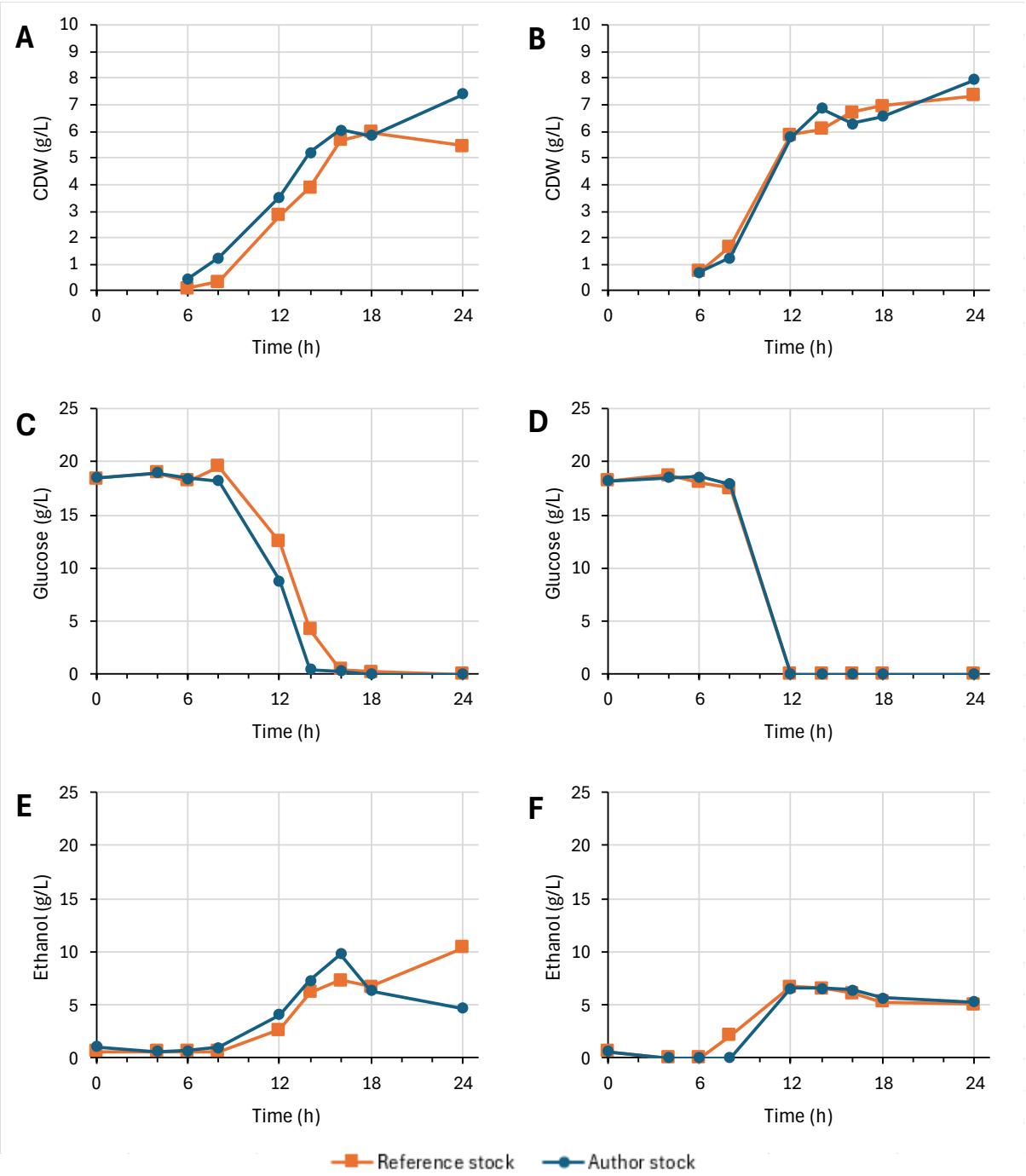

Figure 51. CDW, glucose and ethanol concentrations grown in shake flasks comparing a reference freeze stock to the authors freeze stock. A/C/E = *C. jadinii*, B/D/F = *W. anomalus*.

The results were highly similar to that of the reference stock, and it can therefore be concluded that the -80˚C stocks prepared for this work were not mislabelled or mishandled. This meant that the high ethanol levels were caused by something other than faulty freeze stocks.

3.4 Assessment of k_La

Estimating the k_La value when using different aeration rates and agitation speeds can give insight into the oxygen limitations of the applied bioreactor system. It can also provide the basis for a scale-up procedure. For this experiment, the effect of increasing the aeration rate and agitation speed of the system on the efficiency of the oxygen entering the bioreactor and subsequently dissolve in the media was assessed.

The results generally showed an expected increase in k_La value with increasing aeration rates and agitation speeds (Figure 52). For the Minifors 1 bioreactor the k_La seemed not to be affected by using an aeration rate of either 0.5 vvm or 1.0 vvm when agitation rates were set at 700 rpm or higher. While for the Minifors 2 bioreactor the k_La seemed not to be affected by using an aeration rate of either 1.0 vvm or 1.5 vvm when agitation rates were set at 500 rpm or below. Also, the influence of agitation rates at 800 rpm or above on the k_La were significantly lower than at lower agitation rates for the Minifors 2. This made the Minifors 1 achieving a higher k_La value at higher agitation rates when compared to the Minifors 2, while at lower agitation rates the value was more similar.

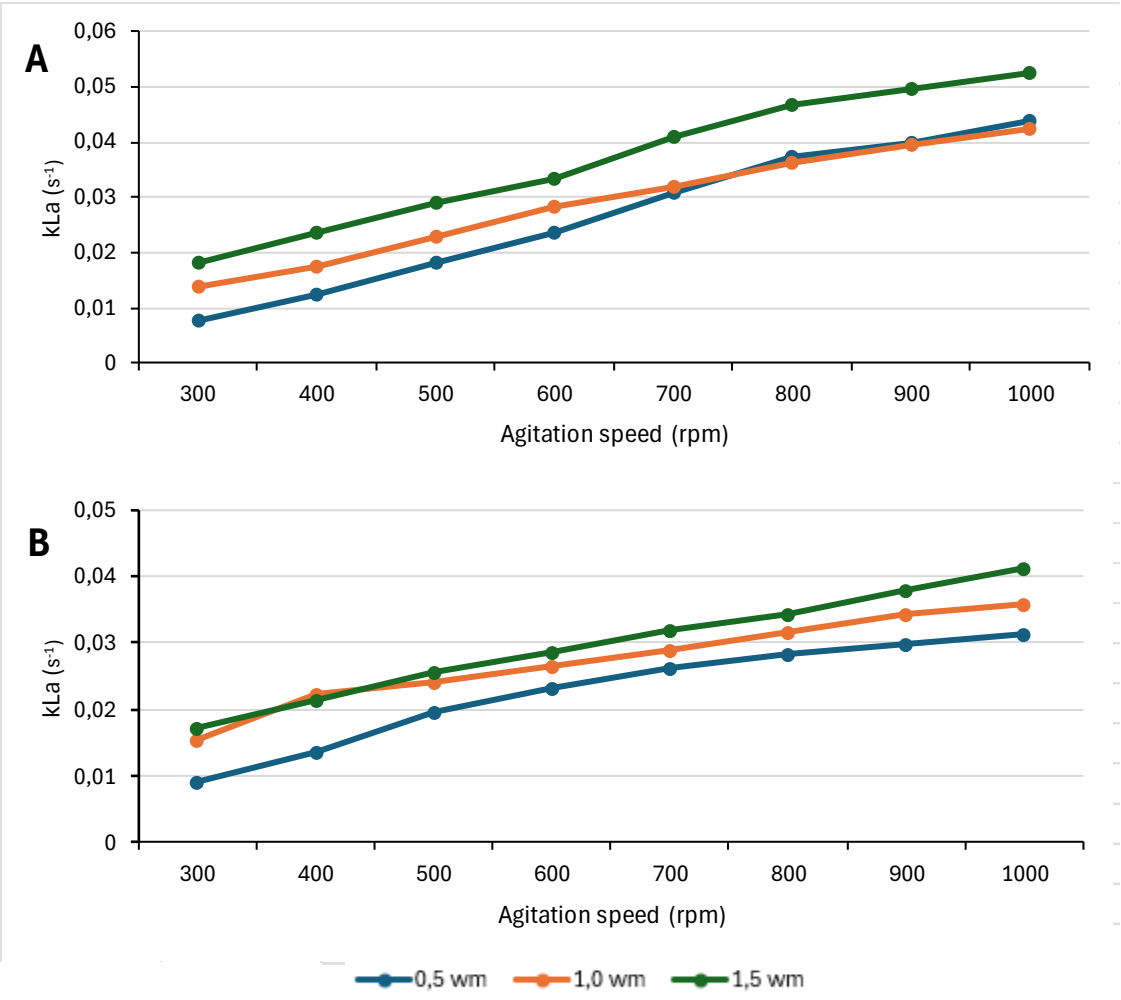

Figure 52. k_La values measured at different stirring speeds and aeration rates. A = Minifors 1 bioreactors, B = Minifors 2 bioreactors.

The control results were highly similar (Figure 53) revealing this to be a reliable method of assessing k_La.

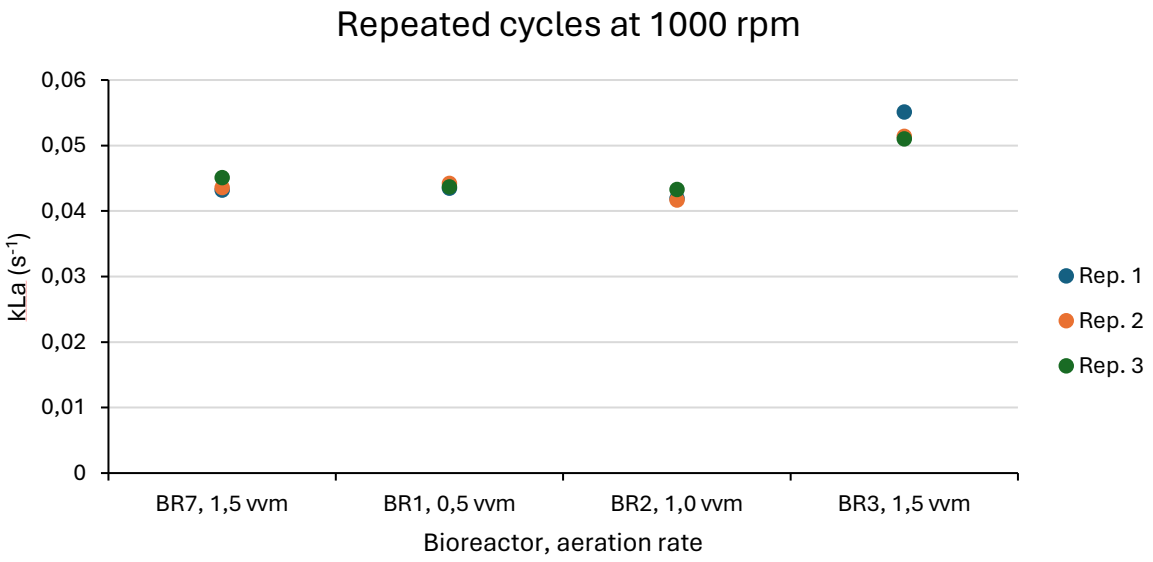

Figure 53. k_La experiments controls by repeated cycles with stirring speeds of 1000 rpm using different aeration rates. BR1/BR2/BR3 = Different Minifors 1 bioreactors, BR7 = A Minifors 2 bioreactor.

4. Conclusion

For an optimized yeast SCP production process, the metabolism of the yeast should be steered towards producing new cells and intracellular protein rather than undesired side-products such as ethanol or organic acids. Side product formation typically takes place under anaerobic conditions and results in a low biomass yield. It is also important to use fast growing yeast strains with a high productivity. In this study, three strains, *B. adeninivorans, C. jadinii* and *W. anomalus,* were screened for their ability to produce SCP. *W. anomalus* was selected as the best candidate because of its limited ethanol production and its relatively high productivity. Medium composition is important when optimizing a SCP production process and several media were investigated in this study with different glucose concentrations, nitrogen sources, C/N ratios, and with the addition of vitamins and trace elements. A medium composition of 100 g/L glucose, a C/N ratio of 3.5 and the addition of a vitamin and trace element solution achieved the highest CDW in the batch cultivations with 40.2 g/L and was therefore also used for continuous cultivation.

As seen in this study, inoculum preparation was really important to avoid side-product formation in the bioreactor cultivations. The shake flask pre-cultures tended to transfer into anaerobic conditions and ethanol production, which continued in the bioreactor cultivations even under well aerated conditions. Ethanol production was efficiently prevented by shortening the inoculum incubation times to around 11 h. Maintaining aerobic conditions during cultivations is therefore highly important. Even when using bioreactors with systems automatically maintaining oxygen levels, keeping the oxygen at the optimal level can be difficult as yeast cells accumulate during the cultivation process demanding increasingly higher oxygen supply. One way to avoid this is by utilizing a continuous cultivation process where the number of cells in the system is kept constant at a concentration that enable the system to keep up with the oxygen demand. In this study, a stable continuous cultivation of yeast at around 35 g/L was achieved with a feed rate of 9.375 mL per hour of a medium containing glucose at 200 g/L. Different feed rates were tried and when increasing to 19.062 mL per hour the system reached its maximum capacity for oxygen supply as the DO dropped below the set value of 30% and the aeration rate and stirring speed reached its maximum of 2.0 vvm and 1250 rpm. The average CDW productivity for the stable cultivation period was

at around 0.5 g/L/h. A carbon mass balance calculation showed that the yeasts utilized all of the glucose being fed into the bioreactor.

Reaching a steady-state process may take time with various adjustments to the process conditions (Doran, 2012). Therefore, it would be of interest to conduct experiments with longer cultivation times and different feed rates to gain more insight into the continuous cultivation experiment conducted in this study so as to assess how and if the selected microbes, media, and conditions would reach this steady-state. Also, detailed analysis and identification of the side products released by the microbes selected in this study and which factors of the process has the most impact on their release would be of interest. Future work would benefit from including more biologically independent replicates to verify the findings of this study. For identifying more of the side products using analytical tools with higher specificity, such as mass spectrometry, would be of interest in regard to further research.

Gaining more knowledge of the metabolic behaviours and side products of the yeasts used in this study could result in more efficient cultivation processes and possible applications of SCP as food and feed.

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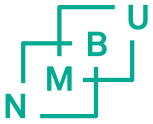

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