Cultivation of *Yarrowia lipolytica* on a hydrolysate based on food waste

Hanna Norrman

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Cultivation of *Yarrowia lipolytica* on a hydrolysate based on food waste

Karakterisering av tillväxten av Yarrowia lipolytica kultiverad på ett hydrolysat baserat på matavfall

Hanna Norrman

Supervisor:	**Jonas Ohlsson, SLU, Department of Molecular Sciences**
Assistant supervisor:	Anna Schnürer, SLU, Department of Molecular Sciences
Examiner:	Volkmar Passoth, SLU, Department of Molecular Sciences

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Swedish University of Agricultural Sciences
Faculty of Natural Resources and Agricultural Sciences (NJ)
Department of Molecular Sciences

Abstract

Interest in replacing fish oil and fish meal as fish feed ingredients with more socially, environmentally, and economically sustainable alternatives has increased in recent years. One such alternative could be the use of yeast biomass. In this report, the growth characteristics of the yeast *Yarrowia lipolytica* on a hydrolysate originating from food waste have been analyzed. The yeast was grown on a filtrated hydrolysate, which was produced in several filtering steps using paper and glass fiber filters, where two degrees of purity were used for the cultivations, e.g., *minimally,* and *sterile filtered hydrolysate*. The yeast was cultivated in small bioreactors where the growth characteristics on the different substrates were analyzed. Another parameter that was investigated was the pH (6.2 and 5.5) effect on the yeast growth. Also, the impact of pH at harvest was analyzed by harvesting the biomass at low and high pH levels. During all cultivations, samples were taken for the analysis of volatile fatty acids and lactate by High-Performance Liquid Chromatography (HPLC). The main findings of this project were firstly, that *Y. lipolytica* could grow on both sterile and minimally filtered hydrolysate. Secondly, the yeast seemed to prefer acetate and propionate over lactate. Thirdly, that the method used for filtration worked well for the preparation of yeast cultivation medium.

Keywords: Yarrowia lipolytica, hydrolysate, food waste, fish feed, volatile fatty acids

Sammanfattning

Användandet av fiskolja och fiskmjöl står under senare tid inför ett skifte för att utvecklas till mer socialt, miljömässigt och ekonomiskt mer hållbara alternativ. Ett av dessa alternativ kan vara att ersätta tidigare råvaror genom biomassan från jäst. I denna rapport har *Yarrowia lipolyticas* tillväxtfaktorer på ett hydrolysat från matavfall analyserats. För att jästen ska ha kunnat växa på hydrolysatet har det först filtrerats i olika steg med hjälp av papper- och glasfiberfilter där två alternativ av renhet valts ut för kultivering (minimalt filtrerat och sterilfiltrerat hydrolysat). Jästen har vuxit i små bioreaktorer där tillväxtfaktorer har analyserats. Andra parametrar som även undersökts är vad olika pH (6.2 och 5.5) har för påverkan på tillväxten. pH-värdens påverkan vid skörd av biomassan har också undersökts. Under alla kultiveringar har prover tagits som analyserats för flyktiga fettsyror och laktat genom High-Performance Liquid Chromatography (HPLC). Resultaten av dessa experiment har för det första varit att det är möjligt för *Y. lipolytica* att växa på de båda substraten. För det andra, verkar jästen föredra de två fettsyrorna acetat och propionat framför laktat. För det tredje, att filtreringsprocessen som togs fram visade sig vara en bra metod för att preparera odlingsmedium.

Nyckelord: Yarrowia lipolytica, hydrolysat, matavfall, fiskfoder, flyktiga fettsyror

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Abbreviations

HPLC	High-Performance Liquid Chromatography
FM	Fish meal
FO	Fish oil
PUFA	Polyunsaturated fatty acids
SCP	Singe cell proteins
TS	Total solids
VFA	Volatile fatty acids
VS	Volatile solids
YPD	Yeast Peptone Dextrose

1. Introduction and aims

Aquaculture is a growing industry that now faces a shift in the source of fish feed origins (FAO 2020). The common sources have been fish oil (FO) and fish meal (FM) which give high-quality nutritional compounds (Hua et al. 2019). However, since FO and FM are problematic from an environmental and sustainable point of view, there is a need for other alternatives that are more environmental, economic, and socially sustainable. (FAO 2022)

One option is to cultivate microorganisms and use the biomass as a protein source. These are called single cell proteins (SCP) and are a cost-effective way to produce high-quality proteins (Gamboa-Delgado & Márquez-Reyes 2018). The yeast *Yarrowia lipolytica* is known to utilize a variety of carbon sources and has therefore been used in a wide range of applications, including production of SCP (Liu et al. 2015). This yeast is, as shown by its name, known to grow in hydrophobic environments (ibid.).

Food waste is a significant problem that has a direct effect on the environment. However, wasted, or spoiled food can, together with other organic materials from agriculture, be recycled to produce environmentally sustainable fuels. This can be performed by producing biogas out of organic waste materials that are degraded to methane in anaerobic environments by microorganisms. The biogas process is divided into four main degradation steps from the organic materials to methane (see section 2.3). Early in the process, the food waste is degraded to a more homogenous liquid, a hydrolysate, containing organic acids (Schnürer & Jarvis 2017). In this project, I have made use of this hydrolysate as a substrate for the cultivation of *Y. lipolytica.* In the future, the biomass of the yeast created through this process is intended for use in fish feed production.

In the project of which this master thesis is a part, the growth characteristics of the yeast *Y. lipolytica* using hydrolysate from food waste as substrate has been analyzed. This has been performed by cultivating the yeast in small laboratory bioreactors. Because the hydrolysate is an unfiltered slurry, it needs to be clarified to enhance the quality of the final product. Therefore, finding a method to filtrate the hydrolysate in a manner that is suitable for cultivations of *Y. lipolytica*, as well as applicable for scaled-up production, has been a necessity for this project.

1.1 Aim and research questions

The aim of this thesis was to investigate the possibility of utilizing hydrolysate, based on food waste, as a nutrient source for the yeast *Y. lipolytica*, for further use as fish feed. Methods for filtration and sterilization of the substrate were also developed and described, followed by analysis of the growth performance of *Y. lipolytica* on this substrate. The consumption of the substrate as well as the influence of pH on growth and biomass was evaluated.

The research questions guiding this thesis was:

- What growth characteristics does *Y. lipolytica* have on hydrolysate with different levels of purity?
- How can sterilization and reduction of particles in the hydrolysate be performed in a suitable way?
- What is the effect of pH on the growth of *Y. lipolytica* on the hydrolysate?
- What is the effect of harvest pH on the biomass?

2. Background

2.1 Aquaculture and the fish industry

Aquaculture is an industry that has been growing more per year compared to other food protein origins. In 2018, 82.1 million tons of fish were produced by the aquaculture industry (FAO 2020). Aquaculture can be divided into unfed and fed systems, where 70 percent of aquaculture is fed using different types of fish feed. The remaining 30 percent is unfed, and in this case, the supply of food in the system is provided naturally by the ecosystem itself (Hua et al. 2019).

For certain feed systems, such as those producing carnivorous fishes, the common fish feed that have been used are fish meal (FM) and fish oil (FO), but other feed origins also exist. The advantages of using FO and FM for aquatic feed are the high-quality proteins and the lipid profile, containing long-chain omega-3 fatty acids and polyunsaturated fatty acids (PUFA) (Hua et al. 2019). This is also important for the human diet since fish containing these fatty acids provide us with essential fatty acids that have been shown to have anti-inflammatory effects (Swanson et al. 2012).

FM and FO can be produced from residual products of the fish food industry, such as heads, skin, and bones that are "inedible" parts for human consumption. However, there is a large amount of FM produced from whole fish by so-called reduction fisheries, fish only caught for the purpose of FM production (Hua et al. 2019). Of 179 million tons of the world's fish production, 156 million tons were used for human consumption, and 22 million tons (12 %) were used for non-human consumption such as FO and FM production (FAO 2020). Examples of captured fish for non-food uses are small pelagic forage fish, like anchovies, mackerel, and others that could be used for human food instead of the production of FM and FO. This type of fish is also an important source of human food with its essential fatty acids and protein content, especially in Africa and the Sub-Saharan region, which makes the aquaculture industry a competitor to food for people in need. Fish products produced by aquaculture are indirectly consumed by wealthier people that consume the fish produced by aquaculture (Metian 2009). It is therefore important to look at this growing industry with regards to an environmental, social, and economically sustainable way.

FM and FO are, as mentioned above, not the only sources that have been used as fish feed. Also, plant-based raw materials have been used to feed fish. Common

plant oils that have been used in fish feed are soybean, rapeseed, sunflower, and palm oil among others. However, FM and FO cannot always be fully replaced but is dependent on what type of plant origin and fish species are farmed (Nasopoulou & Zabetakis 2012). Plant-based feed is for example a problem for carnivorous fish since it can reduce their growth and occur health problems (Gasco et al. 2018). An another aspect is also that plant based oils does not contain long-chain PUFAs (Pickova & Mørkøre 2007). Using plant-based raw material as fish feed occupies arable land and is hence competing with crop production for human food and feed for agricultural livestock production (Foley et al. 2011).

Insect meal is also an alternative to FM and FO that can be used as fish feed. This is especially relevant since a lot of carnivorous fish consume insects as part of their natural behavior and diet. The common types of insects being used for fish feed are crickets, mealworms, and the black soldier fly (Gasco et al. 2018).

An additional source for fish feed is the use of biomass from microorganisms, commonly referred to as single cell protein (SCP). This source includes microorganisms such as microalgae, bacteria, and yeast. The advantage of using microorganisms is that they can be cultivated with less use of land compared to plant-based sources and even be produced on residuals and waste streams. Microorganisms can effectively yield a substantial amount of high-quality proteins and some of them can reach the same levels of protein content as in FM (Gamboa-Delgado & Márquez-Reyes 2018; Agboola et al. 2021). Some yeasts can also be used for the replacement of FO especially yeast species that can produce a lot of lipids, so called oleaginous yeasts (Blomqvist et al. 2018).

The production of SCPs is performed by cultivating the chosen organism on a suitable substrate. Then the biomass is collected and the cells are washed and the biomass can be directly incorporated into feed formulations, or processed further to improve its digestibility (Sharif et al. 2021). The cultivation is performed in a bioreactor depending on the scale. The instruments that are needed for the cultivation are equipment that can regulate temperature, control pH, and an aerator for anerobic fermentation (Nasseri et al. 2011).

2.2 *Yarrowia lipolytica*

Y. lipolytica was at the beginning classified as *Candida lipolytica* before the findings of its sexual state (Barth & Gaillardin 1996). It has two mating types, named *Mat A* and *Mat B* (Liu et al. 2015). *Y. lipolytica* is a dimorphic fungus, meaning it can grow both in a yeast form and in pseudohyphae and septate hyphae state. *Y. lipolytica* is strictly aerobic and does not grow in temperatures above 32–34°C (Barth & Gaillardin 1996).

This yeast can naturally be found in dairy food origins, such as cheese, but also in other hydrophobic oily environments (ibid.). *Y. lipolytica* can with its diverse abilities use different carbon sources as substrates (both hydrophobic and hydrophilic)

and is therefore very useful in different applications (Liu et al. 2015*). Y. lipolytica* is for example a promising microorganism in food processing since it can tolerate low pH, high salt concentrations, and environments with a low water activity (A_W). It also has good enzymatic abilities such as lipases, proteases, and esterases, which makes this yeast suitable for food production as those properties are useful in e.g., ripening of dairy products such as cheese and fermented meat products such as salami (Zinjarde 2014).

Y. lipolytica can also be used for applications other than for food. One example is to use specific strains of this yeast to degrade crude oil which can be useful when taking care of oil pollution (Bankar et al. 2009).

2.3 Food waste and biogas production

Food waste is a significant issue that has direct effects on the environment. According to FAO, up to one-third of global food production is wasted (Gustavsson 2011). However, wasted, or spoiled food can be utilized as a potential resource, such as substrate for biogas production, to contribute to more sustainable fuel production. In the production of biogas, food waste and other organic material such as manure are degraded by microorganisms in an anaerobic environment. The production set-up can differ depending on the main consistency of the substrate and how much water it contains. The different set-ups that are common in the process are to use continuous or batches of either wet digestion, with 2-15 % dry solids, or dry digestion with a low amount of liquids that usually contain 20-35 % of solids (Schnürer & Jarvis 2017).

Although the production of biogas is divided into four main microbial degradation steps it is usually performed in one single process with the four steps happening simultaneously in one bioreactor. First, the organic material (containing proteins, polysaccharides, and fats) is hydrolyzed into amino acids, mono- and short-chain saccharides, and peptides. Second, the hydrolysate is fermented by several reactions into intermediate products such as alcohols, fatty acids, and lactic acid. The third step is a process called anaerobic oxidations where the product from the fermentation is consumed by anaerobic oxidative reactions that produce hydrogen gas and acetic acid, which in the fourth step is consumed by methanogens in the production of methane. The anaerobic oxidative step in the process is controlling the levels of how much methane can be produced and is therefore very crucial (ibid.). In this project, the hydrolysate used as a substrate, has been collected through a two-stage production. This means that the waste material in the first reactor has been degraded to acids and then is transferred to another reactor where the production of methane can proceed (Moestedt et al. 2020). However, in this case, the hydrolysate produced from the first stage has been collected to use as a substrate for yeast cultivation.

Methane is not the only product that can be achieved from a biogas process, common compounds produced in the biogas process are also volatile fatty acids (VFA), possible to recover in a two-stage system. These organic fatty acids can for example

be used in food processing and for food preservation. They can also be utilized in the production of cosmetics, textiles, and pharmaceutical products. VFAs have a maximum carbon length of six carbon atoms. Common examples are acetic acid, propionic acid, and butyric acid (Bhatia & Yang 2017). In recent years, there has been a rising interest in producing VFAs by microbial production instead of chemical synthesis from raw petroleum, due to sustainability and profitability reasons. Different fatty acids can be produced by different types of microorganisms and carbon sources (ibid.). The use of biologically produced VFAs has also increased regarding the use of waste materials as a substrate for the microorganisms since it is cost-effective, has a lower environmental impact, and gives another use for waste materials such as food waste. It also gives biogas production another purpose in addition to the production of methane as biofuel (Atasoy et al. 2018).

3. Materials and method

3.1 Overview of experiments

Three main cultivations were performed to analyze the growth characteristics of the yeast. However, before cultivations, a suitable filtration and sterilization method needed to be developed for the hydrolysate. The filtration steps are therefore the first method described in this section. The first experiment was performed to analyze the growth characteristics of the yeast, using two substrates that were obtained by filtering the hydrolysate on two different degrees of purity (in this report referred to as minimally and sterile filtered hydrolysate). In the second experiment, the yeast was cultivated at different pH and the bioreactors were therefore adjusted to different pH (6.2 and 5.5) by 5M NaOH. Here, the same type of hydrolysate was used. In the last cultivation experiment, the biomass was harvested at different pH, first by harvesting the biomass before the final consumption of the substrate (i.e., at the end of the fed-batch phase, before pH increased due to acid consumption) by taking a sample from the fermenters, and then a second harvest after most of the acids had been consumed and the pH had increased. The harvested biomasses were then further analyzed. During all cultivation experiments, samples were taken for characterization of VFA concentrations and optical density, at set time points during cultivations. VFAs were analyzed using High-Performance Liquid Chromatography (HPLC). All cultivation experiments performed are visualized in a flow chart in Figure 1 below.

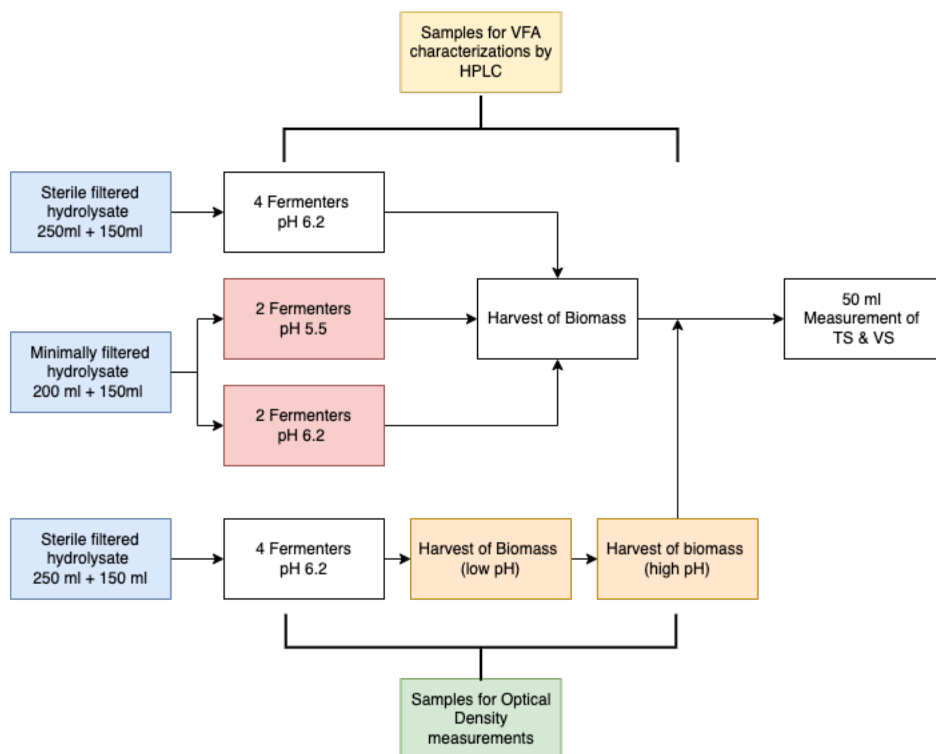

Figure 1. Flow chart of all experiments performed. The blue boxes represent how the hydrolysate has been filtered. The amount of substrate is representing the amount of hydrolysate used in the fermenters (150 ml) and the amount used in the feed bottles. At the next level, it is stated how many fermenters have been used for each growth experiment and their pH settings. The orange boxes represent the experiment where the yeast has been harvested at different pH levels. The yellow and green box surrounding the others with braces represent that samples have been taken and analyzed for optical density and HPLC during these steps. At the end of each fermentation, the biomass has been harvested and investigated by measuring total solids, volatile solids, and ash content. The filtration of the hydrolysate is not visible in this figure but is described further below.

3.2 Filtration and sterilization of hydrolysate

The hydrolysate used for this project was produced as part of a pilot study from IVL Svenska miljöinstitutet AB (Swedish Environmental Research Institute), in Stockholm. The food waste (which is a mixture of food waste from different households) used for producing this hydrolysate is from the Scandinavian Biogas facility in Södertörn, Stockholm.

The goal of this filtration was to find a method to (1) clear a substrate to be able to measure the optical density during the cultivations. (2) create a substrate with low amounts of particles for the yeast to grow in, but without requirement for sterility and optical clarity. The hydrolysate was filtered in several steps starting with centrifugation at 14 000 *g* for 20 minutes at 4 °C in batches of 800 ml bottles. Further on, the hydrolysate was filtered in steps by using a 90 mm Büchner separation funnel (porcelain) together with a Büchner flask and vacuum or suction pump.

The hydrolysate was filtered in two different ways to get two degrees of purity. (1) A sterile filtered hydrolysate and (2) a minimally filtered hydrolysate (where the last step using a sterile filter was omitted). Before filtration, the hydrolysate was autoclaved. Thereafter the filtration was performed through paper filters at temperatures between 40-60°C. Filtration was performed in 6–7 filtration steps (depending on sterile or minimally filtered hydrolysate). The filtration steps can be seen in Table 1, starting with a < 10 µm paper filter, followed by a 5–6 µm paper filter. In the third step, the filter was changed to a 1.6 µm glass fiber filter. This step was performed three times, changing the filter between each repetition. After three repetitions, no more particulate materials were absorbed by the filters. At this point, the minimally filtered hydrolysate had been produced. For the sterile filtered hydrolysate, a PES 0.45 µm sterile filter was used as the last step. The minimally filtered hydrolysate could be autoclaved together with the preparation of the fermenters while the sterile filtered hydrolysate was autoclaved after centrifugation. The sterile filtered hydrolysate was analyzed for VFA content using HPLC (Section *3.5*). This could also have been performed on the minimally filtered hydrolysate. However, since the last sample preparation for HPLC analysis is filtration through 0.2 µm syringe filters, it would be the same as using the sterile filtered hydrolysate directly. In addition, the first samples (at time 0) taken during the fermentations gives an analysis of the VFA content for the minimal filtered hydrolysate.

Table 1. Filtration steps of hydrolysate. This table shows the different filtration steps and equipment used to filter the hydrolysate. For the sterile filtered hydrolysate, the same approach was used except for the last step below the dashed line.

Filtering steps		**Filters**	**Temperature of hydrolysate at filtration**	**Equipment**
Minimally filtered substrate	1	Paper filter (< 10 µm)	40–60 °C	Büchner flask + funnel
	2	Paper filter (5–6 µm)	40–60 °C	Büchner flask + funnel
	3–6 3x times (until no visible particles on the filter)	Glass fiber filters (1.6 µm)	Not temperature dependent step	Büchner flask + funnel
Sterile filtered substrate	7 (Only for the sterile filtered hydrolysate)	0.45 PES sterile filtra-tion	Not temperature dependent step	Sterile Duran flask

3.3 Preparations of yeast culture

The yeast used for this project, *Y. lipolytica* CBS 7504 was cultured on Yeast extract–Peptone–Dextrose (YPD) medium agar plates, YPD broth, and acid growth media. All media prepared was sterilized before use by autoclaving at 121°C. All media used for the yeast preparations are listed in the Appendix.

Before growth in fermenters, the yeast was pre-cultured in YPD broth (150-200ml) in baffled 250 ml Erlenmeyer flasks on a rotary shaker board (150 rpm) at 30°C for three days. To prepare the yeast for conditions related to the hydrolysate, a solution of acids (pH 6.5) was prepared by pouring approximately 25 ml of the active yeast culture in YPD broth into 150 ml of acid growth medium (Appendix). This was performed one day before inoculation into the fermenters in baffled Erlenmeyer flasks and cultivated in the same conditions.

3.4 Preparation of fermenters

The fermenters used for these experiments were 4 Infors HT Multifors 2 bioreactors. The fermenters were loaded with 150 ml of the substrate and with 200–250 ml of the substrate in feed bottles connected to acid pumps. This is called a pH stat fed-batch culture where the substrate is pumped into the fermenting vessel depending on the pH levels in the fermenter that is rising when the acids in the hydrolysate are consumed by the yeast (Fontanille et al. 2012). The temperature was set to 28 °C. Before inoculation of the yeast, pH was adjusted by adding 5 M $NaOH$ with a 0.2 µm sterile filter and needle. The dissolved oxygen was set to 20 %, controlled by

stirring at a rate of 500–1200 rpm. Aeration was accomplished by sparging with 0.3 L/minute of compressed air.

The concentration of the preculture was measured by optical density at 600 nm (OD_{600}) and the volume was added since the inoculum was calculated according to the measured concentration (Equation 1). The volume was adjusted and inoculated into the fermenters using a syringe and needle. The fermenters were inoculated with a target OD_{600} of 5 using yeast cells washed in saline solution. After inoculation, 3 drops of antifoam (polypropylene glycol, PPG 2000) were added to prevent foaming in the fermentation vessels.

$$C_1V_1 = C_2V_2 \tag{1}$$

Total solids and volatile solids

The biomass of all experiments was collected in 50 ml Falcon tubes to measure the total solids (TS) and volatile solids (VS). The sample was prepared by spinning the biomass in a centrifuge at 4000 rpm for 10 minutes and the cells were then washed with deionized water and centrifuged at the same settings twice. The biomass was dried in an oven at 105 °C for approximately 24 h. The dry samples were then weighed before and after incineration at 550 °C to determine ash content.

To calculate TS % and VS % the following equations were used (Equation 2 & 3).

$$\mathrm{TS}(\%) = 100 \times \frac{\text{dry weight at 105 °C}}{\text{wet weight}} \tag{2}$$

$$\mathrm{VS}(\%) = 100 \times \frac{\text{dry weight at 105 °C} - \text{ash content}}{\text{dry weight at 105 °C}} \tag{3}$$

The rest of the 300 ml of the biomass was collected and stored at –20 °C for further analysis.

Characterization of yeast growth performance

In this experiment, four fermenters were loaded with 150 ml of the sterile filtered hydrolysate and the feed flask with 250 ml of the same substrate. The pH was set to 6.2 in all four fermenters.

Effect of pH on yeast growth

Four fermenters were loaded with 150 ml of the minimally filtered hydrolysate and autoclaved together with the fermenters and 200 ml of the same substrate. The pH was set to 5.5 on two fermenters and 6.2 on the other two fermenters.

Effect of harvest pH on biomass

Four fermenters were loaded with 150 ml of sterile filtered hydrolysate and 250 ml of the same substrate in the feed flasks. The pH was set to 6.2.

Bioreactor operation

Samples were taken continuously at several time points during the fermentation, including the start of the inoculation. The samples were collected through a sampling port with a sterile syringe to avoid contamination. All samples were collected in Eppendorf tubes for HPLC analysis and OD_{600} measurements. Samples collected for further biomass analysis were kept in 50 ml Falcon tubes kept in –20°C.

3.5 High-Performance Liquid Chromatography (HPLC) analysis

At different time points of the cultivations, samples were taken to analyze the concentrations of VFA in the samples. Samples were collected throughout the cultivation. All samples were put in the freezer before the preparation of the HPLC run. The samples were frozen at –20°C and then centrifuged at 11 498.63 *x g* for 15 minutes. The supernatant was decanted and put in new Eppendorf tubes, inverted and then 700 µl of the samples were mixed with 70 µl of 5 M H_2SO_4. The samples were put in the freezer again and later centrifuged at 11 498.63 *x g* for 10 minutes. After this step, each sample was collected with a syringe and filtered through a 0.2 µm syringe filter into a glass HPLC vial. The column of the HPLC machine was an ion exchange column, and a refractive index detector was used for detection. During the run, the column was set to 60 °C and the samples were eluted with 5 mM H_2SO_4 with 0.6 ml min^{-1} as a flow rate (Westerholm et al. 2010).

3.6 Optical density measurements

During cultivations, samples were taken at different time points to analyze the growth rate of the yeast. For each sample, the OD_{600} was measured. The samples were diluted in deionized water to have an absorbance below 0.6. This was to measure the concentration of yeast. OD_{600} was also measured before inoculation of the yeast in the fermenters to calculate the volume added in the fermenters, to ensure equal concentrations of yeast cells and volume in all fermenters.

3.7 Statistical comparisons

All statistical analyses were performed in Microsoft Excel using the Student's t-test. In all t-tests, a two-tailed distribution of equal variance (homoscedastic) was used.

4. Results

4.1 Filtration and sterilization of the hydrolysate

The filtration steps described above proved to be a suitable method to get a filtered hydrolysate. However, during the filtrations, it was noticed that heat treatment of the hydrolysate was necessary before using the paper filters since the hydrolysate probably has a high-fat content that is not permeable to hydrophilic paper filters. During filtration at lower temperatures, the filter papers clogged faster and it was thereby very time-consuming. The glass fiber filters, on the other hand, could be used without heat treatment since they are more usable for hydrophobic liquids. The PES sterile filters have the same properties as the glass fiber filters in this regard. However, there were sometimes problems with filtering the hydrolysate if there were too many particles in the solution that clogged the 0.45 µm pores.

The appearance of the hydrolysate shifted depending on temperature treatments. The centrifuged hydrolysate had a yellow-green color compared to the autoclaved substrate, which turned more orange yellow at high temperatures. The filtered substrates kept in colder temperatures (in a refrigerator) had a browner color.

Figure 2. Centrifugation of hydrolysate. The hydrolysate before (to the left) and after centrifugation.

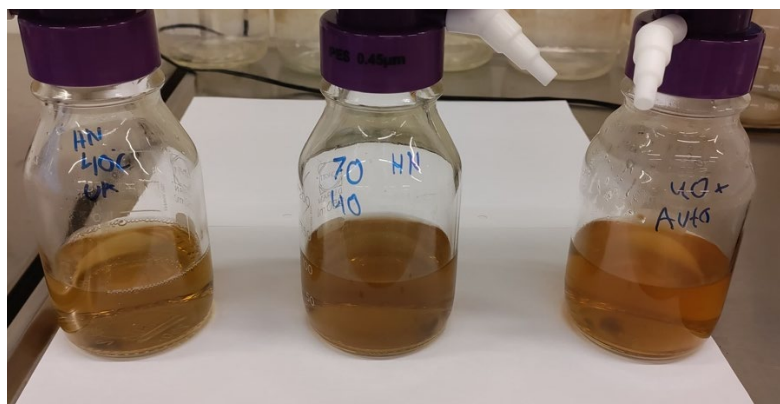

Figure 3. Three different heat treatments of the sterile filtered hydrolysate. To the left, the hydrolysate was filtered after incubation in a water bath at 40 °C. In the middle, the hydrolysate has been kept in a water bath at 70 °C and thereafter 40 °C. To the right, the hydrolysate has been autoclaved which clearly shows a more orange color compared to the other heat treatments.

As described in the previous chapter, the hydrolysate was also analyzed for the composition and level of VFAs to see the concentrations of these acids in the substrate. It was only the sterile filtered hydrolysate that was investigated in this way, and this is presented in the table below (Table 2). The mean values of the three dominating acids were in the following order of magnitude: lactate > propionate > acetate.

Table 2. HPLC results of the (VFA) characterizations of the sterile filtered hydrolysate. Lactate has the highest concentration of the VFAs in the hydrolysate followed by propionate and acetate.

	Acetate g/L	Lactate g/L	Propionate g/L
Mean value	5.56	13.76	7.33
Standard deviation	0.104	0.099	0.079

4.2 Characterization of yeast growth performance

4.2.1 Cultivation on sterile filtered hydrolysate

The cultivation on the sterile filtered hydrolysate took 26 hours and samples were taken at 0 h, 15 h, 18 h, 21 h, and 26 h before harvesting of the biomass. As described in the Methods section, the bioreactors were set by pH-stat fed-batch to feed the yeast with more hydrolysate. The volume of the substrate in the fermenter is represented by the gray area in the graph and was increasing with the growth of the yeast biomass (Figure 4). As can be seen in the diagram, OD_{600} increased with the growth of the yeast biomass. All acids were consumed with the growth of *Y. lipolytica*. E.g., for the measurement at 26 h, acetate decreased down to 0–0.1 g/L and propionate down to 0.6 g/L. The concentration of lactate, on the other hand, in-

creased until 18 h (17.5 g/L), at which point the concentration of acetate and propionate was significantly lower. At harvest time lactate was measured to 14.2 g/L. The biomass was collected after harvest and calculated to be 11.1 g/L of dry weight.

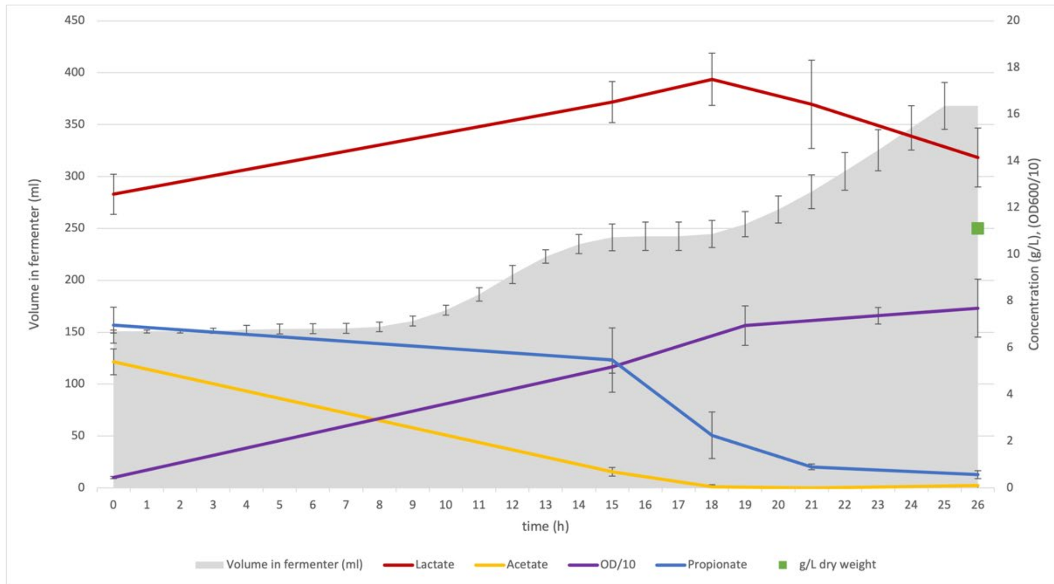

Figure 4. Growth of the yeast on the sterile filtered hydrolysate using 4 fermenters. All values used are the mean values of all replicates together with error bars representing the standard deviation of these values. The gray area represents the amount of substrate present in the fermenters. Red, blue, and yellow lines indicate concentrations of individual VFAs. The purple line represents the OD_{600}, *divided by a factor of 10. The green square represents the amount (g/L) of the dry weight of the collected biomass after harvest.*

4.2.2 Cultivation on minimally filtered hydrolysate

The cultivations using the minimally filtered hydrolysate were finished after 24–26 hours depending on the fermenters since they were harvested when the feed was consumed. Optical density could not be measured in this experiment since the hydrolysate was too opaque and is therefore not represented in the graph. The consumption of acids had a similar pattern as for the growth on the sterile filtered substrate. At harvest, the concentrations of VFAs were 0 g/L for acetate and 0.65 g/L for propionate. Lactate was as in the previous experiment higher at the end (12.5 g/L). The biomass was collected and measured to be 13.4 g/L.

The ash content (%) (100 - VS %, i.e., the biomass minus the volatile solids) of the biomass from the sterile filtered hydrolysate was calculated to constitute 3.7 % and for the yeast grown on the minimally filtered hydrolysate, the ash content was 5.5 % (AC %, + 49 %, p= 0.0016). These results are composed of two replicates from the minimally filtered data and four replicates from the sterile filtered data since the experiment performed with the minimally filtered hydrolysate were divided into two groups with different pH at 5.5 and 6.2. If the results from cultivations performed at different pHs are put together, the following values would be obtained: Ash content of the minimally filtered hydrolysate with four replicates (5.7 %), and

the ash content of the sterile filtered experiment would be the same as mentioned above (3.7 %). This gives a p-value of 0.0009. (AC %, + 52 %, *p= 0,0009*). The dry weight of the biomass at harvest was higher in the minimally filtered hydrolysate (13.4 g) compared to the sterile filtered hydrolysate (12.4 g).

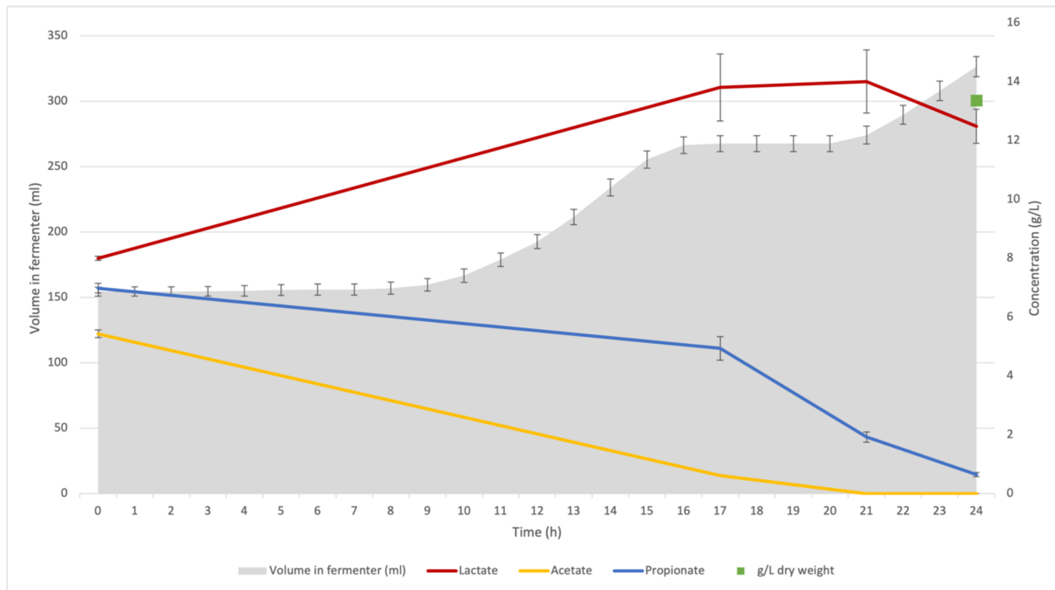

Figure 5. Growth of the yeast on the minimally filtered hydrolysate using 2 fermenters with a pH of 6.5. All values used are the mean values of all replicates together with error bars made with the standard deviation of these values. The gray area represents the amount of acid substrate in the fermenters. Red, yellow, and blue lines indicate concentrations of individual VFAs. The green square represents the dry weight (g/L) of the collected biomass after harvest.

4.3 Effect of pH on yeast growth

In this experiment, the aim was to see the differences in the growth characteristics of the yeast at different pH (6.2 and 5.5). At both pH levels, the VFA consumption patterns were similar, with acetate and propionate being preferentially consumed. There was no apperent inhibition at this pH level, with cultivations finishing at approximately the same time at both levels. (See Figure 6)

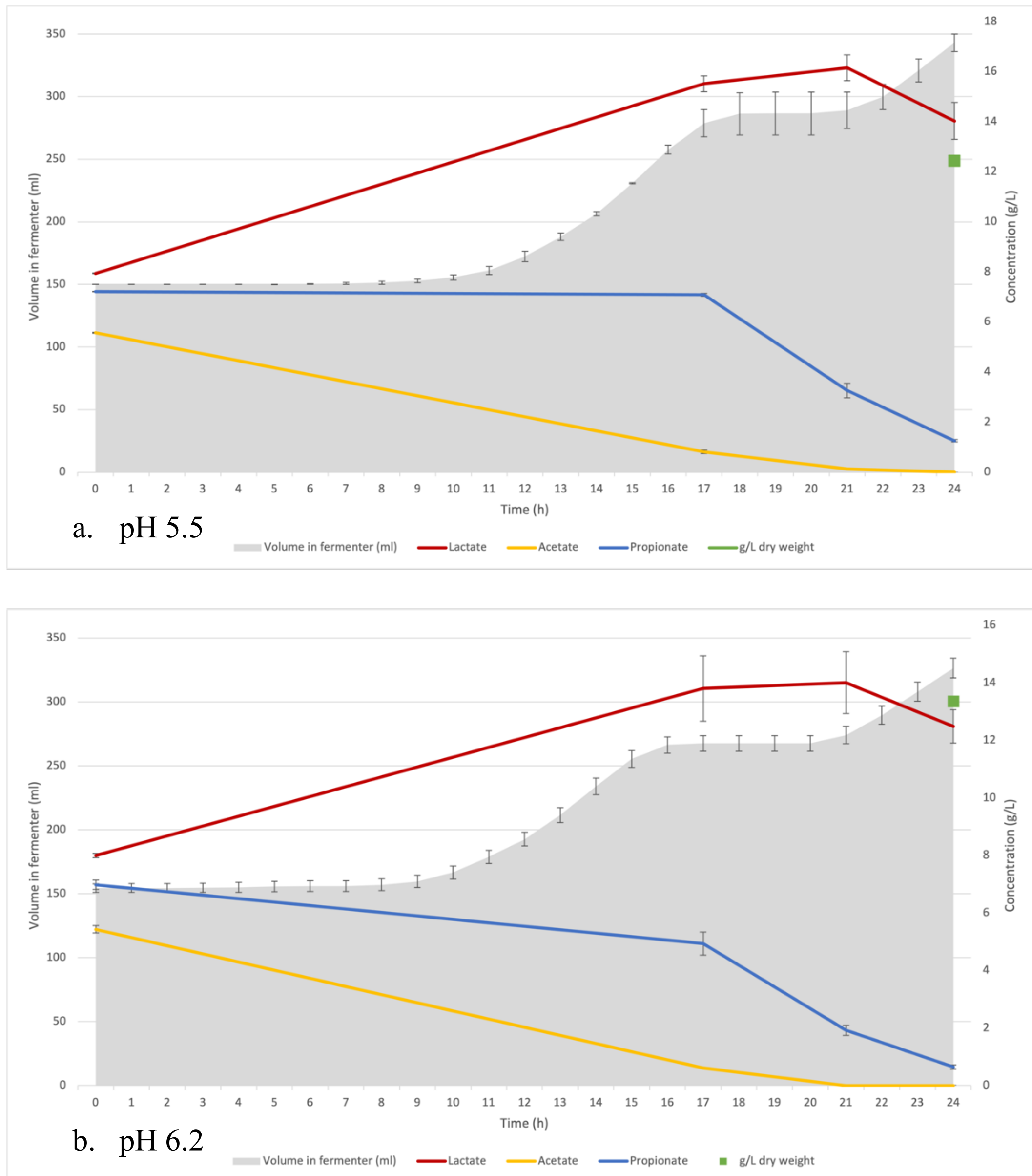

Figure 6. Effect of pH on growth performance, showing growth characteristics of the yeast grown at pH 5.5 (panel a) and pH 6.2 (panel b). Both are grown at the minimally filtered substrate using 2 fermenters. All values used are the mean values of all replicates together with error bars made with the standard deviation of these values. The gray area represents the acid pump of the amount of acid pumped into the fermenters during the growth. The red line shows the concentration of lactate during growth, the blue line shows the concentration of propionate, and the yellow line shows the consumption of acetate. The data point (green square) to the right represents the concentration (g/L) of the dry weight of the collected biomass after harvest.

The fermenters were harvested when the fed batch medium was fully consumed in this experiment. The mean batch time of the two fermenters at pH 5.5 was 25 h 26 min 29 s, which is very similar to the fermenters at pH 6.2 which had a mean batch time of 26 h 45 min 42 s. The dry weight of the biomass produced at pH 6.2 (13.4

g/L) is almost the same as the biomass from pH 5.5 (12.4 g/L). The same patterns is also visible in the volatile solids and ash content.

4.4 Effect of harvest pH on biomass composition

This experiment aimed to analyze the biomass before the consumption of residual acids after the fed batch phase and concomitant rising pH to see how that affected the biomass composition. The dry weight of the biomass is presented in Table 3. The biomass harvested at higher pH had a higher ash content and dry weight compared to the biomass harvested at lower pH (ash content + 728 %, p = 0.014; dry weight + 76 %, p = 0.0007).

Table 3. Ash content (g/L) and (Weight %), dry weight (g/L) and VS (g/L) of the harvested biomass at high and low pH. Values are given as means of three replicates, with standard deviations in parentheses.

	Ash content (Wt.%)	Ash content (g/L)	Dry weight (g/L)	VS (g/L)
Low pH	5,5 (0,5)	0.58 (0.07)	10.5 (0.42)	9.93 (0.35)
High pH	18,8 (4)	3.53 (1.01)	18.5 (1.56)	15.0 (0.63)

5. Discussion

5.1 Characterization of yeast growth performance

From the cultivations using different substrates (sterile and minimally filtered hydrolysate) it could be established that the yeast can grow on both the substrates tested. The growth patterns shown by the two figures regarding sterile and minimally filtered substrates (Figure 4 & Figure 5) are relatively similar.

The calculated dry weight of the biomass was slightly higher for the yeast grown on the minimally filtered hydrolysate and the ash content (%) differed between 3.7 % and 5.5 % (AC %, + 49 %, $p = 0.0016$) between the different experiments. This shows that there is a lower ash content if the sterile filtered substrate is used. These calculations were performed with four replicates of the sterile filtered experiment and only two replicates of the minimally filtered samples. In the experiment performed with the minimally filtrated substrate, two different pH settings were used which seems to not have a large impact on the results. Hence, it could be interesting to see what would happen if these replicates were pooled together into four replicates. This gave a result of 3.7 % and 5.7% ash content between the sterile and minimally filtered samples (AC %, + 52 %, $p = 0.0009$). This again suggests that the sterile filtered substrate should be used over the minimally filtered substrate to have as low ash content as possible left in the biomass. However, the question is how much ash content that is acceptable in the biomass when it comes to fish feed. In addition to this matter, it would be statistically favorable to perform more experiments with the same settings as this would give more replicates.

By looking at the HPLC samples, the yeast in all experiments seemed to prefer the VFAs in the following order: acetate > propionate > lactate. This, because the consumption of lactate, seemed to start only when the concentration of acetate and propionate was significantly lower. The capacity of these small bioreactors and thus short cultivation times, a full consumption of lactate was not seen. However, if this would have been performed in larger batches that allow more time, lactate would probably have been consumed more at later stages. In other studies where *Y. lipolytica* has been growing on VFAs as a substrate, they indicate that higher concentrations of VFAs inhibit cell growth. In a study by Gao *et al. (2017),* their results showed an inhibition of cell growth in the following order: butyric acid > propionic acid > acetic acid. However, after 30 days, the yeast adapted to grow on 20g/L acetic acid (Gao et al. 2017). Another study by *Llamas et al. (2020)* showed that *Y.*

lipolytica favors short-chained VFAs over long-chained (Llamas et al. 2020). In a study by *Pereira et al (2021),* they also concluded that acetate was consumed at higher rates compared to other VFAs. They also discuss that one reason could be the differences in metabolic pathways for example acetate and propionate (Pereira et al. 2021). In this experiment, the HPLC results of the sterile filtrated hydrolysate showed to have a low concentration of acetate (5.56 g/L) compared to lactate (13.76 g/L) and propionate (7.33 g/L). If instead, the concentration of the shorter chained acetate would be proportionally higher than the other two, perhaps a higher biomass could be obtained as implied by (Llamas et al. 2020). In each cultivation, an apparent metabolic shift was evident around the 17th hour. During this period, medium alkalinization subsided for a few hours, after which lactate started being consumed. Further studies using larger reactors could bring more clarity to this phenomenon. The pH-stat feeding of the hydrolysate was a good strategy for the cultivations in these experiments with this substrate since a pH-stat fed-batch minimizes the amount of titrant, which improves the financial prospects of large-scale production.

5.2 Effect of pH on yeast growth

In this experiment, both fermenters set at pH 5.5 and 6.2 had similar results in all parameters. This makes it possible to argue that it is an important result since it could indicate that the chosen setting of different pH was not enough to see any clear difference and therefore need more investigation to conclude if lower pH affects the growth characteristics. Cultivating the yeast at lower pH can be regarded as beneficial since it may decrease the risk of contamination and minimizes the amount of titrant required. Only two replicates were used for these experiments, which is not enough for a statistical evaluation, however, the apparent similarity between cultivations suggests that even lower pH values should be evaluated.

5.3 Effect of harvest pH on biomass composition

When harvesting the yeast at a lower pH (before the consumption of all VFAs), there was considerably less ash content compared to the yeast harvested at a higher pH level, with the yeast harvested at high pH containing over sevenfold more ash. This result, therefore, suggests that the yeast should be harvested at lower pH to yield a biomass containing a lower inorganic content. In aspects of fish feed, it is important to find a biomass that has lower levels of inorganic content in aspects of quality. With this method, the biomass would be purer containing organic materials with higher protein and lipid content. One solution could be to harvest the yeast when there is a little bit of substrate left in the feed bottles of the fermenters to ensure to have a low pH at harvest time.

5.4 Filtration of hydrolysate

For production at a larger scale, it is beneficial to have fewer filtration steps since this does not only save time but an extra filtration step together with the high purity would consume more time require more energy for filtration and would thereby be more costly . Since the growth characteristics were quite similar on both types of substrates it is also arguable that there is no need for the extra filtration step on a larger scale production. However, it would be interesting to further investigate if the number of particles would increase with less pure substrates and how that would accumulate in the yeast biomass and its effect as fish feed.

Centrifugation was an important step to clear out particles before the filtration steps. At the beginning of the trial, different filters were evaluated to find a good solution. One problem was that a higher temperature affected the filtration through paper filters. For all experiments, it was necessary to do some heat treatment by either sterilizing or pasteurizing the hydrolysate. The heat treatment did also have a positive impact on the filtration. It was thereby decided to heat the hydrolysate to approximately 40–70 °C in a water bath before filtration. In the experiment when the hydrolysate was autoclaved before filtration (for the sterile filtered hydrolysate), it was even easier to filter the hydrolysate, and after cooling down of the substrate, the filtration through paper filters was harder. When shifting to glass fiber filters, the temperature did not affect filtration anymore. For a larger scale production, it would be necessary to heat the substrate before filtration if paper filters are used. Filtrations of the hydrolysate at larger volumes were not analyzed in this project. This means that problems that could occur with larger quantities are not visible in these experiments. In this study, the glass fiber filters used were only available in one pore size, and the last filtering steps were therefore performed three times to clear the hydrolysate. For further experiments, it would be more convenient to have different filter pore sizes in the last steps instead of using a 5–6 µm and then going down to 1.6 µm, more filtering steps in between would facilitate filtration. It might not be necessary to use paper filters at all to be able to filter this substrate. To improve the method of filtration for this substrate it would have been interesting to investigate more alternative filter materials (such as nylon) and sizes compared to those used in this method.

5.5 Yeast biomass suitability as fish feed

The biomass harvested from the experiments was sent for chemical analysis to investigate its nutritional composition and toxicity. These results were not included in this thesis due to scheduling conflicts. They would however be of interest to discuss the yeast biomass suitability as fish feed. This thesis on the other hand did not have its main focus on this theme but instead evaluated the growth characteristics of *Y. lipolytica* on the used hydrolysate and how this substrate should be filtered. However, other studies have investigated other applications of *Y. lipolytica* as an SCP- source for feed, but also for the use as an oleaginous yeast that can create important lipids by having hydrophobic substrates as carbon sources. One study on

this matter is performed by Yan *et. al*, (2018). They investigated how *Y. lipolytica* could be used to produce SCPs and acid-resistant lipases for use in fish feed additives. The substrate used in this investigation was similar to the food waste sources from this project, where agro-industrial wastes (sugarcane molasses, waste cooking oil, and crude glycerol) were used. The production of SCPs was most successful using molasses as a substrate where a 10 L batch gained 151.2g/L of SCPs. Diets with 2% of SCP added had a positive effect on the growth promotion of the marine fish *Cynoglossus semilaevis*. Even though this yeast strain was manipulated for an increased lipase expression, the use of *Y. lipolytica* as a protein source for fish feed was successful (Yan et al. 2018).

6. Conclusions

This thesis aimed to investigate the possibility to use food waste-based hydrolysates as a substrate for the yeast *Yarrowia lipolytica*, to be used as fish feed. From the findings of the experiments performed in this project, the following conclusions can be established: first, the growth of the yeast *Y. lipolytica* on the hydrolysate from food waste was successful. It was also possible to grow the yeast on the minimally filtered substrate that is suitable for the larger production of this biomass. The method for the filtration of the hydrolysate was also successful, however since it has not been investigated in large quantities, more research is needed in this area. The yeast grew well on both pH 6.2 and 5.5 and might grow at even lower levels although these were not evaluated. The yeast needs to be harvested at lower pH to avoid a precipitate and a higher ash content of the biomass. The pH-stat fed-batch is a good strategy for this production since it would need less titrant compared.

The findings from this project have been part of developing methods for filtration and growth characteristics of *Y. lipolytica* that will be a part of the more sustainable production of fish feed compared to the raw materials used today. Since the substrate is a part of biogas production it also creates a regenerative system where food waste and other waste materials can be used not only to drive our vehicles but also be a part of the production of fish feed.

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Popular science summary

In aquaculture, a huge part of the fish feed is originating from fish oil and fish meal. These are nutritious and specifically, contain omega-3 fatty acids that will stay in the fish meat which is beneficial for human health. However, in recent years fish oil and fish meal have been facing a shift to other sources that are more environmentally, economically, and socially sustainable. Other origins of fish oil and fish meal that have been investigated are plant-derived products such as soybeans etc. but also insects that for some carnivorous fishes are a natural part of their diets in the wild. Another option is also the use of microorganisms to produce protein sources. These are called single-cell proteins since they are produced by single-cell organisms. In this project, the use of a yeast called *Yarrowia lipolytica* has been used. In the production of single-cell proteins, microorganisms are growing in tanks called fermenters on a substrate which is a nutrient source for the microorganism. In this case, the yeast cells will consume the nutrients in the substrate and develop into more cell units which are called biomass. This biomass can further be processed into powders or pellets that have a high amount of protein.

Biogas production is a common way to produce more environmentally friendly fuel for vehicles. Biogas is a process that uses manure or other agricultural organic materials, but also food waste from households. The organic material is degraded in anaerobic environments by microorganisms that in the end produce methane. However, a slurry called hydrolysate can be collected if the process is stopped. In this project, a hydrolysate produced from food waste has been used as a substrate for the yeast. In this way, the use of biogas production gives other applications than just the production of biogas.

In this project, the aim was to analyze how well the yeast *Y. lipolytica* could grow on this particular substrate. This was performed by analyzing different parameters during the growth of the yeast. In the end, the biomass was collected and dried to see how much organic materials it contained to discuss this yeast's suitability as a possible fish feed origin that is more sustainable compared to fish meal and fish oil.

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Appendix

Culture media

YPD agar plates and broth (1L)

20% Bacterial peptone
10% Yeast extract
20% D+ - Glucose
(10-20% Agar)
1L tap water

Acid growth medium pH ca 6.2

Sodium acetate 21.6g
Sodium lactate 26.4g
Ammonium sulphate 4g
Sodium propionate 2.076g
YNB (Yeast nitrogen base without amino acids) 1.36g
d H_2O 800ml

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