Cultivating oleaginous yeast on spent mushroom substrate and logging residues hydrolysate

An assessment of yeast growth on side product substrates from the food, agriculture, and forestry industry

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Cultivating oleaginous yeast on spent mushroom substrate and logging residues hydrolysate. An assessment of yeast growth on side product substrates from the food, agriculture, and forestry industry.

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Abstract

There is an interest in finding alternatives to vegetable oils because of negative environmental impact. Many green sectors generate an abundant amount of side products that are inedible and consist in large of lignocellulose rich materials. Oleaginous yeast can ferment inedible substrates and accumulate a high amount of lipids that have a similar fatty acid profile to that of vegetable oils. Various screening methods exist to assess microbial growth with the aid of colour changing redox indicators. Two yeast strains, *Rhodotorula toruloides* CBS14 and *Rhodotorula babjevae* DVPG8058, were screened in a microtiter plate (MTP) system (Applikon Micro-Flask) with a tetrazolium salt as indicator (TTC). The strains were also cultivated on spent mushroom substrate hydrolysate (SMSH) and logging residues hydrolysate (LRH) as substrates. The TTC indicator likely caused inhibition at 1.0 g/l. It was visible at lower concentrations (0.1 and 0.05 g/l) but permanently stained the MTPs. The SMSH had the most positive impact on yeast growth while the cellulose fraction (CF) and hemicellulose fraction (HF) of the LRH showed less overall growth and inhibitory effects. The HF caused high inhibition in both yeast strains, but they could grow at 30% dilution. The CF had similar concentrations of glucose, xylose, and acetic acid as the SMSH but showed less growth. The SMSH could need less pre-treatment than LRH and possibly not need to be supplemented with nutrients because of mushroom residues. The Applikon-Micro Flask system proved sufficient for screening of yeasts on several substrates.

Keywords: Lignocellulose, side products, oleaginous yeast, yeast oil, microtiter plate system, redox indicator, yeast growth, spent mushroom substrate hydrolysate, logging residues hydrolysate

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Abbreviations

ACA	Acetic acid
CCS	Consumed carbon source
CDW	Cell dry weight
CF	Cellulose fraction
GLC	Glucose
HF	Hemicellulose fraction
LR	Logging residues
LRH	Logging residues hydrolysate
MTP	Microtiter plate
SLU	Swedish University of Agricultural Sciences
SMS	Spent mushroom substrate
SMSH	Spent mushrooms substrate hydrolysate
TTC	2,3,5-triphenyltetrazolium chloride
XYL	Xylose

1. Introduction

1.1. Background

With the growing awareness of negative environmental impact by human activity there is an emerging interest in finding alternative sources to vegetable oils in production of food, animal feed, biofuels and other relevant applications (Tiukova *et al.* 2019; Martín-Hernández *et al.* 2023). Production of vegetable oil requires huge areas of land for oil crops and is predominately based on fossil fuel as energy source. Furthermore, many biofuels are based on vegetable oils from crops that would otherwise be consumed by humans and it creates a competition between the use of agricultural land for food and fuel production (Brandenburg *et al.* 2021; Chmielarz *et al.* 2021). Some microorganisms in nature, e.g., bacteria, fungi (filamentous and yeasts), and microalgae, are able to break down and ferment organic matter that is otherwise inedible by humans and produce high-value compounds, such as lipids (Kosa *et al.* 2018; Valdés *et al.* 2020).

An abundant by-product from the agriculture and forestry industries is lignocellulose-based biomass, e.g., wheat straw and wood. Some of these side products could be used as a substrate for oil producing microbes (Kosa *et al.* 2018; Valdés *et al.* 2020). Lignocellulose is a large network that constitutes the backbone of plant structure and consist of several polymers: cellulose, hemicellulose, and lignin (Brandenburg *et al.* 2016). One group of microorganisms that are able to metabolize a huge variety of substrates are yeasts: unicellular fungi that can be found amongst the *Ascomycota* and *Basidiomycota* phylum (Kurtzman 1994). Oleaginous yeasts derive their name from the ability to accumulate lipids upwards to 20 % or more of their cell dry weight (CDW). Many oleaginous yeast species can produce oils with similar fatty acid profiles to that of vegetable oils. (Chmielarz *et al.* 2021).

Side product substrates rich in lignocellulose must be subjected to pre-treatment to achieve breakdown of the recalcitrant polymer network. It is usually done with high heat, e.g., steam expansion. Afterwards, the residues are enzymatically treated to achieve hydrolysis (Taherzadeh *et al.* 2001). The result of this process is various hydrolysates that contain numerous different organic compounds. Hydrolysates who contain large concentrations of free sugar monomers, such as glucose (GLC)

or xylose (XYL), can be utilized as a carbon source for oleaginous yeasts (Brandenburg *et al.* 2021). There are unfortunately other compounds released in the pre-treatment of lignocellulosic biomass that can act as inhibitors on yeast growth, such as phenolic compounds (Chen *et al.* 2022b).

Oleaginous yeasts grow fast and can be cultivated regardless of season, they can also use low-value substrates to accumulate high quality lipids (Ageitos *et al.* 2011; Valdés *et al.* 2020). There are issues with costs of yeast oil production that need to be resolved. Therefore, it would be of interest to investigate multiple yeast strains on several potential substrates. It could be beneficial for finding new strain properties that exhibit fast growth on abundant substrates, this would help to streamline costing operations (Brandenburg *et al.* 2021; Chmielarz *et al.* 2021). By using biomass that would otherwise be destroyed or thrown away, it could help to mitigate greenhouse gas emission if we can reuse side products from resource heavy industries for food, feed, or biofuel production. Microbial oils could be a future vital part of an emerging bio-economy based on sustainable resource management (Passoth *et al.* 2023).

1.2. Oleaginous yeasts

There are about 30 known species of oil producing yeasts that can accumulate lipids from 25 % or more of their CDW (Ageitos *et al.* 2011), two among these are *Rhodoturula toruloides* and *Rhodotorula babjevae* which are investigated in this thesis.

Oleaginous yeasts store lipids in the form of triglycerides. The lipid profile of most species consists of myristic, palmitic, stearic, oleic, linoleic, and linolenic fatty acids (Li *et al.* 2008). Apart from lipids, several oleaginous yeasts can also produce other applicable compounds, such as carotenoids (Rapoport *et al.* 2021; Nagaraj *et al.* 2022). *R. toruloides* can accumulate lipids up to 76% of their CDW and can utilize several types of crude substrates. The species can be found in many different places in the environment, such as soil and water (Ageitos *et al.* 2011; Tiukova *et al.* 2019). Some strains of *R. babjevae* can produce a high lipid yield in substrates that have shown inhibitory effects on other strains, e.g., one strain could utilize an undiluted hemicellulose based hydrolysate made from wheat straw to produce a high amount of lipids (Brandenburg *et al.* 2021). Furthermore, (Brandenburg *et al.* 2021) have shown that *R. toruloides* strain CBS14 and *R. babjevae* strain DVPG8058 (named J195 in this thesis) were among the best performing yeast strains in terms of lipid accumulation with around 65% for J195 and around 40% for CBS14 in CDW. These two yeast strains were analysed in this thesis.

1.3. Spent mushroom substrate and logging residues

There are many potential substrates to be investigated if they are suitable for production of microbial oils. Two substrates of curiosity are logging residues (LR) from the forestry sector and spent mushroom substrate (SMS) from edible mushroom production.

SMS consists of leftover material after harvest of edible mushrooms (fruit bodies) that have been cultivated on crop or wood substrate. The by-product contains traces of mushroom mycelium and partly degraded lignocellulose. Many edible fungi species can degrade lignocellulose rich material by secretion of enzymes. This makes desired monosaccharides in the biomass more available for microbial fermentation. The degree of degradation of lignocellulose varies between mushroom species, cultivation conditions and nutrient availability (Chen *et al.* 2022a). The SMS hydrolysate (SMSH) is made of post-harvest material from fungi cultivation through hydrolysis (preparative enzymatic saccharification) (Chen *et al.* 2022b). There has been little to no studies conducted of SMSH effects on oleaginous yeast fermentation to the author's knowledge.

LR are forestry by-products, such as treetops, branches, bark, and needles. Compared to SMS they require pre-treatment (steam expansion) to break down the lignocellulose before hydrolysis because they are not subject to mushroom degradation. The hydrolysate is named LR hydrolysate (LRH) in this thesis.

Cellulose and hemicellulose are polysaccharides that consist of different sugar monomers: cellulose is a chain of D-glucose units and hemicellulose is made of hexose and pentose monosaccharides (Hertzog *et al.* 2021). Many lignocellulosic hydrolysates contain a multitude of other organic substances like phenylic compounds, furans, and aliphatic carboxylic acids. Some of these compounds can act inhibitory on yeast growth (Jonsson & Martin 2016). There have been several studies that have showed some oleaginous yeast species are able to ferment lignocellulose based hydrolysates as substrates and convert it to lipids (Brandenburg *et al.* 2016; Blomqvist *et al.* 2018; Brandenburg *et al.* 2021)

1.4. Microtiter plate system and redox indicator

Microtiter plate (MTP) systems are often used in various scientific fields to screen compound effects on microorganisms. These plate systems consist of anywhere between 24 and 96 deep wells in each unit, making it possible to screen large quantities of compounds for anti/pro-microbial properties. Establishing an MTP system-based screening method for measuring growth in yeast strains could be a more time efficient method for initial screening of strain properties (Kosa *et al.* 2018).

Several redox indicators have been developed and applied in studies for microbial growth assessment for a long time. One indicator group are tetrazolium salts, they act as an electron acceptor and cause colour change in the presence of oxidative enzymatic systems. They are colourless in their un-oxidized state and switch colour in response to microbial growth. One such indicator is 2,3,5-triphenyltetrazolium chloride (TTC) which turns red-purple when oxidized (Gabrielson *et al.* 2002).

1.5. Aim of thesis

The research group is establishing a screening method to cultivate yeasts in an MTP system (Applikon Micro-Flask) to test promising substrates and select yeast strains based on growth for future experiments. The aim of this thesis project is to investigate if the MTP system can make screening tests easier, quicker, and more efficient and if it would be more suitable for high-throughput screening than the current screening methods in use at the Department of Molecular Sciences, Swedish University of Agricultural Sciences (SLU), Uppsala. Simultaneously, assessing the SMSH and LRH as substrates for the oleaginous yeast strains *R. toruloides* CBS14 and *R. babjevae* J195.

2. Materials and methods

2.1. Materials

2.1.1. Yeast strains

The *R. toruloides* CBS14 strain was sourced from the CBS culture collection of fungi and yeasts, Westerdijk Fungal Biodiversity institute, Utrecht, The Netherlands. The *R. babjevae* J195 strain was originally isolated and stored at the former Department of Microbiology, SLU Uppsala.

Henceforth, the yeasts will be referred to their strain name, CBS14 and J195, throughout the text.

2.1.2. Culture media

All culture media was prepared by weighing the dry ingredients separately and adding deionized water. All the flasks were autoclaved except where noted.

YM-medium was made with the following concentration: 3 g/l yeast extract (Yeast Extract, Merck, Darmstadt, Germany), 3 g/l malt extract (Malt extract, Merck, Darmstadt, Germany), 5 g/l peptone (Peptone from casein, pancreatic digest, Merck, Darmstadt, Germany) and 10 g/l GLC (D-(+)-Glucose, Merck, Darmstadt, Germany).

YM-agar was made with the following concentrations: 3 g/l yeast extract (Yeast Extract, Merck, Darmstadt, Germany), 3 g/l malt extract (Malt extract, Merck, Darmstadt, Germany), 5 g/l peptone (Peptone from casein, pancreatic digest, Merck, Darmstadt, Germany), 10 g/l GLC (D-(+)-Glucose, Merck, Darmstadt, Germany) and 16 g/l agar (Agar powder, VWR International, Leuven, Belgium).

Test media (TM) was made with the following concentrations: 20 g/l ammonium sulfate (Ammonium sulfate, Merck, Darmstadt, Germany), 7.5 g/l yeast extract (Yeast Extract, Merck, Darmstadt, Germany) and 17 g/l yeast nitrogen base (Yeast Nitrogen Base without Amino Acids, Sigma, St: Louis, USA). This was a 10-time concentration of the original recipe. The TM was filtered through a 0.20 µm membrane filter.

2.1.3. MTP system

The MTP system Applikon Micro-Flask (distributed by Getinge AB, Gothenburg, Sweden) was used for cultivation of the yeasts in which different test series were developed to test various substrates. The MTP unit consisted of 24 deep wells with an individual well volume of 15 ml and a Sandwich-type lid cover: the dissolved oxygen levels in the deep wells were comparable to traditional shake flasks (Applikon Micro-Flask - Cultivation in microtiter plates (getinge.com)). The MTPs were mounted onto a holder and secured by a clamp. The holder was from the same manufacturer, and it was placed onto a horizontal orbital shaker and incubated at different temperatures and revolutions per minute (rpm).

2.1.4. Hydrolysates, redox indicator, and pH-buffer

The SMSH was sourced from the Department of Biotechnology, Inland Norway University of Applied Sciences, Hamar and the LRHs were sourced from the Department of Forest Biomaterials and Technology, SLU Umeå.

A stock solution of 5 ml 10 g/l TTC (2,3,5-Triphenyltetrazolium chloride, T8877-5G, Sigma, St. Louis, USA) was made with deionized water and filtered through a 0.20 µm membrane filter.

The 2-(N-Morpholino)ethanesulfonic acid hydrate (MES) was added in TM for the hydrolysate experiments as a pH-buffer: 9.726 g MES (MES hydrate, M8250-250G, Sigma, St. Louis, USA) was added to 100 ml TM.

2.2. Method

Each of the substrate tests (test series) were performed in triplicates against both positive and negative controls except where noted. The test series for each experiment setup were divided between five to eight series depending on how many wells were used at that time. A well row of three deep wells was assigned to each test series. All laboratory work requiring sterile work environment were performed under a laminar flow hood.

2.2.1. Preparation of pre-culture and cell wash

The yeasts were thawed from deep freeze storage at -81 °C and streaked onto agar plates which were incubated for 48 h at 25 °C prior to pre-culture inoculation. The pre-culture was prepared by pouring 50 ml of YM-medium in a 250 ml shake flask. The YM-medium was inoculated with yeast culture from the agar plate using a sterile loop and incubated on a desktop shaker between 48-72 h at 25 °C at 150 rpm.

After incubation, a sample of 0.1 ml from the pre-culture was taken for optical density (OD) assessment at 600 nm. The OD in the pre-culture was measured before

and after cell washing. The pre-culture was poured into a 50 ml tube and centrifuged at 4000 xg for 5 min. The supernatant was discarded, and the tube was refilled with 50 ml of sterile 9 g/l NaCl (aq) and re-suspended. The tube was centrifuged a second time at 4000 xg for 5 min and the supernatant was discarded. Saline solution was added again for a final resuspension. The final re-suspended solution with the washed yeast cells was the one used for inoculating the MTP experiments.

2.2.2. MTP inoculation and OD analysis

To calculate the volume of washed cell solution to be added in the MTP experiments a 0.1 ml sample was taken, and the desired OD value was divided by the measured OD value from the final re-suspended cell solution and multiplied by 5 ml (the total volume of media in each deep well) to get the volume of washed cell suspension to be added in the MTP.

The start OD for all experiments was OD at 1.00. The samples were analyzed in a WPA biowave CO8000 Cell Density Meter (Biochrom Ltd) at a wavelength of 600 nm. Samples with an OD between 0.00 and 0.60 were only prepared with one dilution series while samples with a higher OD than 0.60 were prepared with two dilution series. A saline solution (9 g/l NaCl) was used as test reference in all OD measurements except where noted.

2.3. TTC calibration tests

The 5 ml 10 g/l TTC stock solution was kept refrigerated and wrapped in aluminium foil to decrease light exposure due to the indicator being photo and temperature sensitive. The TTC stock solution was prepared as close in time to the MTP plating as possible to avoid degradation. A stock solution of 70 g/l GLC was made as substrate in TTC calibration tests 1 and 2.

2.3.1. TTC calibration test 1

The MTP setup was prepared according to table 1. The MTP was incubated for 72 h in a dark room on a desktop shaker at 30 °C at 220 rpm and then visually observed each day during the time of the experiment. Afterwards, the MTP was placed back in the shaker for another 72 h of incubation. At day six (144 h) the MTP was removed for a final OD analysis of the yeast growth, the TM was used as a test reference for the OD measurement in this experiment.

Table 1. Setup of TTC calibration test 1 with GLC as substrate.

Compounds (ml)	GLC	TM	Water	TTC
Test series				
1.0 g/l TTC	4.0	0.5	-	0.5
0.1 g/l TTC	4.0	0.5	0.45	0.05
No TTC	4.0	0.5	0.5	-
No TTC no GLC	-	0.5	4.5	-
1.0 g/l TTC no yeast	4.0	0.5	-	0.5
0.1 g/l TTC no yeast	4.0	0.5	0.45	0.05
TTC 1.0 g/l no GLC	-	0.5	4.5	0.5
TTC 0.1 g/l no GLC	-	0.5	4.45	0.5

2.3.2. TTC calibration test 2

The setup of TTC calibration test 2 was designed in the exact same way as the first one except the TTC concentrations were changed from 1.0 and 0.1 g/l to 0.1 and 0.05 g/l, table 2. The MTP was incubated for 72 h in a dark room on a desktop shaker at 30 °C at 220 rpm and the OD was measured each day for three consecutive days before the final measurements were done after six days.

Table 2. Setup of TTC calibration test 2 with GLC as substrate.

Compounds (ml)	GLC	TM	Water	TTC
Test series				
0.1 g/l TTC	4.0	0.5	0.45	0.5
0.05 g/l TTC	4.0	0.5	0.475	0.025
No TTC (positive control)	4.0	0.5	0.5	-
No TTC no GLC (negative control)	-	0.5	4.5	-
0.1 g/l TTC no yeast	4.0	0.5	0.45	0.05
0.05 g/l TTC no yeast	4.0	0.5	0.475	0.025
0.1 g/l TTC no GLC	-	0.5	4.45	0.5
0.05 g/l TTC no GLC	-	0.5	4.475	0.025

2.3.3. TTC calibration test 3 with XYL and acetic acid (ACA) as substrates

The test setup was designed in a similar way as the previous TTC calibration tests. A stock solution of 70 g/l XYL and 2 g/l ACA was made. The pre-culture was incubated for 24 h instead of 48 h in this experiment. The MTP was prepared with 0.05 g/l TTC, TM, water, XYL, and ACA according to table 3. Another difference with this experiment was all the test series were inoculated with yeast because the concentration for TCC had been decided by this stage.

Table 3. Setup of TTC calibration test with XYL and ACA as substrate with 0.05 g/l TTC.

Compounds (ml)	XYL	ACA	TM	Water	TTC
Test series					
TTC w. XYL	4.0	-	0.5	0.475	0.025
No TTC w. XYL	4.0	-	0.5	0.5	-
No TTC no XYL	-	-	0.5	4.5	-
TTC no XYL	-	-	0.5	4.475	0.025
TTC w. ACA	-	4.0	0.5	0.475	0.025
No TTC w. ACA	-	4.0	0.5	0.5	-
No TTC no ACA	-	-	0.5	4.5	-
TTC no ACA	-	-	0.5	4.475	0.025

2.4. SMSH tests

The SMS came from Shiitake mushroom (*Lentila edodes*) grown on a birch-based substrate: dry material birch sawdust (80% (w/w)) and wheat bran (20%). The SMS was dried at 45 °C post-harvest until constant mass and milled to particle size <0.5 mm before enzymatic saccharification.

The hydrolysate was prepared by adding 20 g of SMS to a media consisting of 175 ml buffer and 4.7 ml enzyme. It was left at 45 °C at 150 rpm on a desktop shaker for 72 h. The final step was to centrifuge the hydrolysate at 8500 rpm for 30 minutes. The liquid phase, SMSH, was kept and frozen while the solid fraction was discarded. The SMSH contained approximately 30 g/l GLC and 10 g/l XYL.[1]

A stock solution with GLC and XYL was prepared with deionized water to mimic the sugar concentration in the SMSH. The TM was adjusted to pH 6.00 by adding NaOH (aq) dropwise and MES (s) was added as a buffer. The pH of the undiluted SMSH was also adjusted to pH 6.00 with NaOH for SMSH test 1b and 2.

The SMSH test series were diluted into four concentrations: 90%, 70%, 50% and 30%. The experiment setup was designed so the amount of hydrolysate concentration would decrease from 90% to 30% while the sugar concentration was kept at 27 g/l GLC and 9 g/l XYL in each hydrolysate test series (to mimic the 90% concentration). This was done to investigate if the hydrolysate itself had an inhibitory effect on yeast growth independent from the monosaccharide concentration.

[1] Email correspondence with Carlos Orestes Martin Medina from Inland Norway University of Applied Sciences, Hamar, 2023-05-17.

2.4.1. Preparation of SMSH

The hydrolysate was centrifuged at 20 000 xg at 4 °C for 20 minutes. The supernatant was first filtered through a 0.45 µm membrane filter in a non-sterile environment and then through a 0.20 µm membrane filter under a sterile hood. The two-step filtration was necessary due to the presence of large particles in the SMSH. The hydrolysate clogged the membrane filter, which needed to be replaced two to three or more times during filtration. A small amount of hydrolysate (a couple of millilitres) was lost for each filtration stage.

2.4.2. SMSH test 1a and 1b

Only CBS14 was tested in this experiment to see how one yeast strain would respond to the SMSH. Test 1a was done without prior alteration to pH-level. In test 1b, the SMSH and TM was adjusted to pH 6.00, table 4. The MTP was incubated at 30 °C at 220 rpm. The OD was measured every 24 h over the following three days.

Table 4. Setup of SMSH test 1a and 1b.

Compounds (ml)	**Hydrolysate**	**GLC + XYL**	**TM (+MES) ***
Test series			
90 %	4.5	-	0.5
70 %	3.5	1.0	0.5
50 %	2.5	2.0	0.5
30 %	1.5	3.0	0.5
Positive control	-	4.5	0.5

**MES was added to TM in SMSH test 1b.*

2.4.3. SMSH test 2

The second SMSH test was expanded with testing two yeast strains, CBS14 and J195, in two MTPs at the same time. A big difference was the addition of a test series with no TM and 90 % dilution, a negative control and a water and MES buffer test series. The MTP setup is displayed in table 5. The MTPs were incubated at 25 °C at 220 rpm for four days and were monitored by OD analysis every 24 h mark.

Table 5. Setup of SMSH test 2.

Compounds (ml)	Hydrolysate	GLC + XYL	TM	Water + MES	Water
Test series					
90 %	4.5	-	0.5	-	-
70 %	3.5	1.0	0.5	-	-
50 %	2.5	2.0	0.5	-	-
30 %	1.5	3.0	0.5	-	-
90 % no TM	4.5	-	-	0.5	-
Positive control	-	4.5	0.5	-	-
Negative control	-	-	0.5	-	4.5
Water + MES	-	-	0.5	0.5	4.5

2.5. LRH tests

LR from Norway spruce (*Picea abies*) were sorted and milled at the Department of Forest Biomaterials and Technology, SLU Umeå. The LR were thermo-chemically treated and made into LRH at Lund University.

The LR were soaked with 1% ACA and then pre-treated by steam expansion. The LR were separated into a liquid fraction and a solid fraction by pressing out the liquid during pre-treatment. The first liquid fraction was the hemicellulose fraction (HF) and the solid fraction consisted mainly of cellulose and lignin. The solid fraction was later enzymatically hydrolysed and consisted of a liquid cellulose fraction (CF) and a solid lignin fraction; these were separated at the Department of Molecular Sciences (described later). The steam expansion pre-treatment was performed as described by (Blomqvist *et al.* 2018).

The approximate sugar concentrations in the undiluted fractions were CF: 30 g/l GLC and 6 g/l XYL, HF: 4.5 g/l GLC and 10.40 g/l XYL. These concentrations were obtained from a preliminary HPLC analysis of the fractions at the department. Stock solutions of GLC and XYL were prepared to have the same saccharide concentrations as the undiluted LRH fractions in the experiments.

2.5.1. Preparation of CF and HF

After enzymatic hydrolysis, the cellulosic hydrolysate (CF) was separated from the solid lignin by centrifugation at 6500 xg at 4 °C for 30 minutes. The CF was kept while the solid fraction, consisting mostly of lignin, was discarded. The CF was first vacuum filtrated through a ceramic Buchner funnel with a large diameter through a 1.0 µm filtration paper. The filtered hydrolysate was filtered again through a vacuum 0.20 µm membrane filter and placed in a freezer for storage.

The HF hydrolysate was first filtered through a 0.70 µm paper filter, then directly through a 0.20 µm membrane filter.

2.5.2. CF and HF tests

The setup for this experiment was like SMSH test 2 apart from test series with 90 % diluted hydrolysate and no TM and the test series with only water and MES buffer were removed, table 6. The MTPs were inoculated with yeast strains CBS14 and J195 and were incubated at 25 °C at 220 rpm for three to four days. An OD analysis was carried out at each 24 h mark.

Table 6. Setup of CF and HF tests.

Compounds (ml)	Hydrolysate	GLC + XYL	TM + MES	Water
Test series			0.5	-
90 %	4.5	-	0.5	-
70 %	3.5	1.0	0.5	-
50 %	2.5	2.0	0.5	-
30 %	1.5	3.0	0.5	-
Positive control	-	4.5	0.5	-
Negative control	-	-	0.5	4.5

2.6. Preparation of samples for HPLC and CDW analysis

Samples were collected and prepared for later High-performance liquid chromatography (HPLC) and CDW analysis in the following experiments: SMSH test 2 and the LRH fraction tests. At the end point of each experimental cycle, samples from test series containing hydrolysate and the positive control were collected in 15 ml test tubes. Each tube was individually marked and weighed beforehand to calculate the CDW after freeze drying. The total volume of each collected sample was 3.5 ml. The samples were centrifuged at 6500 xg at 4 °C for 10 minutes. The supernatant was saved and poured into new marked 15 ml test tubes and kept at -18 °C for later HPLC analysis. The remaining yeast cell pellet in the pre-weighed test tubes were washed by pouring deionized water (3.5 ml) and centrifuged at 6500 xg at 4 °C for 10 minutes. After each centrifugation cycle the water was discarded. This process of washing the yeast cells with deionized water was repeated three times. After the final cell wash the yeast cell pellet samples were kept at -18 °C for later CDW analysis.

2.6.1. HPLC analysis

The compound detection range was between 10.0 and 0.1 g/l and six standard samples within this concentration range were made. One standard sample was prepared with only Milli-Q water and 5 M H_2SO_4 (aq), sulphuric acid, making a total of seven standards used as reference for the calibration curve in each analysis sequence. A stock solution of 20 g/l GLC, XYL and ACA was made. The stock solution was diluted with both Milli-Q and 5 M sulphuric acid (final concentration of 0.5 M sulphuric acid in each standard) into six tubes with the following concentrations: 10.0, 7.5, 5.0, 2.5, 1.0 and 0.1 g/l with a total volume of 1 ml.

The test samples were prepared by diluting them with Milli-Q and 5 M sulphuric acid for a total volume of 1.0 ml. These dilution series were based on the initial HPLC analysis of the undiluted hydrolysates; SMSH: 30 g/l GLC and 10 g/l XYL. CF LRH: 30 g/l GLC, 6 g/l XYL and 2.1 g/l ACA. HF LRH: 4.5 g/l GLC, 10.41 g/l XYL and 6.3 g/l ACA. The SMSH and the CF samples were diluted ten times and the HF was diluted two times.

Each of the standards and samples were pipetted into individual wells on a well-plate filter (AcroPrep Advance 96 Well 350 µL 0.2 µm GHP Shirt Tip NaturalPP Base 10/pk, Pall Corporation, New York, USA) and were filtrated through centrifugation at 3000 xg for 1 min to remove any potential precipitations before HPLC analysis. The standards and samples were pipetted to HPLC vials with inserts and marked.

The samples were analysed in a 1100 Series system (Agilent Technologies) with 5 mM sulphuric acid as mobile phase, flow rate at 0.6 ml/min, Rezex-ROA-Organic Acid H+ 300 × 7.80 mm column (Skandinaviska Genetec AB), 60 ℃ and 5 µl of injection, as described by (Brandenburg *et al.* 2016).

2.6.2. CDW analysis

The test tubes were removed from freeze storage and the caps were unscrewed then parafilm was placed upon the open tubes to cover them to prevent any unwanted sample leakage during the freeze-drying process. The parafilm was punctured with a pipette tip once at each tube opening to enable water vapour to pass through when sublimation occurs. The samples were placed back into freeze storage to ensure the samples were frozen before being placed in the freeze-drying machine.

The samples were freeze dried in a Scanvac CoolSafe Touch 110-4 Freeze Dryer (Labogene ApS) under vacuum during 48 h at -101 °C. Each sample tube was individually weighed afterwards and a final CDW assessment was made.

3. Results and discussion

3.1. TTC calibration

It was investigated if the TTC firstly would work as an indication of yeast growth (substrate fermentation) at all, and secondly which concentrations would be sufficient to indicate yeast growth, and thirdly if the TTC would inhibit yeast growth. Unfortunately, the indicator did not function as expected in the first experiment and showed signs of inhibition on yeast growth, so another experiment was made with lower concentration ranges. The lower concentrations showed less inhibition on yeast growth and the indicator shifted color in the medium. The TTC also permanently stained the deep well walls of the MTPs. Therefore, the TTC redox indicator was not used in the later experiments with the hydrolysates.

3.1.1. TTC calibration test 1 with 1.0 and 0.1 g/l TTC

All test series were inoculated with CBS14 and had GLC as substrate except the series with names that display no yeast and no GLC. After three days of incubation the MTP was visually investigated where a light reddish hue could be distinguished in test series 0.1 g/l TTC and a light-yellow hue could be seen in the positive control (no TTC), top of photograph in figure 1. The MTP was placed back for another three days of incubation.

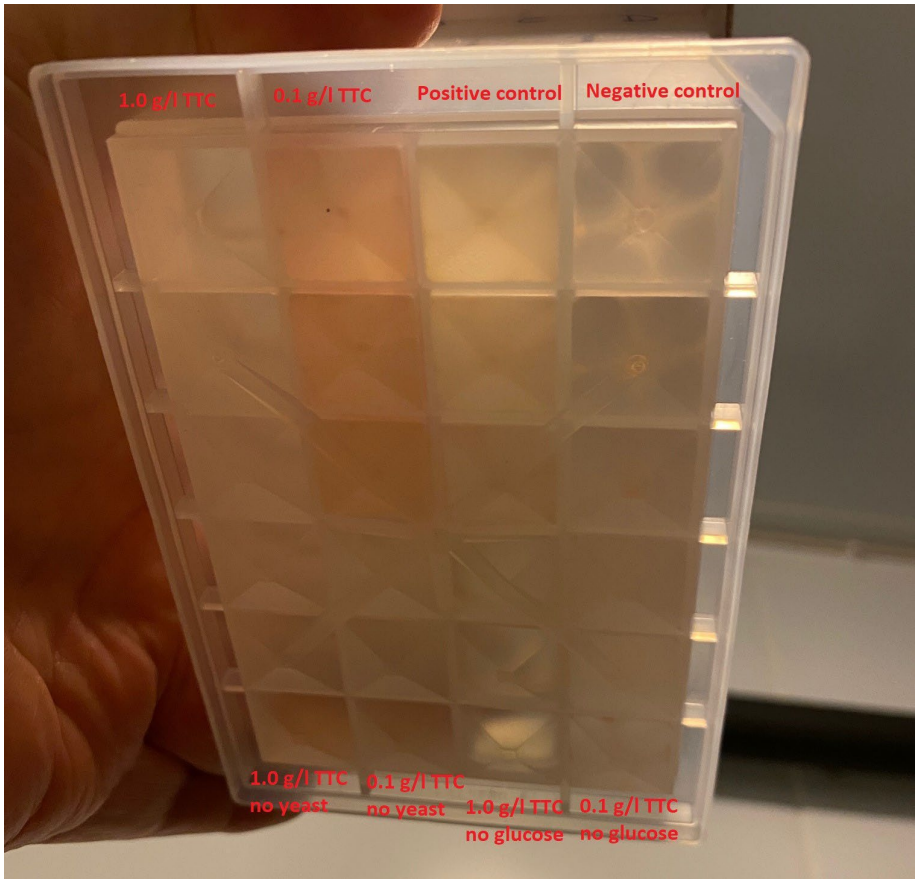

Figure 1. Photograph of MTP with CBS14 from TTC calibration test 1 after 72 h of incubation at 30 °C at 220 rpm. Every test series was inoculated with CBS14 and GLC was added except the ones at the bottom in the picture that display no yeast and no glucose. Credit: Johanna Blomqvist.

After six days (144 h) the MTP was retrieved for a final visual observation. Test series 1.0 g/l TTC was still uncoloured (visually), and it was hard to distinguish any yeast growth at the bottom of the wells. Series 0.1 g/l TTC was coloured in a light red "grapefruit juice-like" colour and yeast growth could be observed at the bottom of the well row. The positive control (no TTC) was coloured with a light-yellow "lemon juice-like" hue and there was also yeast growth observed at the well bottoms. The negative control (no TTC no GLC) was also coloured with a light-yellow hue but was more transparent than the positive control. Test series 1.0 g/l TTC no yeast and 0.1 g/l TTC no yeast did not have any visible colouration. Series 1.0 g/l TTC no GLC showed no change of colour as well. Test series 0.1 g/l TTC no GLC also had a light-yellow hue and similar colour as the negative control.

It appeared as if the TTC concentration of 1.0 g/l inhibited the growth of CBS14, which explains the lack of obvious coloration in series 1.0 g/l TTC. In contrast, the lower concentration of indicator appears to have worked in the test series 0.1 g/l TTC (grapefruit red) where the yeast was given GLC. Compared to the other test series, 0.1 g/l TTC with no GLC and series 0.1 g/l TTC with GLC showed a red/pink

color, which confirmed the redox indicator worked. The TTC series without yeast appeared to show no visually observable color.

The yeasts in the negative control and test series 0.1 g/l TTC no GLC was not given any substrate and thereby used their lipid reserves and did not proliferate as much as series 0.1 g/l TTC and the positive control. When comparing test series 1.0 g/l TTC, 0.1 g/l TTC and positive control with each other, only 0.1 g/l TTC and the positive control showed proficient growth despite GLC being added in all the test series. This further suggests that a TTC concentration of 1.0 g/l could inhibit yeast growth.

After six days the OD was assessed. Series 0.1 g/l TTC and the positive control had the highest OD at 5.90 and 6.90, table 7. They were followed by 0.1 g/l TTC no GLC at 0.29 and negative control at 0.45 respectively. This enforces the conclusions drawn from the visual observations about indicator concentration of 1.0 g/l was probably inhibiting yeast growth.

Table 7. OD analysis of CBS14 from TTC pilot test 1 after six days (144 h) of incubation. Mean value and standard deviation of each test series triplicate.

Time	**144 h**
Test series	
1.0 g/l TTC	0.01 ± 0.01
0.1 g/l TTC	5.90 ± 0.36
Positive control	6.90 ± 0.12
Negative control	0.29 ± 0.02
1.0 g/l TTC no yeast	0.00 ±
0.1 g/l TTC no yeast	0.00 ±
1.0 g/l TTC no GLC	0.00 ±
0.1 g/l TTC no GLC	0.45 ± 0.18

3.1.2. TTC calibration test 2 with 0.1 and 0.05 g/l TTC

The pre-culture was incubated for less than 24 h because of time constraints and proved to have a sufficient OD at 14 after cell wash. The coloration in test series 0.1 g/l TTC and 0.05 g/l TTC was like TTC calibration test 1 with its “grapefruit juice-like” coloration, figure 2. The positive control showed a slight yellow hue, and the negative control was transparent.

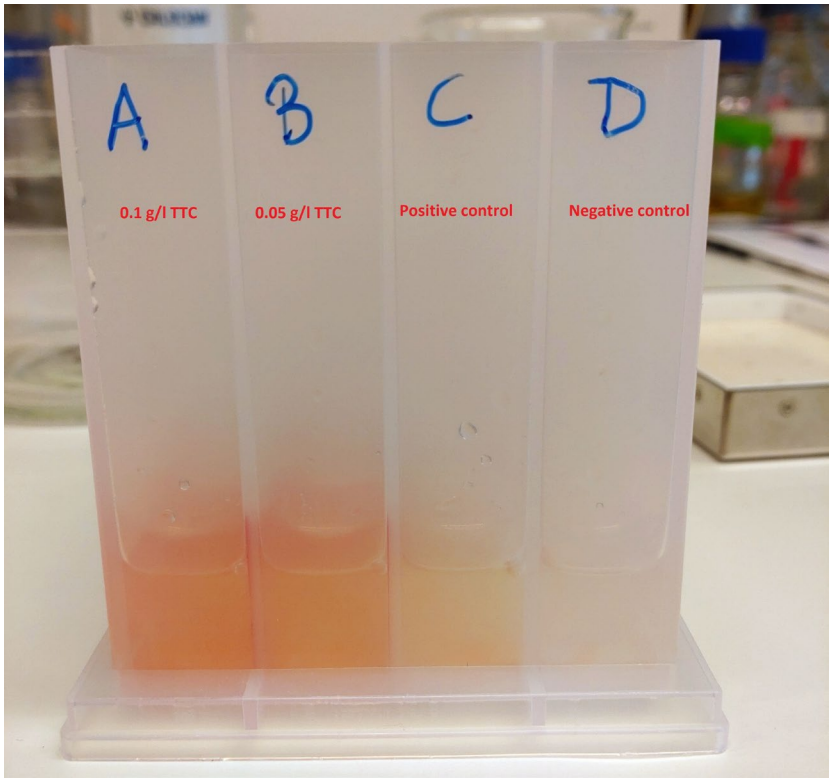

Figure 2. Photograph of MTP with CBS14 from TTC calibration test 2 after 144 h of incubation at 30 °C at 220 rpm. Test series one through four (A to D). Every test series was inoculated with CBS14 and GLC was added except the ones that display no yeast and no glucose. Credit: Robin Lukic.

The liquid media in the subsequent test series with no yeast and no glucose showed no observable color change and were transparent, figure 3. The series with no GLC and TTC had a slight orange/red coloration at the well bottoms, this is probably due to the CBS14 cells from the pre-culture producing carotenoids (Rapoport *et al.* 2021; Nagaraj *et al.* 2022), but it did not color the liquid media itself.

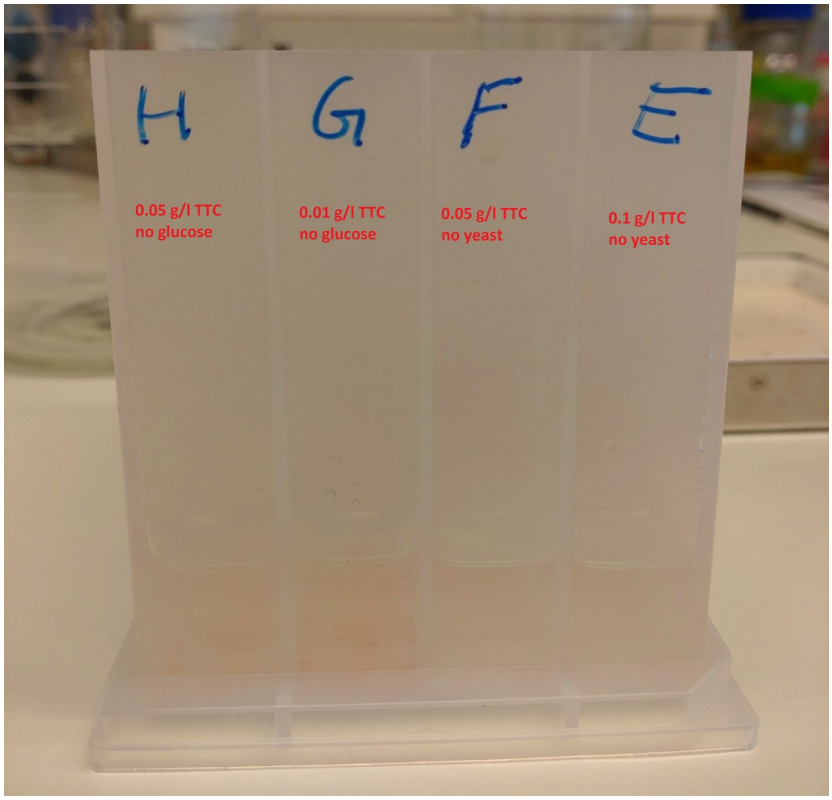

Figure 3. Photograph of MTP with CBS14 from TTC calibration test 2 after 144 h of incubation at 30 C at 220 rpm. Test series five through eight (E to H). Every test series was inoculated with CBS14 and GLC was added except the ones that display no yeast and no glucose. Credit: Robin Lukic.

The OD was only analyzed for the first four test series because the rest were identical to the previous experiment (TTC calibration test 1). The series with the highest OD after six days was 0.05 g/l TTC at 2.20 followed by 0.1 g/l TTC at 1.66 and the positive control at 1.63, table 8.

Table 8. OD analysis of CBS14 from TTC pilot test 2. Mean value and standard deviation of each test series triplicate.

Time	**24 h**	**48 h**	**72 h**	**144 h**
Test series				
0.1 g/l TTC	1.73 ± 0.21	1.03 ± 0.15	0.96 ± 0.49	1.66 ± 0.32
0.05 g/l TTC	2.30 ± 0.10	1.26 ± 0.51	2.03 ± 0.46	2.20 ± 0.30
Positive Control	1.73 ± 0.49	2.00 ± 1.95	0.83 ± 0.59	1.63 ± 0.59
Negative Control	0.40 ± 0.00	0.23 ± 0.00	0.20 ± 0.00	0.20 ± 0.00

3.1.3. TTC calibration test 3 with XYL and ACA as substrates with 0.05 g/l TTC

The mixture of monosaccharides in the LRH fractions and SMSH consists of many other sugars besides GLC. In the previous TTC experiments, GLC was used as the sole substrate (carbon source). Before experimenting on the hydrolysates could begin a test run was conducted with only XYL or ACA as substrates to see how CBS14 would respond. The experimental setup changed from the previous TTC experiments in the removal of the test series with no yeasts. All the test series in this experimental run were inoculated with CBS14. The first OD was measured less than 24 h after incubation due to time constraints while the following OD analyses were done after the MTP had been incubated over the weekend, beginning at the 96 h mark and being repeated every 24 h until the final OD analysis at 144 h.

None of the test series showed any visual colour change except series 0.05 g/l TTC with ACA (barely visible slightly pink) after 96 h. It was not until the 120 h mark that the rest of the test series media colours started to alter. After 144 h it was even more visibly distinguishable, figures 4 and 5. When comparing the elapsed time for the redox indicator in the previous TTC calibration tests, it was much longer delayed in visible change of colour.

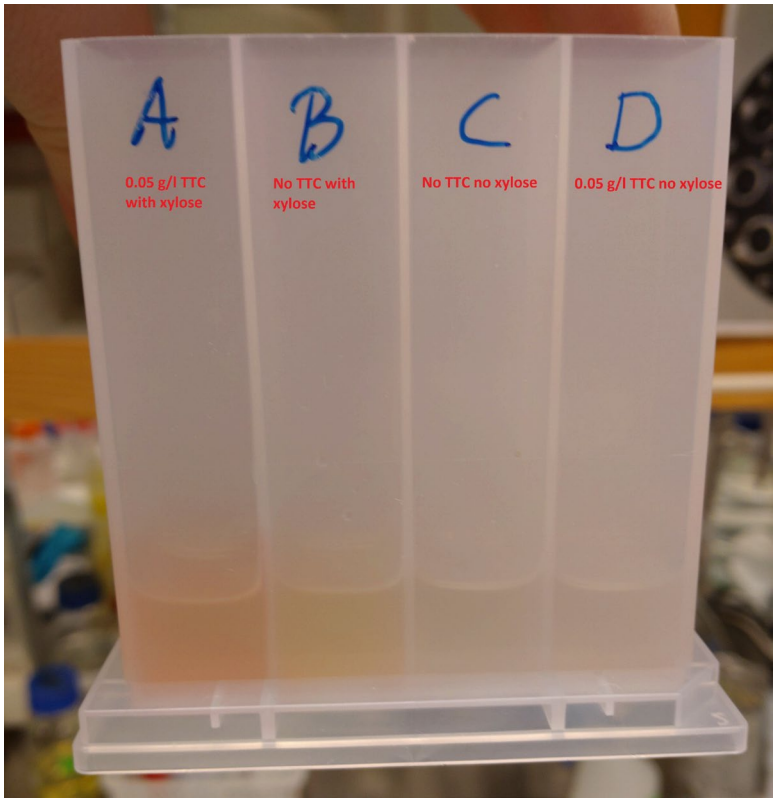

Figure 4. Photograph of MTP with CBS14 from XYL and ACA with 0.05 g/l TTC test after 144 h of incubation at 30 °C at 220 rpm. Test series one through four (A to D). Credit: Robin Lukic.

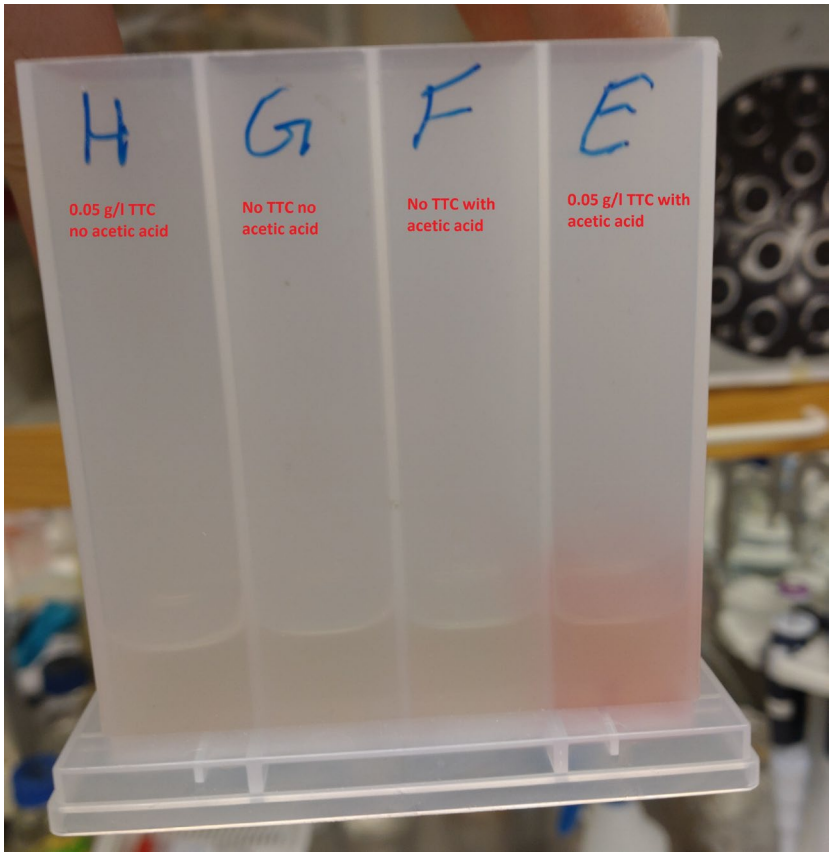

Figure 5. Photograph of MTP with CBS14 from XYL and ACA with 0.05 g/l TTC test after 144 h of incubation at 30 C at 220 rpm. Test series five through eight (E to H). Credit: Robin Lukic.

The OD was the highest in test series TTC with XYL and no TTC with XYL at 2.57 and 1.63 after 144 h, table 9. The other series had an OD between 0.37 and 0.27. The delay in yeast growth, can be seen in OD vales from the 24 h to 96 h mark, corresponds with the longer latency period of TTC indicator change as mentioned earlier.

This could be due to CBS14 taking longer time to switch substrate metabolism from GLC to XYL (Martín-Hernández *et al.* 2023).

Table 9. OD analysis of CBS14 from XYL and ACA test with 0.05 g/l TTC. Mean value and standard deviation of each test series triplicate.

Time	24 h	96 h	120 h	144 h
Test series				
TTC w. XYL	0.06 ± 0.06	0.40 ± 0.17	1.70 ± 0.61	2.57 ± 0.49
No TTC w. XYL	0.16 ± 0.06	0.76 ± 0.15	2.03 ± 0.21	1.63 ± 0.21
No TTC no XYL	0.56 ± 0.06	0.23 ± 0.06	0.30 ± 0.00	0.30 ± 0.00
TTC no XYL	0.63 ± 0.06	0.23 ± 0.06	0.27 ± 0.06	0.27 ± 0.06
TTC w. ACA	1.56 ± 0.06	0.16 ± 0.06	0.27 ± 0.06	0.37 ± 0.15
No TTC w. ACA	1.90 ± 0.10	0.30 ± 0.00	0.37 ± 0.15	0.37 ± 0.15
No TTC no ACA	0.46 ± 0.06	0.26 ± 0.06	0.33 ± 0.06	0.27 ± 0.06
TTC no ACA	0.50 ± 0.00	0.13 ± 0.00	0.30 ± 0.00	0.30 ± 0.00

3.2. SMSH substrate tests

The initial SMSH tests, 1a and 1b, were performed with only CBS14 because it was unknown how the strain would respond. The positive control had the same sugar concentration (27 g/l GLC and 9 g/l XYL) as the hydrolysate dilution test series. SMSH test 1a showed limited yeast growth and consequently the pH-level was changed to pH 6.00 in the subsequent experiments (both SMSH and LRH). SMSH test 1b showed positive results and by this stage the experiments were expanded by testing the CBS14 and J195 strains simultaneously in SMSH test 2 and later in the LRH fraction experiments.

3.2.1. SMSH test 1a and 1b

In SMSH test 1a, the growth of yeasts was the highest in the 30 % hydrolysate and positive control series, ranging from OD at above 5.0 to slightly below 3.0. The lowest growth was measured in the 90 %, 70 % and 50 % test series (data not presented). It was hypothesized that this could be due to a non-optimum pH-level or some other unknown inhibitory factors in the hydrolysate. The pH-level in the undiluted SMSH was measured to pH 4.31 at 28 °C with a pH-meter after SMSH test 1a.

The pH-level in all test series from SMSH test 1a were measured to be approximately between pH 2.00-4.00 with standard pH-paper after five days of incubation. Unfortunately, it was not possible to measure the samples with the more precise pH-meter because of the small total volume of sample, therefore, the values are not precise. Nonetheless, it indicated an unwanted pH-level decrease.

It was demonstrated in a study by (Taskin *et al.* 2016) that another yeast species in the same genus *Rhodotorula* cultivated on a sugar beet molasses based-substrate,

produced the highest amounts of lipids at a pH between 5.0 and 5.5. Similar pH-levels have been used in other studies as well (Blomqvist *et al.* 2018; Chmielarz *et al.* 2021). Therefore, the pH-level of the undiluted SMSH was set to pH 6.00 for the next experiment.

In SMSH test 1b (pH 6.00) the yeast grew better than in SMSH test 1a. The hydrolysate dilution series displayed similar growth regardless of concentration and grew much more than the positive control (same sugar concentration), table 10.

Table 10. OD analysis of CBS14 in SMSH test 1b after 96 h of incubation. Mean value and standard deviation of each test series triplicate.

Time	**96 h**
Test series	
90 %	35.67 ± 6.81
70 %	28.00 ± 5.29
50 %	31.00 ± 6.56
30 %	35.00 ± 3.00
Positive Control	3.87 ± 0.90

3.2.2. SMSH test 2

The yeasts were incubated at 25 °C instead of 30 °C to see if the overall growth would change. *R. babjevae* strains show variable growth at 30 °C while *R. toruloides* could grow at 30 °C. Similar descriptions of growth conditions and temperatures were found at an online yeast database (T. Boekhout 2022). Other studies have used similar temperatures (Blomqvist *et al.* 2018; Chmielarz *et al.* 2021). From this point onward, the temperature and rpm were not changed throughout the project.

It was hypothesized that the yeasts could proliferate without adding TM, and this was tested in the series with 90 % dilution and no TM in SMSH test 2, table 5. Moreover, a negative control and a test series with only water and MES was added.

The growth was the highest in all hydrolysate dilution test series and there was no distinguishable difference in proliferation between the hydrolysate series for both CBS14 and J195, figures 6 and 7.

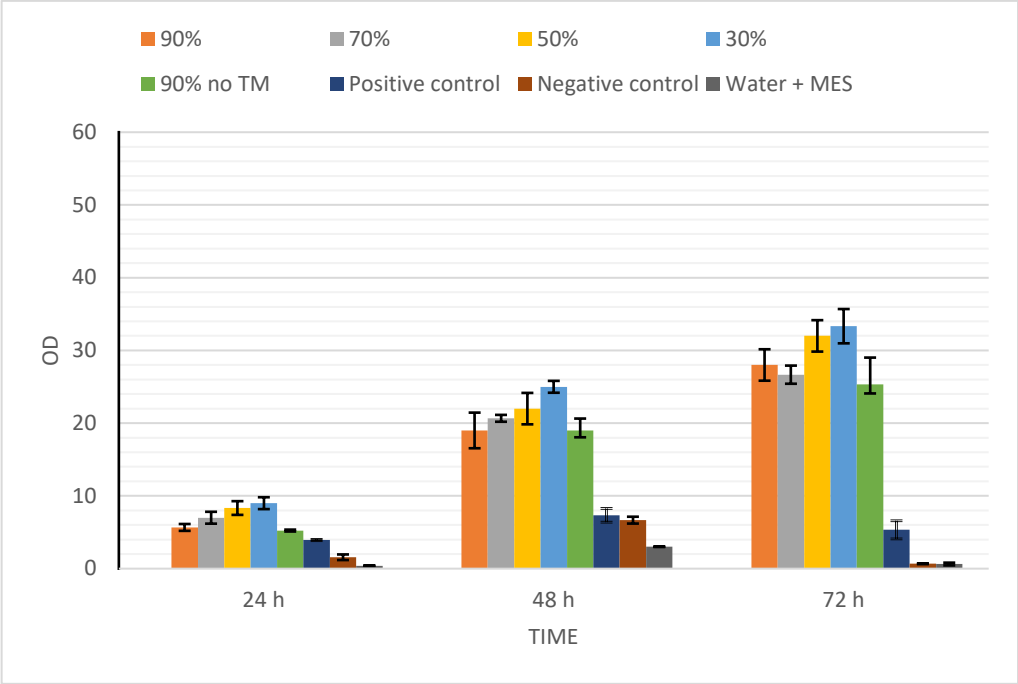

Figure 6. Chart of OD analysis of SMSH test 2 for CBS14. Hydrolysate dilution series from 90% to 30% and positive control have the same GLC and XYL concentrations. The negative control and water and MES have no GLC and XYL. Mean value and standard deviation of each test series triplicate.

J195 had higher overall OD than CBS14. The OD of positive control series for J195 was much higher than CBS14, figure 7. There was less difference between the positive control and the dilution series with J195 compared to CBS14.

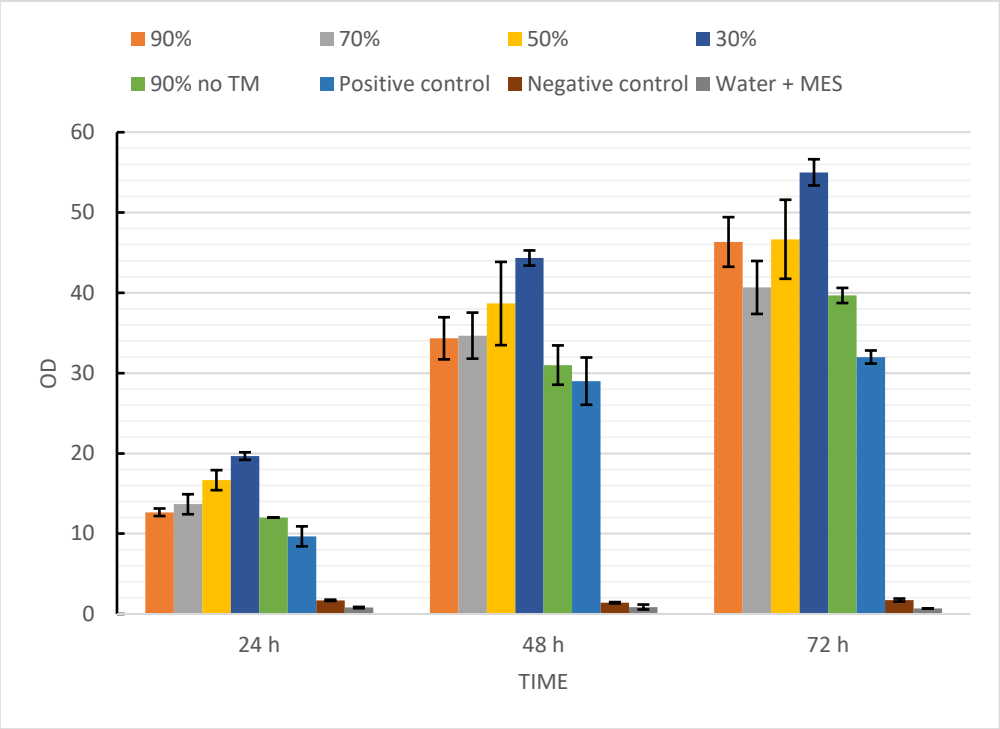

Figure 7. Chart of OD analysis of SMSH test 2 for J195. Hydrolysate dilution series from 90% to 30% and positive control have the same GLC and XYL concentrations. The negative control and water and MES have no GLC and XYL. Mean value and standard deviation of each test series triplicate.

J195 had higher CDW concentration in all test series compared to CBS14, figure 8. There was a slight difference between the dilution series and positive control for J195 while there was no large distinction between the dilution series and positive control for CBS14.

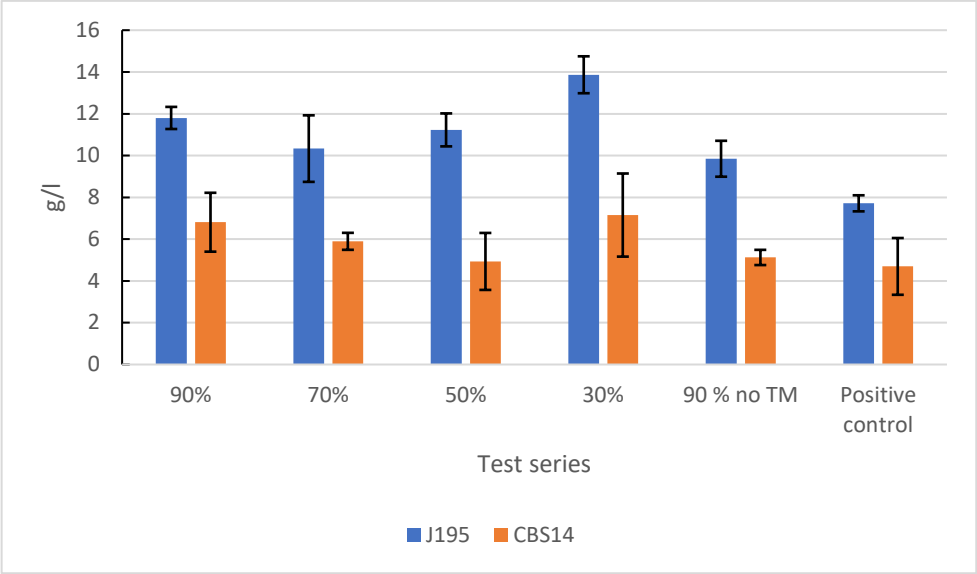

Figure 8. Chart of CDW concentration from SMSH test 2 for J195 and CBS14 after 48 h of freeze drying. Mean value and standard deviation of each test series triplicate.

There was no large difference in-between the hydrolysate dilution test series in yield, CDW in g/l per consumed carbon source (CCS): the summation of GLC, XYL and ACA in g/l. There was some difference with CBS14 between the 50% and the rest of the dilutions but none such with J195, table 11. All hydrolysate test series gave a yield around <0.5 g CDW per g CCS.

Table 11. HPLC analysis of SMS test 2 on J195 and CBS14 after 72 h of incubation. Yield of CDW per consumed carbon source (CCS) g/g. Mean values and standard deviations of CDW (g/l) from CDW analysis divided by total CCS (g/l) from HPLC analysis.

Yield (CDW g/CCS g)	**J195**	**CBS14**
Test series		
90%	0.48 ± 0.02	0.37 ± 0,07
70%	0.45 ± 0.01	0.34 ± 0.04
50%	0.45 ± 0.01	0.26 ± 0.05
30%	0.45 ± 0.02	0.33 ± 0.07
90% no TM	0.40 ± 0.02	0.37 ± 0.02

3.3. LRH substrate tests

The overall OD from the LRH substrate tests was lower than SMSH test 2, below OD at 30 compared to OD near 60 in the SMSH. The CDW concentration was lower in the HF compared to the CF in both strains and there was less accumulated CDW in both LRH fraction tests compared to SMSH test 2.

3.3.1. HF test

The pH-level was pH 3.78 at 25 °C in the undiluted HF prior to pH-calibration. The CBS14 grew only in the 30 % dilution test series and did not show any growth in the higher HF concentrations, figure 9. The rest of the dilution series had a much lower OD and there was no makeable difference between them. The positive control had the next highest OD.

The lower yeast growth could be due to the much higher XYL and ACA concentrations at approximate 10.40 and 6.3 g/l compared to GLC at 4.47 g/l, the yeasts switching from mainly GLC as substrate to XYL and the inhibitory effects of other organic acids as described by (Martín-Hernández *et al.* 2023). At 30 % dilution the concentration of inhibitors could possibly be low enough to cause less inhibition.

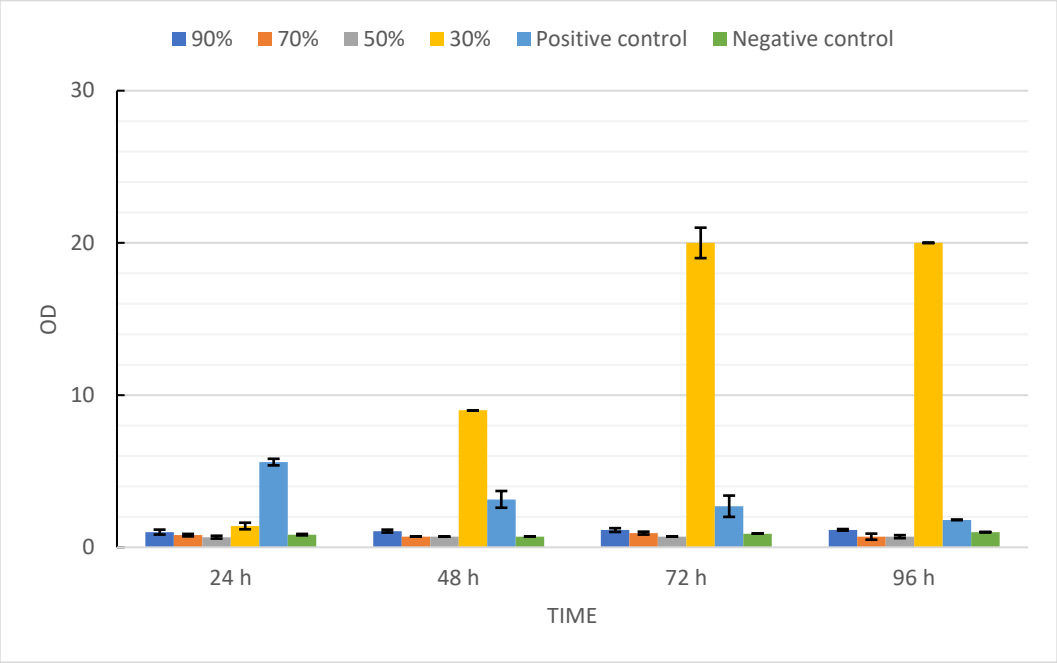

Figure 9. Chart of OD analysis of HF test for CBS14. Hydrolysate dilution series from 90% to 30% and positive control have the same GLC and XYL concentrations. The negative control has no GLC and XYL. Mean value and standard deviation of each test series triplicate.

Like CBS14, J195 had the best performance in the 30 % dilution and positive control series, with a big difference that positive control was initially much higher for J195 and then overtaken by the 30 % dilution series at the endpoint after 96 h, figure 10. The difference between 30 % dilution and positive control at the end point of J195, figure 10, were not vast as in CBS 14, figure 9.

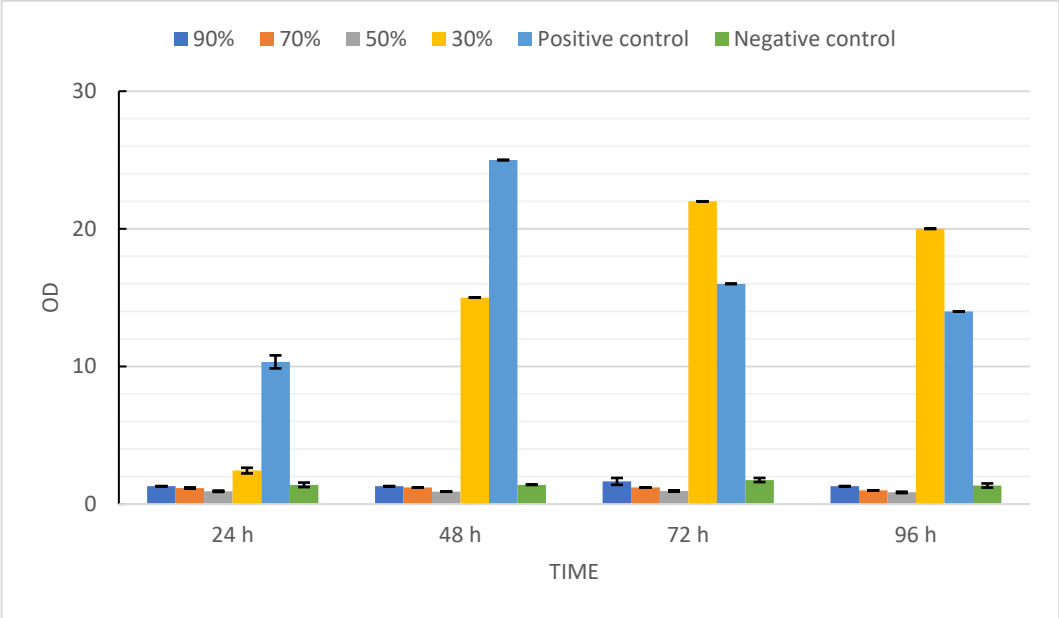

Figure 10. Chart of OD analysis of HF test for J195. Hydrolysate dilution series from 90% to 30% and positive control have the same GLC and XYL concentrations. The negative control has no GLC and XYL. Mean value and standard deviation of each test series triplicate.

There was no large difference between the strains in CDW concentration in the HF test except the positive controls, figure 11. The overall largest CDW was seen in the 30% dilution series in both strains followed by the positive controls. In the higher dilution series (90%, 70% and 50%) the CDW was very miniscule in both strains.

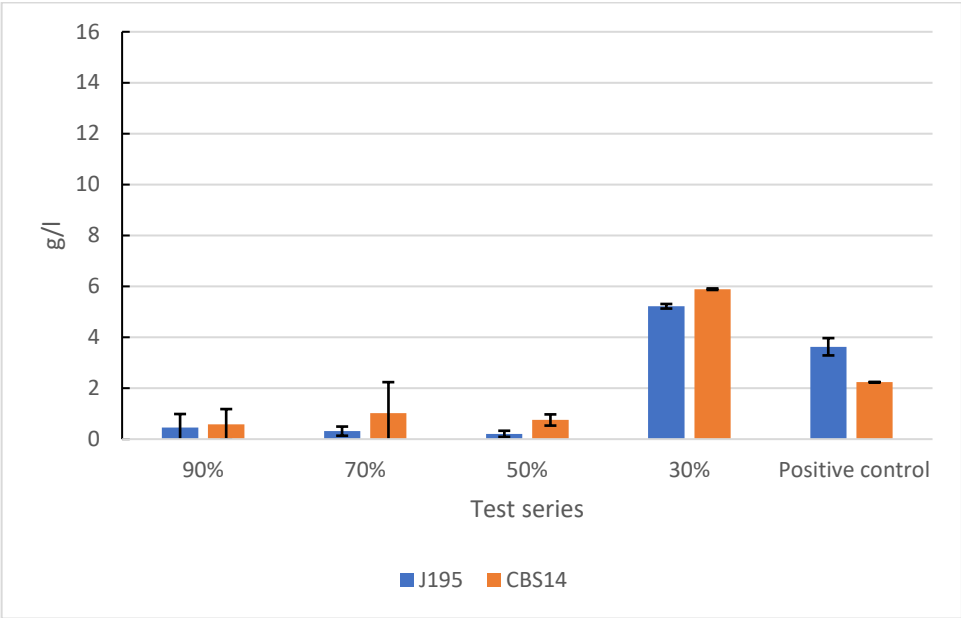

Figure 11. Chart of CDW concentration from HF test with J195 and CBS14 after 48 h of freeze drying. Mean value and standard deviation of each test series triplicate.

There was virtually no CCS through the 90% to 50% dilution series from the HF test. Therefore, the data from 90% to 50% were kept out of the results table, table 12. The yield for 30% test series J195 was slightly lower than CBS14. The yield was approximately 0.33 g CDW per g CCS for J195 and 0.37 g CDW per g CCS.

Table 12. HPLC analysis of HF test on J195 and CBS14 after 96 h of incubation. Yield of CDW per consumed carbon source (CCS) g/g. Mean values and standard deviations of CDW (g/l) from CDW analysis divided by total CCS (g/l) from HPLC analysis.

Yield (CDW g/CCS g)	**J195**	**CBS14**
Test series		
30%	0.33 ± 0.01	0.37 ± 0.00

3.3.2. CF test

The pH level was 4.96 at 16 °C in the undiluted CF before calibration. The general observation is that OD values for CBS14 and J195 in the CF test were greater than the HF test. There was not any noticeable difference between the CF dilution series in both CBS14 and J195, figures 12 and 13. With CBS14, the CF dilution series had a higher OD than the positive control after 48 and 72 h, figure 12.

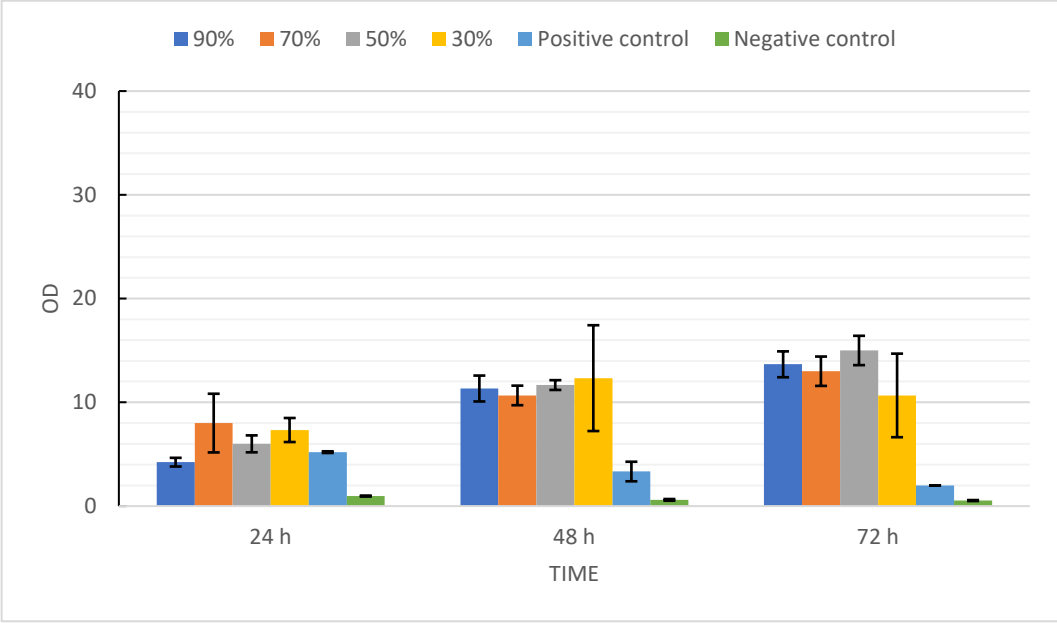

Figure 12. Chart of OD analysis of CF test for CBS14. Hydrolysate dilution series from 90% to 30% and positive control have the same GLC and XYL concentrations. The negative control has no GLC and XYL. Mean value and standard deviation of each test series triplicate.

J195 had less difference in OD between the CF dilution series and positive control, figure 13, compared to CBS14, figure 10. The 30% dilution series proliferated more than the rest of the dilution series after 48 and 72 h.

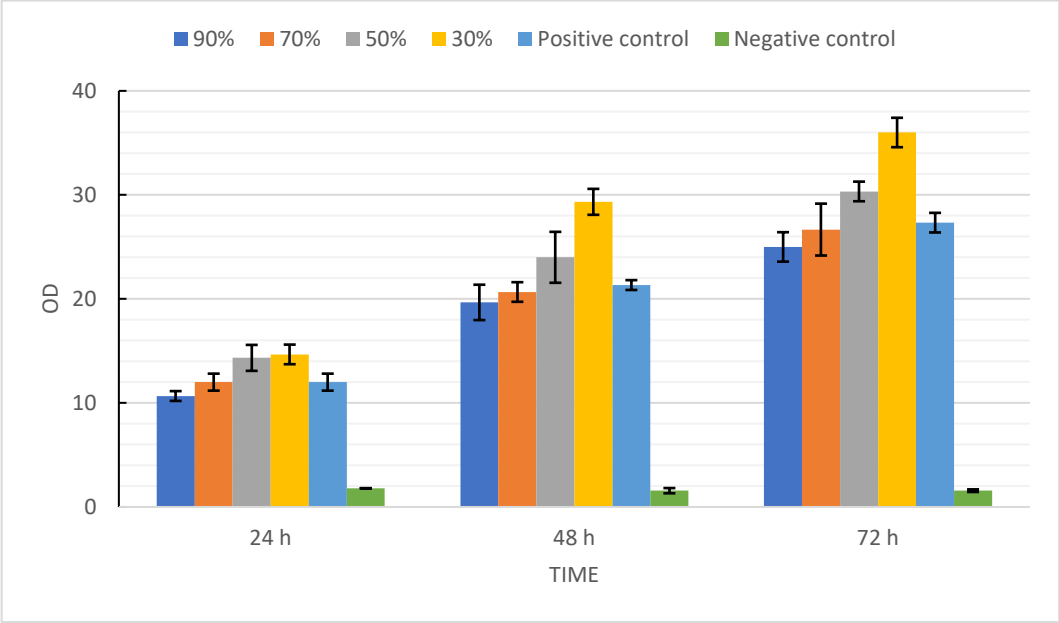

Figure 13. Chart of OD analysis of CF test for J195. Hydrolysate dilution series from 90% to 30% and positive control have the same GLC and XYL concentrations. The negative control has no GLC and XYL. Mean value and standard deviation of each test series triplicate.

The CDW concentration was in general higher for J195 than for CBS14, figure 14. There was no remarkable change between the CF dilution series and positive controls in both strains. The CDW was overall higher for both strains compared to the HF test, figure 11.

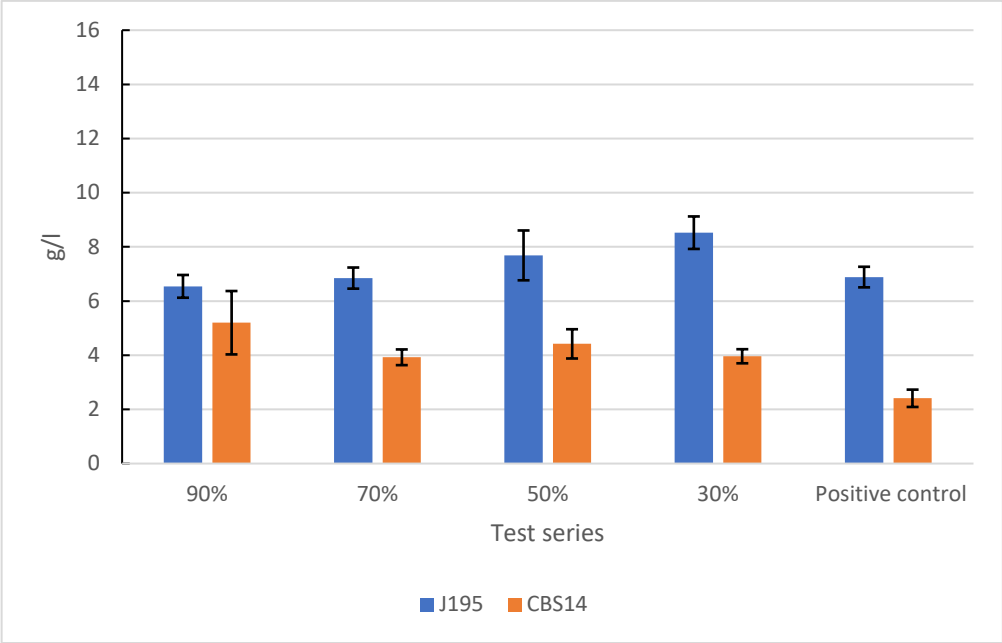

Figure 14. Chart of CDW concentration from CF test with J195 and CBS14 after 48 h of freeze drying. Mean value and standard deviation of each test series triplicate.

There was no distinguishable change in yield between the yeast strains, all the hydrolysate dilution test series performed approximately 0.30 to 0.35 g CDW per g CCS, table 13. Almost all GLC was consumed by both strains in SMSH test 2 compared to the CF test were overall less GLC was consumed despite similar start point levels (data not presented). It could explain why the growth was greater with the SMSH compared to the CF as substrate. In general, GLC was the most preferred carbon source in both strains in all tests.

Table 13. HPLC analysis of CF test on J195 and CBS14 after 72 h of incubation. Yield of CDW per consumed carbon source (CCS) g/g. Mean values and standard deviations of CDW (g/l) from CDW analysis divided by total CCS (g/l) from HPLC analysis.

Yield (CDW g /CCS g)	**J195**	**CBS14**
Test series		
90%	0.35 ± 0.00	0.35 ± 0.08
70%	0.34 ± 0.01	0.30 ± 0.01
50%	0.35 ± 0.02	0.35 ± 0.04
30%	0.35 ± 0.02	0.41 ± 0.10*

*ACA not detectable

3.4. Discussion

3.4.1. TTC experiments

Redox indicators can be used in absorbance measurements to aid the quantification of microbial growth. Precise values of OD are limited within certain concentration ranges and an addition of an indicator has been shown to increase the reliability of spectrophotometric assessments. In bacterial growth, it has been shown that the color change (precipitated salt) is proportional to the growth (Gabrielson *et al.* 2002).

The purpose was to investigate if the TTC indicator could give a visual signal that the yeasts were metabolically active. This was achieved in the TTC calibrations tests overall but inhibitory effect on yeast growth was also observed in the higher TTC concentrations (1.0 g/l), figure 1 and table 7, and in some degree the lower (0.1 and 0.05 g/l), figures 2 and 3 and table 8. The switch of main carbon source from GLC to XYL and ACA in calibration test 3 caused a delayed indicator change (several days), figures 4 and 5. It could be CBS14 is taking longer time to adjust to XYL and ACA with a longer lag phase.

There are issues described with XYL as sole substrate among some yeast strains that have resulted in lower growth and lipid yields. In mixed substrates with both GLC and XYL, many strains grew better and accumulated more lipids. Yeast grown on GLC had the highest overall growth when it was a sole substrate. Specifically, the CBS14 strain has been shown to have lower OD and lipid yield than the J195 strain when using XYL as a sole substrate (Martín-Hernández *et al.* 2023). It should be noted that the purpose of this thesis was not to ascertain the lipid accumulation in the strains but to assess the overall yeast growth. In future studies, the carbon source in the YM-medium (pre-culture) could be changed from GLC to XYL or ACA to investigate if would have a different impact on the yeast.

It should be noted that the pH-level was not set in either of the TTC calibration experiments, this could explain the lower observed OD values throughout the tests compared to the hydrolysate assays. Furthermore, the TTC caused irreversible staining in the deep wells of the MTPs in the test series where the yeasts were metabolically active. This presents an issue if the goal is to have visual signals in deep wells when substrate fermentation occurs. For replication studies, the pH-level could be adjusted, and the type of redox indicator could be changed to see if the staining problem disappears, or another MTP could be tested with TTC. Consequently, the TTC redox indicator was abandoned during the rest of the project because of the staining.

3.4.2. SMSH experiments

According to (Chen *et al.* 2022b) fungal pre-treatment increased the solubility of nitrogen sources and nitrogen accumulation in three different Shiitake cultivation SMSs on aspen, alder, and birch sawdust as substrates. This could explain why the overall OD was higher in SMSH test 2 compared to the LRH fractions. Further, the yeast consumed more GLC in the SMSH than the CF during the same incubation time.

The test series with 90 % dilution no TM showed no relevant difference when compared to the other dilution series with TM added, figures 6 and 7. It suggests there could be enough nutrients present in the SMSH itself without the addition of TM. If so, it could save costs when no supplemented nutrients are needed but it is early to say since the lipid amount was not quantified in this practical. Nevertheless, these preliminary results are promising.

Furthermore, (Chen *et al.* 2022b) also described a higher content of glucan and lower content of xylan and lignin (polysaccharides part of the cellulose structure) in the SMSs compared to the non-mushroom treated wood substrates. The GLC, XYL and ACA concentration in the study's SMSs was like the SMSH in this practical, around 30 g/l GLC, 10 g/l XYL and 2 g/l ACA. The presence of more glucan, consisting of linked GLC units, explains the overall higher concentration of GLC.

The general yield (CDW per CCS) was higher in the SMSH test for J195 compared to both J195 and CBS14 in the LRH tests, tables 11, 12 and 13. The CDW assessment was only based on end point measurement in SMHS test 2 and the LRH fraction tests. Therefore, it was not known which CDW starting concentration the strains had in the pre-cultures.

The HPLC analysis was unfortunately imprecise because of a technical issue with the calibration curve. Therefore, the yield values should be carefully evaluated when compared to the other results. That said, the OD and CDW analyses, figures 6, 7 and 8, indicate that the overall yeast growth was larger with both yeast strains on SMSH as substrate compared to both LRH fractions.

An issue with preparation of SMSH was the loss of hydrolysate in the filtration stage after centrifugation because of blockage in the membrane filters, both 0.45 and 0.20 µm. In the first SMSH tests, tests 1a and 1b, approximately 12 ml was lost during sterile filtration out of approximately 60 ml of hydrolysate. This can present and issue if the goal is to minimize loss of SMSH and preparation stages.

3.4.3. LRH fractions experiments

There was a remarkable difference between the HF and CF hydrolysates in terms of CDW and yield, figures 11 and 14, tables 12 and 13. This was partly expected for the HF because of the already described repressive effects by higher

concentrations of inhibitory compounds by (Chen *et al.* 2022b) and others (Brandenburg *et al.* 2016).

The initial concentration of ACA, XYL, and GLC in the undiluted HF was around 6.3, 10.41, and 4.47 g/l. It was a higher ACA and XYL and lower GLC amount compared to the CF. The overall concentration of these carbon sources was lower in the HF, and it could help explain why the yeast did not grow as much.

Inhibitory compounds are released during pre-treatment (steam expansion) of LR and there are more in the HF because of the breakdown of hemicellulose. Compounds such as furfurals and other organic acids are freed when steam expansion occurs (Jonsson & Martin 2016).

These compounds probably impaired the yeast proliferation in both strains with the HF as substrate, particularly in the 90%, 70%, and 50% dilution series. This resulted in a very low OD, CDW, and yield in the other dilution series compared to the 30% dilution and positive control, figures 9, 10, and 11, and table 12. It could be that the number of inhibitors were low enough in the 30% dilution.

It was mentioned in a previous discussion point that the HPLC results were not precise. Even if the margin of error could be high, the CCS in the HF test could be negligible in comparison with the CF and SMSH tests when taking the other assays into account.

The CF hydrolysate showed higher growth with both CBS14 and J195 and it could be due to less amounts of inhibitors and more GLC than the HF. The GLC concentration was much higher in the CF, around 30 g/l, and the ACA and XYL were lower than in HF, around 2.1 g/l and 6 g/l. Another interesting detail was the CF had less yeast growth than the SMSH despite similar concentrations of carbon sources. It is possible the SMSH has more available nutrients (nitrogen sources) than the CF because of mushroom cultivation. The 90% dilution no TM series in SMSH test 2 did show that the yeast could growth without addition of TM and the yeast consumed almost all GLC in the SMSH while they consumed less in the CF during the same incubation time.

3.4.4. Comparison between SMSH and LRH as substrates for oleaginous yeasts

The SMSH gave the best performance in both strains in terms of growth, CDW and yield compared to the LRH fractions. Strain J195 had an overarching theme of higher OD and CDW in all hydrolysate tests except in the HF test.

One advantage with the SMS based hydrolysate is the ability to grow food (edible mushrooms) on side products from the agricultural and forestry sectors. The fungal pre-treatment in the SMS can give a more accessible nutrient availability for fermenting yeast as described by (Chen *et al.* 2022a) without the extensive pre-treatment of steam expansion in LR which contributes to the release of many inhibitors.

A wood substrate can first be used directly in food production, then serve as SMS for further food or other biotechnological applications with oleaginous yeast fermentation. It could also help to diminish the greenhouse gas emissions if the same organic biomass is utilized several times before finally being discarded.

The LRH has a potential in yeast fermentation since lignocellulosic hydrolysates have been shown to be promising substrates (Brandenburg *et al.* 2016; Blomqvist *et al.* 2018; Brandenburg *et al.* 2021). This was a first attempt at using LRH as substrate and there was an initial uncertainty if an unseparated LRH (mixture of both HF and CF) would be too inhibitory. Therefore, the LRH was separated into two liquid fractions. Although there may be issues with the HF because of the high concentrations of inhibitory compounds, it could be worth to attempt cultivation with an unseparated LRH.

The economic viability of LRH as a substrate depends if the costs associated with pre-treatment overweigh the potential lipid yields (Chmielarz *et al.* 2021). Compared to the SMS, the crude LR are not enzymatically pre-treated by mushroom enzymes and probably do not possess equal amounts of nutrients, like fungi mycelium, in the final hydrolysate.

It is a fascinating possibility to be able to convert the abundant lignocellulosic biomass and turn it into high value commodities such as food, animal feed or biofuels. It would be interesting to conduct further studies on both strains CBS14 and J195 on SMSH and LRH in the future with other analytical measurements, e.g., lipid quantification.

3.4.5. Establishing a screening method

The original plan for the screening method was to enable running more parallels simultaneously with low volumes of substrate and make it more time efficient. This plan involved using both stepper and multichannel pipettes to diminish individual pipetting steps. Unfortunately, it was not possible to implement these other pipette types and techniques in the project because of technical issues and time restraints. This resulted in a routine which solely involved individual pipetting steps. Consequently, the MTP plating took longer to perform, OD analysis sometimes involved upwards to a hundred single pipetting steps, and the margin of individual error was high. The different volumes in each test series meant constant change of pipette volume settings and pipettes with different volume capacity. To establish a high-throughput screening method, stepper and multichannel pipettes should be implemented if time and effort is to be optimized. This practical was in large focused on yeast growth and the MTP system proved to be sufficient for this.

4. Conclusion

There were issues with the TTC indicator at 1.0 g/l because of inhibition of yeast growth. The indicator worked at lower concentrations (0.1 and 0.05 g/l) but the MTPs were permanently stained. Another indicator or MTP system could be considered for coming experiments. The Applikon-Micro Flask system was suitable for screening yeast growth on different substrates. Stepper and multi-channel pipette routines should be implemented if a high-throughput screening is to be achieved.

The SMSH was the most promising hydrolysate compared to the LRH fractions in terms of overall yeast growth. The SMSH showed yeast proliferation could be achieved without supplement of nutrients in the 90% dilution no TM test series. The HF severely inhibited yeast production in both strains at all dilution series except the 30 % dilution. The CF showed less inhibition of yeast growth than the HF but did not have as high growth as the SMSH despite a similar concentration of GLC, XYL, and ACA.

The investigated hydrolysates should be considered for further inquiry as they may be suitable substrates for yeast oil production. Other lignocellulosic side products from the food, agriculture and forestry sectors could also be of interest in forthcoming studies. These screening methods could be based on the Applicon-Micro flask system or another MTP system.

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Kan vi odla lipidackumulerande jäst med hjälp av förbrukat svampsubstrat och avverkningsrester?

Det har på senare tid uppkommit ett intresse av att hitta alternativ till vegetabiliska oljor på grund av negativ påverkan av miljön. Många gröna sektorer, som skogs- och jordbruket, genererar en riklig mängd biprodukter som är oätliga och består i stora delar av lignocellulosa-rika material. Lignocellulosa är ett nätverk som består av flera kolhydrater: cellulosa, hemicellulosa, och lignin. Det är en huvudsaklig del av växtcellens och plantors struktur.

Vissa mikroorganismer, som bakterier och jästsvampar, kan producera värdefulla ämnen. Lipidackumulerande jäst är en grupp jästsvampar som kan producera oljor som har en liknande sammansättning av fetter som vegetabiliska har. Dom kan fermentera (jäsa) många olika substrat, däribland flera oätliga biprodukter från jordbruket, och omvandla det till ätbara oljor.

En utmaning med dessa sidoprodukter (substrat) är att lignocellulosa är väldigt svårt att bearbeta och lösa upp till dess beståndsdelar (sockerarter). Det går att tillverka en vätska (hydrolysat) av många biprodukter. Hydrolysatet innehåller fria sockerarter (kolkällor), som glukos, men har också många andra ämnen som hämmar jäst, tillexempel organiska syror. Jästen kan livnära sig på fria socker men behöver också annan näring (kvävekälla) som ofta behöver tillsättas.

Ett substrat som kräver mycket bearbetning är avverkningsrester (LR) från skogsbruket. Dom består av trädkronor, grenar, bark och barr. Det krävs en omfattande värmebehandling av LR för att bryta ned lignocellulosa. Förbrukat svampsubstrat (SMS) är rester från ätbar svampodling, tillexempel champinjoner, som kan odlas på sågspån och halm. Fördelen med detta substrat är att svamparna frisätter ämnen som kan bryta ned lignocellulosan och att ingen förbehandling krävs (likt den för LR) samt att det finns kvar svamprester som näringskälla.

Det förbrukade svampsubstrats-hydrolysatet (SMSH) var gjort på SMS från Shiitakesvampodling på björkspån och vetekli (4:1 förhållande). Avverkningrest-hydrolsatet (LRH) kom från gran och separerades i två vätskedelar: en cellulosafraktion (CF) och en hemicellulosafraktion (HF).

Målet med detta mastersarbete var att utforska möjligheten att odla lipidackumulerande jäst på dessa substrat i mikrotiterplattor (MTP). Sådana plattor

har flera små djupa brunnar för att testa olika ämnens positiva eller negativa påverkan på jästens tillväxt. När detta utreds i större skala kallas det för screening. Olika screeningmetoder finns för att bedöma mikrobiell tillväxt med hjälp av färgskiftande indikatorer (ämnen) som byter färg när organismerna växer. I detta projekt användes en typ av MTP system (Applikon-Micro Flask) med 24 djupa brunnar och ett vattenlösligt salt (TTC) som indikator (den byter färg till röd-lila när jästen är aktiv). Två jäststammar, *R. toruloides* CBS14 och *R. babjevae* DVPG8058 (som kallas för J195 i detta arbete), screenades på olika substrat (hydrolysat).

Testen med indikatorn visade att TTC troligtvis hämmade jästen att växa i högre koncentrationer (1.0 g/l) men inte i lägre koncentrationer (0.1 och 0.05 g/l). Även om indikatorn fungerade i de lägre koncentration och skiftade färg blev MTP permanent missfärgade och användes därför inte under resten av arbetet.

SMSH hade den mest positiva effekten på jästtillväxt medan CF och HF visade mindre total tillväxt och hämmande effekter. HF orsakade störst hämning hos båda jäststammarna men de kunde växa vid 30 % utspädning. CF hade liknande koncentrationer av kolkällorna glukos, xylos och ättiksyra som SMSH men visade mindre tillväxt.

Tillverkning av SMSH behöver mindre förbehandling än LRH pga. svampnedbrytning av lignocellulosa. SMSH har också troligtvis nog med näringsämnen från början (svamprester) och behöver möjligen inte komplettares med ytterligare näring innan det används. Applikon-Micro Flask-systemet visade sig vara tillräckligt bra för screening av jäst på flera olika substrat.

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