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Characterisation of non-conventional yeast *Kluyveromyces marxianus* in lignocellulosic hydrolysate

Bachelor's Thesis (12 ECTS)

Curriculum Science and Technology

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Tartu 2021

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Abstract:

Lignocellulosic biomass is a globally available non-edible renewable feedstock, the hydrolysate of which can be used in microbial cell factories for the production of industrially relevant compounds. However, a successful microbial host should possess certain traits as the environment of lignocellulosic hydrolysates is challenging. It requires the ability to consume or, preferably, co-consume different substrates (mainly xylose), and to tolerate growth-inhibiting compounds. This thesis is focused on characterisation of a thermotolerant fast-growing non-conventional yeast, *Kluyveromyces marxianus*, in a lignocellulosic hydrolysate derived from birch. Growth in shake flasks experiment was firstly assessed and appropriate hydrolysate dilution for further characterisation in bioreactors was chosen. For the fastest-growing eukaryote, *K. marxianus* performed surprisingly poorly in the bioreactor experiments with the maximum specific growth rate reaching only 0.03 h^{-1} and final biomass of 5 gdw/L. An important point to consider is the nature of the nitrogen source which is one of the key factors affecting the performance. In a different experiment with organic nitrogen – a solution of yeast extract and peptone – the yeast demonstrated higher specific growth rate (0.11 h^{-1}), in contrast to the main experiment with ammonium sulphate. A known 2-PE producer, *K. marxianus* produced low amounts of 2-PE (up to 0.032 g/L was detected) which can also be linked to the nature of the nitrogen source used.

Keywords:

Lignocellulosic hydrolysate, xylose, *Kluyveromyces marxianus*, non-conventional yeast, biotechnology

CERCS:

T490 Biotechnology

Mittekonventsionaalse pärmi *Kluyveromyces marxianus* iseloomustamine lignotsellulooshüdrolüsaadis

Lühikokkuvõte:

Lignotselluloosne biomass on ülemaailmselt kättesaadav mittesöödav taastuv lähteaine, mille hüdrolüsaati saab kasutada mikroorganismidel põhinevatel rakuvabrikutel tööstuslikult relevantsete ühendite tootmiseks. Kuna lignotsellulooshüdralüsaatide keskkond on keeruline, edukal mikroobsel peremeesorganismil peaks olema teatud omadused, nagu erinevate substraatide (peamiselt ksüloosi) tarvitamine või koos tarvitamine ning taluvus kasvu pidurdavate ühendite suhtes. Käesolev bakalaureusetöö on keskendunud termotolerantse kiiresti kasvava mittekonventsionaalse pärmi *Kluyveromyces marxianus* iseloomustamisele kasest saadud lignotsellulooshüdralüsaadis. Kõigepealt määrati kasvu raputuskolbides ja valiti bioreaktorites edasiseks iseloomustamiseks sobiv hüdrolüsaadi lahjendus. Kõige kiiremini kasvava eukarüoodi jaoks demonstreeris *K. marxianus* bioreaktorikatsetes üllatavalt halva tulemuse, saavutades vaid 0.03 h^{-1} maksimaalse spetsiifilise kasvumäära ja 5 gdw/L lõpliku biomassi. Bioreaktorikatses orgaanilise lämmastiku – pärmiekstrakti ja peptooni lahuse – lisamisega näitas pärm kõrgemat spetsiifilist kasvumäära (0.11 h^{-1}), mis viitas lämmastikuallika olemuse rollile kasvu mõjutava võtmetegurina. Tuntud 2-PE tootjana *K. marxianus* tootis 2-PE väikestes kogustes (oli tuvastatud kuni 0.032 g/L), mille põhjust võiks samuti seostada kasutatud lämmastikuallika olemusega.

Võtmesõnad:

Lignotselluloosi hüdrolüsaat, ksüloos, *Kluyveromyces marxianus*, mittekonventsionaalne pärm, biotehnoloogia

CERCS:

T490 Biotehnoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

QPS – qualified presumption of safety; EU safety assessment system for a general use of a microorganism

GRAS – generally recognised as safe; USA safety assessment system for a specific application of a microorganism

μ – specific growth rate; expressed in per hour (h^{-1})

Yxs – biomass yield on substrate; expressed in gram dry weight per gram of substrate (gdw/gs)

rs – specific substrate uptake rate; expressed in gram per gram dry weight per hour (g/gdw*h)

INTRODUCTION

As human-caused climate change is becoming ever more prominent each year, diversion from fossil fuels to alternative resources is of the utmost importance for the sustainable future (Haines *et al.*, 2006). The most abundantly available solid alternative resource is lignocellulose, which hydrolysate can be used for microbial production of chemicals and energy sources, presently derived from fossil-based resources (Abdel-Hamid, Solbiati and Cann, 2013).

Lignocellulosic biomass is a widely available renewable plant-based resource. Polysaccharides, namely cellulose and hemicellulose, as well as aromatic polymer lignin, are the main constituents of lignocellulosic biomass. Until recently, hemicellulose was considered as waste and often used for energy generation by burning (Anwar, Gulfraz and Irshad, 2014). However, with recent advances in biomass hydrolysis, pre-treated hemicellulose can be converted into pentose and hexose sugars, with xylose being the dominating sugar. Hydrolysate solution of these monomers may later be used as substrates for microbial cell factories to obtain the desired biochemicals and biofuels.

Conventionally, genetically modified baker's yeast, *Saccharomyces cerevisiae*, has been employed in the bioproduction of ethanol using lignocellulosic hydrolysate as a substrate. Still, unmodified *S. cerevisiae* is not able to utilise xylose naturally due to the absence of xylose catabolizing enzymes. On the other hand, several non-conventional yeasts, one of them being *Kluyveromyces marxianus*, have the advantage of naturally present metabolic pathways allowing for more efficient xylose utilisation.

This thesis focuses on the characterisation of the non-conventional yeast *K. marxianus* in hemicellulosic hydrolysate derived from birch.

1 LITERATURE REVIEW

1.1 Lignocellulosic biomass

One of the main goals of a biobased economy is the development of sustainable and economically viable biotechnological processes as feasible alternatives to fossil-based chemistry (Kavšček *et al.*, 2015). Renewable feedstocks can be used in microbial cultivation for the sustainable production of bulk and fine chemicals. Conversion of lignocellulosic biomass is of particular interest as it is a non-edible plant matter, hence no involvement in the human food chain, and, unlike the use of crops such as corn and sugarcane in fermentation processes, no compete for arable land (Karen O. Osiro *et al.*, 2019).

Lignocellulosic biomass is a renewable natural resource, and its harvest is fairly inexpensive compared to crude oil (Ge *et al.*, 2018). Lignocellulosic biomass is also theoretically carbon-neutral as the carbon cycle is closed when CO_2 taken up by the plant in the course of its life is released during harvesting and processing, resulting in zero net emission of carbon dioxide (Potters, Goethem and Schutte, 2010). One of the advantages of lignocellulosic biomass is that it can be found in abundance globally as a potential source of biomass. Agricultural residues, energy crops, forestry residues, and human waste are some of the examples.

Lignocellulosic biomass is comprised of carbohydrate polymers: cellulose, hemicellulose, and lignin, though exact fractions of each vary depending on the source of lignocellulosic biomass. Polysaccharide cellulose is comprised of linearly linked glucose monomers and is the most abundant organic polymer. Cellulose contributes to the stability of plant cell wall structure. Hemicellulose is a cross-linked polymer that includes diverse species of monomers: pentoses, hexoses and acetyl groups. Along with cellulose, hemicellulose's role is to strengthen the cell wall, tethering the cellulose microfibrils (Cosgrove, 2000). Lignin is a cross-linked polyphenolic material and is the second most abundant polymer in nature. Lignin

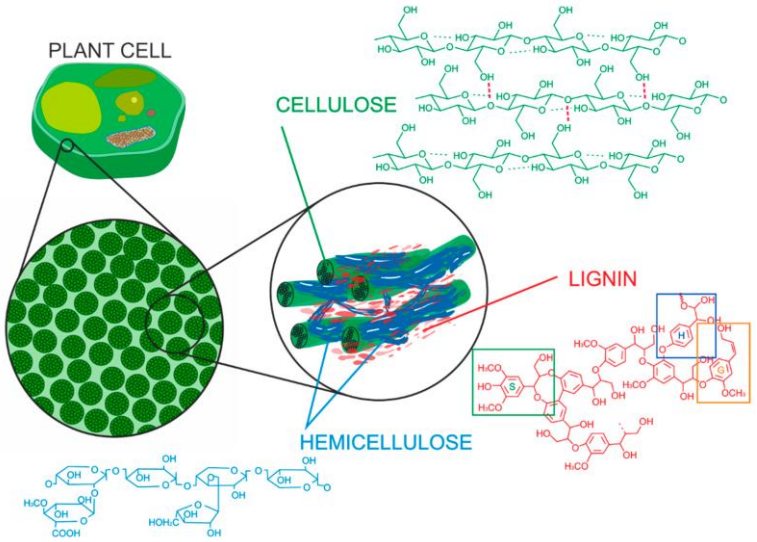

Figure 1. Composition of lignocellulosic biomass (Hasanov, Raud and Kikas, 2020)

provides compressive strength to the plant cell wall by filling up the spaces between cellulose and hemicellulose (Figure 1).

To make monomers of lignocellulosic biomass available for microbial fermentation, the biomass structure needs to be broken open to loosen the structural polymer matrix and enable better depolymerisation of the polysaccharides. This is achieved during the pre-treatment process. The pre-treatment methods can be categorised into four groups: physical, chemical, physico-chemical, and biological (Figure 2). Pre-treatment of lignocellulosic biomass can result in the formation of several different microbial growth inhibitors. Depending on the used biomass source, the amount and type of inhibitors vary. Inhibitor formation also depends on temperatures, reactive chemicals, and the duration of the pre-treatment process. The inhibition mechanisms may include the deactivation of enzymes or decline of vital cell structures. Each inhibitor's toxicity depends on its chemical and physical properties. The main inhibitory compounds found in lignocellulosic hydrolysates are furfural, acetic acid, 5-hydroxymethylfurfural (HMF) as well as phenolic compounds. Moreover, it has been shown that lignocellulosic hydrolysates containing many different inhibitors have a more pronounced inhibitory effect on microbes and enzymes compared to the presence of one inhibitor only (Sjulander and Kikas, 2020).

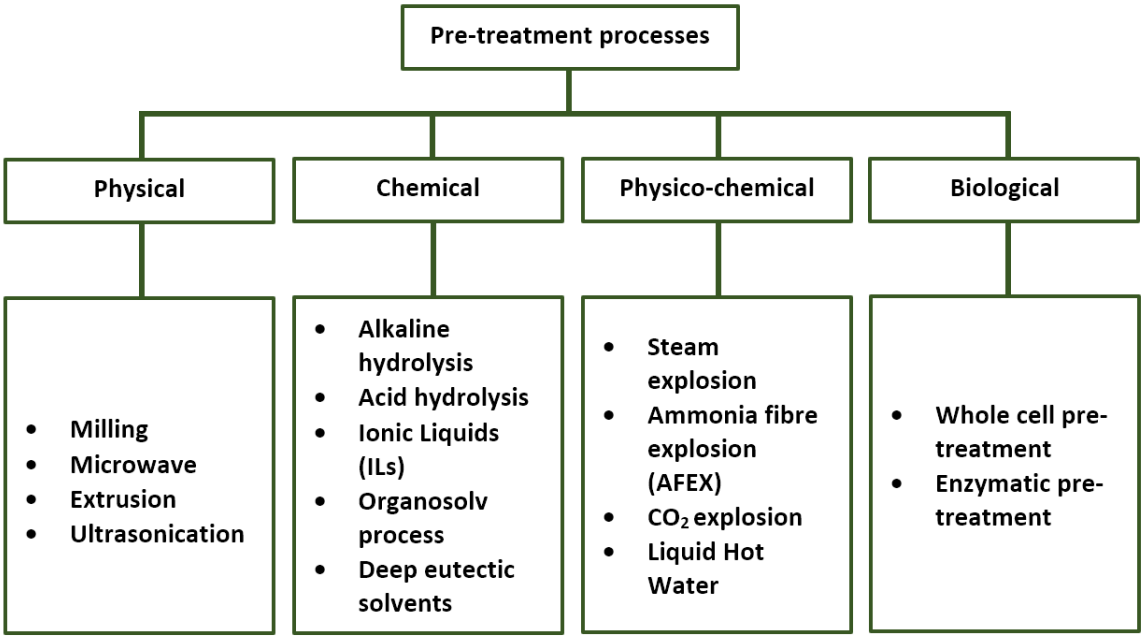

Figure 2. Pre-treatment processes (Baruah *et al.*, 2018)

Among various options for lignocellulosic biomass choice, birch is of particular interest for Nordic countries and Estonia as birch biomass is widespread and available throughout

Northern Europe. A representative of hardwood species, birch lignocellulose is comprised of 40-46% cellulose, 17-23% hemicellulose, and 18-25% lignin (Schutyser *et al.*, 2017).

1.2 *Saccharomyces cerevisiae*

1.2.1 *S. cerevisiae* as a microbial cell factory

S. cerevisiae is one of the oldest and the most extensively studied unicellular eukaryotic microorganisms. Traditionally used in alcohol fermentation, baking, and bio-ethanol production, the yeast is also one of the most frequently used microbes in biotechnology for the industrial production of bulk and fine chemicals as well as heterologous expression of enzymes and therapeutic proteins (Li and Borodina, 2015). A plethora of available *S. cerevisiae* heterologous recombination techniques allows for both the addition and deletion of new genes (Chen *et al.*, 2018). Combined with a wide range of knowledge about various aspects of yeast biology, yeast becomes an attractive research tool and the host of choice in many cases. Nevertheless, the microbial *S. cerevisiae* factory may be hindered by species-specific traits in the development of hosts with high productivity and yields close to theoretical limits (Kavšček *et al.*, 2015).

1.2.2 *S. cerevisiae* xylose consumption

Despite many advantages, *S. cerevisiae* has one major drawback for its successful use as a lignocellulosic microbial biorefinery – this yeast is not able to naturally utilise xylose. To address this problem, there have been successful attempts at developing a xylose utilisation pathway in *S. cerevisiae*. Currently, two xylose pathway implementation strategies are successfully realized: the oxido-reductive pathway and the isomerase pathway (Peng *et al.*, 2012). The oxido-reductive pathway employs two key enzymes in xylose metabolism - xylose reductase to convert xylose to xylitol and a xylitol dehydrogenase to convert xylitol into xylulose. However, cellular redox balance has to be taken into account as these reactions are NAD(P)H-dependent. The latter strategy, the isomerase pathway, relies on xylose isomerase that is responsible for the direct conversion of xylose to xylulose, bypassing the need to consider any cofactors (Figure 3). Despite

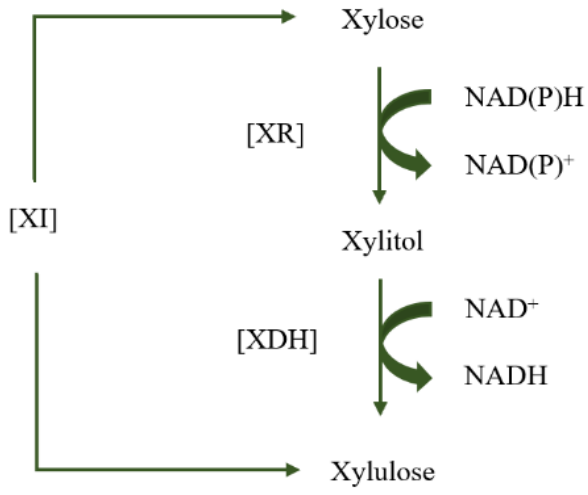

Figure 3. Xylose assimilation pathways. XR - xylose reductase; XDH – xylitol dehydrogenase; XI – xylose isomerase.

successful implementation of both strategies, neither provided the microbe with xylose uptake rates comparable to that of *S. cerevisiae* preferred sugar – glucose (Cunha *et al.*, 2019).

Another challenge for the efficient xylose utilisation by *S. cerevisiae* is the lack of specialised xylose transporters. The uptake takes place through hexose transporters with some xylose affinity, although the presence of glucose impedes the uptake (Subtil and Boles, 2012). A considerable number of studies have pointed towards the xylose paradox where *S. cerevisiae* does not sense that xylose is a fermentable carbon source, although being able to grow on it (Osiro *et al.*, 2018; Karen O Osiro *et al.*, 2019). This paradoxical behaviour indicates that engineering targets should be sought not only in the metabolic but also in signalling networks.

1.3 Non-conventional yeasts

S. cerevisiae limitations for utilisation as a lignocellulosic biorefinery have turned research interest towards non-Saccharomyces yeast species in hopes of finding suitable candidates. Some yeasts not belonging to *Saccharomyces* genus – so-called non-conventional yeasts – have an innate ability to consume xylose. Another non-Saccharomyces yeasts' advantage comes from the fact that they provide alternative metabolic routes for substrate utilisation and product formation (Kręgiel, Pawlikowska and Antolak, 2017). Tolerance to hydrolysate inhibitors and stress factors is also an important criterium which some non-conventional yeasts possess. In addition, some yeasts of non-conventional classification exhibit thermotolerance along with tolerance to ethanol which is important for bioethanol production (Tikka *et al.*, 2013). The employment of simultaneous saccharification and fermentation (SSF), a process that performs enzymatic hydrolysis with fermentation at the same time, is also made possible with thermotolerant yeasts (Choudhary, Singh and Nain, 2016). These special qualities and abilities make non-conventional yeasts attractive systems for lignocellulosic biorefinery.

One of the most well-known non-conventional yeasts with the ability to metabolise xylose, *Rhodotorula toruloides*, has emerged as a promising organism for growth on lignocellulosic hydrolysate owing to co-utilisation ability of different sugars as well as tolerance to certain hydrolysate growth inhibitors such as phenols. *R. toruloides* is an oleaginous yeast meaning that it can accumulate lipids and carotenoids under nutrient stress conditions (Pinheiro *et al.*, 2020). Lipids can later be used for the production of biofuels and oleochemicals while carotenoids can be used in nutraceuticals and cosmetics (Anunciato and da Rocha Filho, 2012; Sharma *et al.*, 2020).

Another prominent xylose-consuming non-conventional yeast system is *Scheffersomyces stipitis*, the fastest xylose fermenter among known microbes (Jeffries *et al.*, 2007). Unlike *S. cerevisiae*, it is a Crabtree-negative yeast, implying that *S. stipitis* does not switch to fermentation when the concentration of glucose in the environment is high (Papini *et al.*, 2012). This ability can be of use for more efficient substrate consumption (Dashko *et al.*, 2014). *S. stipitis* can also be used in the production of xylitol and ethanol (Rodrigues, Kenealy and Jeffries, 2011; Löbs, Schwartz and Wheeldon, 2017).

Kluyveromyces marxianus, a *K. lactis* close relative, is known for its thermotolerance with a high growth rate at elevated temperatures which is beneficial for reduced costs of cooling systems during the cultivation of this microorganism in the bioreactor and aids in contamination prevention. *K. marxianus* is also capable of xylose utilisation as well as other sugars that can be found in lignocellulosic hydrolysates (Leonel *et al.*, 2021).

1.4 *Kluyveromyces marxianus*

1.4.1 *K. marxianus* as a microbial cell factory

Recently, *K. marxianus* has attracted research interest due to its industrially beneficial traits such as the ability to assimilate a wide range of sugars. Thermotolerance (grows on temperatures over 50°C (Lane and Morrissey, 2010)) renders this yeast particularly suitable for biotechnological processes with elevated temperatures as well as preventing the growth of unwanted microorganisms. One of the biggest advantages is the highest specific growth rate (~0.8 h^{-1}) among eukaryotes (Groeneveld, Stouthamer and Westerhoff, 2009), which is of use for faster production of relevant growth-coupled chemical compounds. *K. marxianus* is of QPS (EU) and GRAS (USA) status, owing to its historical use with regular dairy products, which is of special importance for the production of pharmaceuticals and food-grade proteins.

Unlike *S. cerevisiae*, most *K. marxianus* strains are of Crabtree-negative or aerobic-respiring nature, meaning that it does not undergo aerobic alcoholic fermentation (Yu *et al.*, 2021). This feature comprises a beneficial phenotype for industrial biomass-directed applications as ethanol exhibits microbial toxicity. Although there are some conflicting reports of the Crabtree status of this yeast, these contradicting findings may be attributed to the strain variability, pointing to a high degree of intra-species disparity for this yeast in terms of its physiology in addition to its genetics. This variability may be attributed to the fact that *K. marxianus* strains have been isolated from a great variety of habitats (Fonseca *et al.*, 2008). Another beneficial property is the ability to secrete enzymes (Ha-Tran *et al.*, 2021).

One of the highlights of *K. marxianus* studies is its potential to be used for the production of value-added metabolites employing engineering manipulations. 2-Phenylethanol (2-PE), aromatic alcohol with a rose scent, was shown to be successfully produced in high quantities via the Ehrlich pathway in genetically engineered *K. marxianus* (Kim, Lee and Oh, 2014). Recently, a broad-range promoter set for metabolic engineering has been added to *K. marxianus* engineering tools (Lang *et al.*, 2020). In addition, the development of metabolic engineering tools such as CRISPR/Cas9 systems and Golden Gate Assembly dedicated platform (Rajkumar *et al.*, 2019) has enriched *K. marxianus* set of available genetic manipulation methods.

1.4.2 *K. marxianus* xylose consumption

As was mentioned earlier, *K. marxianus* has a native ability to utilise xylose as a carbon source along with being able to consume a broad range of other sugars present in lignocellulosic hydrolysate. In recent years, *K. marxianus* ability to assimilate xylose has been used for xylose-ethanol conversion for the production of second-generation biofuels. A recent study has reported that *K. marxianus* achieved an ethanol yield as high as 0.402 g/g in a xylose/glucose co-fermentation at 40°C (Suzuki, Hoshino and Matsushika, 2019). Another chemical compound that can be derived from xylose consumption is xylitol, which is a by-product of xylose metabolism.

Although *K. marxianus* is capable of xylose utilisation, glucose catabolite repression poses certain problems for efficient co-fermentation of xylose and glucose solutions. Several strategies have been attempted to alleviate carbon catabolite repression in this yeast. One of them made use of a directed evolutionary approach using glucose analogue 2-deoxyglucose which does not undergo further glycolysis (Kim *et al.*, 2019). Another strategy has successfully attempted xylose and glucose co-consuming platform strain creation with the use of genetic engineering (Hua *et al.*, 2019). With the help of these genetic modifications, the foundation for efficient co-fermentation of sugar solution present in lignocellulosic hydrolysate is established.

2 THE AIMS OF THE THESIS

The aims of the thesis are to:

1. Choose the appropriate C5-Birch hydrolysate dilution for *K. marxianus* growth (screen in shake flasks)
2. Characterise *K. marxianus* growth profile and external metabolite fluxes on diluted hydrolysate in bioreactors under aerobic conditions
3. Analyse the potential of *K. marxianus* to be used as a cell factory in hemicellulose-based biorefineries

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Lignocellulosic hydrolysate

Graanul Biotech OÜ (Tallinn, Estonia) provided a lignocellulosic hydrolysate from birch (*Betula pendula*) enriched with pentose sugars, here referred as C5-Birch. Pre-treatment included diluted acid hydrolysis. The hydrolysate composition is presented in Table 1.

	Concentration (g/L)	SD
Xylose	298.1	3.9
Glucose	87.1	9.7
Galactose	29.8	2.8
Mannose	21	0.1
Arabinose	14.1	1.6
Acetic acid	20.5	0.5
Total phenols	33	4.1
Furfural and 5-HMF	< 0.10	

Table 1. C5-Birch hydrolysate constituents and their concentrations

The pH was adjusted to 6.0 using NaOH. The hydrolysate was filtered using a polyethersulfone membrane with 0.2 um pore size Thermo ScientificTM Nalgene Filter (Waltham, USA). Minimal medium (monopotassium phosphate, 3 g/L; magnesium sulphate, 0.5 g/L) was used for hydrolysate dilution. Ammonium sulphate was added for the carbon/nitrogen (C/N) ratio adjustment. The final medium contained no added minerals nor vitamins.

3.1.2 Microorganism

Kluyveromyces marxianus CBS6556 was acquired from Westerdijk Fungal Biodiversity Institute (Utrecht, Netherlands). The strain was maintained at -80°C with 10% (v/v) glycerol according to the collection instruction.

3.1.3 Suitable C5-Birch hydrolysate concentration determination

Overnight incubation at 30°C and 200 rpm in YPD medium (dextrose, 20 g/L; peptone, 20 g/L; yeast extract, 10 g/L) was used for pre-inoculum preparation. Inoculum was prepared by cultivation in mineral medium (magnesium sulphate, 0.5 g/L; monopotassium phosphate, 3 g/L; ammonium sulphate, 5 g/L) with 20 g/L of glucose and a 1 mL per litre solutions of vitamins and trace elements as reported in (Lahtvee *et al.*, 2017) for 6 hours using same conditions as for pre-inoculum preparation. The cells were washed and concentrated in a 0.9% NaCl solutions soon after. The inoculation took place with an initial absorbance at 600 nm (OD 600 nm) of 0.1 in varying concentrations of diluted C5-Birch hydrolysate.

The yeast shake flask cultivation experiment in diluted C5-Birch hydrolysate was performed to assess the optimal hydrolysate concentration for future experiments. The total sugar (glucose, xylose, galactose, mannose, and arabinose) concentrations of diluted hydrolysate were 15, 28, 43, and 58 g/L. Respective C/N ratios were 7, 12, 22, 30 (mol/mol), including only ammonium sulphate for N-content calculations. Shake flasks volume was 125 mL and cultivation working volume was 25 mL. The experiments were carried out in triplicates. The yeast incubation environment was aerobic at 30°C and carried out at 200 rpm. OD 600 nm was determined with a spectrophotometer U-1800 (Hitachi, Tokyo, Japan).

3.1.4 Growth and substrate profiling in C5-Birch hydrolysate

For the detailed characterization of the yeast, the hydrolysate diluted to 50 g/L of total sugars (regarding glucose, xylose, galactose, mannose, and arabinose) was used in controlled bioreactors. Pre-inoculum and inoculum preparation was performed as described in 3.1.3. NaCl 0.9% (m/v) solution was used for washing and concentrating. The initial optical density at 600 nm was 0.5. There were three biological replicates for this experiment. One L MiniBio 1000 bioreactors (Applikon Biotechnology, Delft, The Netherlands) with an initial volume of 800 mL were used. One M HCl and 2 M KOH were used for pH maintenance at 6.0. The varying agitation between 400-800 rpm was employed for the partial pressure of dissolved oxygen (pO_2) to be kept above 25%. An online biomass probe (absorbance at 1300 nm) BugLab BE3000 Biomass Monitor (Bug Lab, Concord, CA, USA) was used for microorganism growth monitoring. CO_2 and O_2 were monitored by off-gas sensors (BlueInOne, BlueSens, Herten, Germany). The data acquisition was performed by the use of BioXpert V2 software v. 2.95 (Applikon Biotechnology, Delft, The Netherlands). For dry

biomass measurement, OD 600 mm, substrates, and metabolites quantification, the samples were taken regularly.

3.1.5 Analytical methods

Gravimetrical analysis was used for dry cell biomass measurement after the cultivation broth filtration using the membrane of 0.45 um pore size (Merck Millipore, Darmstadt, Germany). The membrane was dried overnight at 65°C. For substrates and metabolites concentrations as well as hydrolysate composition determination, the samples were centrifuged (5 min at 18.000 *g*), followed by HPLC analysis (Prominance-I LC-2030C Plus, Shimadzu, Japan) with a Refractive Index Detector RID-20A (Shimadzu, Japan) at 45°C. Rezex ROA Organic Acid column (Phenomenex, Torrance, USA) at 45°C with 5 mM sulfuric acid (>99.5%) as a mobile phase was employed for organic acids, ethanol, and glycerol measurement. Quantification of sugars, xylitol, and arabitol was performed at 85°C using a Rezex RPM Monosaccharide column (Phenomenex, Torrance, USA) and mobile phase employed LC-grade H_2O at a flow rate of 0.6 mL/min. Phenolic compounds concentration was estimated by a colourimetric method according to (Hodge *et al.*, 2009) employing different phenol concentrations for the calibration curve (0.025 – 1.0 g/L) construction. Assessment of 2-PE quantities was performed by using a Kinetex RP-C18 column (Phenomenex, Torrance, USA). UV-Visible Detector (SPD-10A, Shimadzu, Japan) at 258 nm was used for detection. The column was kept at room temperature. 35% sterile water and 65% methanol solution was used at a flow rate of 0.5 mL/min.

3.2 RESULTS

This thesis was part of a larger study screening different non-conventional yeasts in industrial birch hemicellulosic hydrolysate (de Oliveira *et al.*, 2021). The current thesis work is focussed on the detailed characterization of thermotolerant non-conventional yeast *K. marxianus* ability to consume a variety of substrates present in the hydrolysate.

3.2.1 Characterisation in shake flasks

In comparison with mineral media used in laboratory conditions, industrial lignocellulosic hydrolysates may contain a variety of growth-inhibiting compounds, including acetic acid, furfural or 5-hydroxymethylfurfural.

In order to get an estimation of *K. marxianus* behaviour in terms of C5-Birch hydrolysate inhibitors as well as to analyse the yeast growth in different hydrolysate dilutions of total sugars, shake flasks experiments were carried out. These experiments were performed to assess the dilution to be further used in the more detailed characterization in the bioreactor. The most important criterium used for the assessment of microbial growth in shake flask experiments was the specific growth rate, µ. *K. marxianus* showed an average µ of 0.13 ± 0.0 h^{-1} when grown in hydrolysate dilutions with total sugars concentration between 15 – 43 g/L. At the total sugar concentration of 58 g/L, however, µ decreased to 0.05 ± 0.0 h^{-1} (Figure 4).

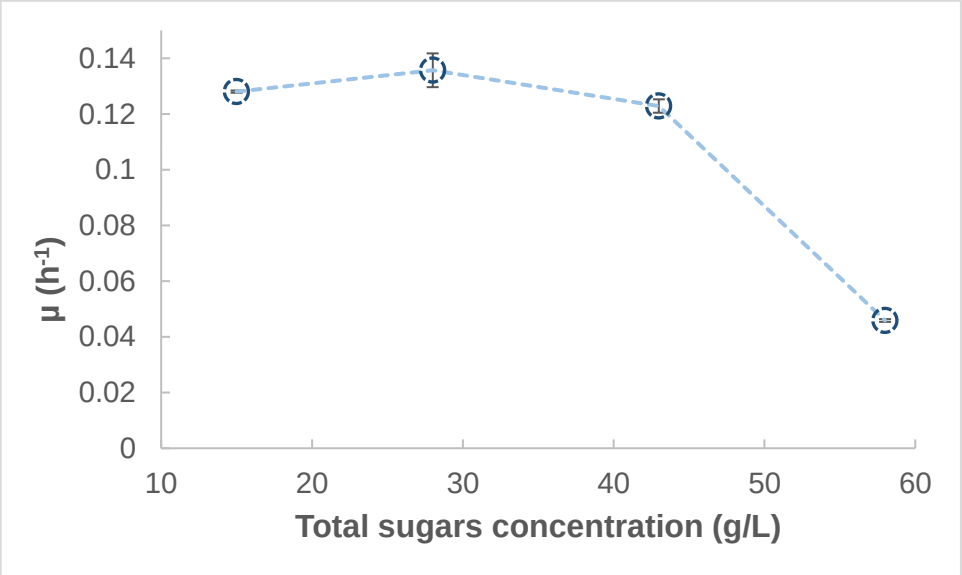

Figure 4. The specific growth rate of *K. marxianus* in varying dilutions of C5-Birch hydrolysate. Errors are expressed in standard deviation from triplicates.

To account for the more controlled environment parameters (such as constant pH and pO_2) in the bioreactors, hydrolysate total sugar concentration of 50 g/L was selected. The C/N

ratio of 5 mol/mol was chosen to avoid nitrogen limitation conditions throughout the fermentation.

3.2.2 Detailed characterisation of *K. marxianus* growth in bioreactors

The cultivation of *K. marxianus* in C5-Birch hydrolysate at the total sugar concentration and C/N ratio specified earlier was carried out in the bioreactor under a constant pH of 6.0. Despite frequently used anaerobic conditions for the production of ethanol and xylitol in *K. marxianus*, fully aerobic environment (dO_2 > 30%) was maintained throughout the cultivation. The reason for this is the higher values of xylose consumption rate in the presence of oxygen (Signori *et al.*, 2014), which were required for the comparison with other non-conventional yeast strains (de Oliveira *et al.*, 2021). Online monitoring of key parameters such as biomass formation, O_2 consumption and CO_2 production spanned the whole cultivation period. Uptake of xylose, glucose, galactose, mannose, and arabinose – the sugars present in the hydrolysate – along with acetic acid which was used as a substrate, were monitored as well. Not including 20-hour long lag phase, the growth of the yeast was divided into four distinctive growth phases based on the consumption profile of listed substrates and supporting online parameters. The first growth phase corresponded to the growth on glucose as the sole carbon source, while in the second phase mannose, galactose and the remaining glucose consumption was observed with minimal production of biomass. In the third phase, mainly acetic acid was consumed, and the last phase was characterised by xylose consumption.

Phases	Substrate consumed	Biomass (gdw/L)		Yxs (gdw/gs)		rs (g/gdw*h)		μ (h-1)	
		Average	SD	Average	SD	Average	SD	Average	SD
P1	Glucose	1.74*	0.23	0.24	0.03	-0.13	0.02	0.03*	0.00
P2	Mannose, galactose, and glucose	2.10*	0.25	0.06	0.00	-0.21	0.01	0.01*	0.00
P3	Xylose, acetic acid, mannose, and xylitol	3.50*	0.20	0.10	0.01	-0.19	0.01	0.02*	0.00
P4	Xylose, arabitol, xylitol	5.08*	0.52	0.16	0.07	-0.07	0.02	0.01*	0.00

Table 2. Yxs – biomass yield on substrate(s); rs – specific substrate(s) uptake rate; μ – specific growth rate. Errors (SD) are expressed in standard deviation unless stated otherwise. * calculated using average deviation based on duplicate measurements.

The first phase was conditionally referred to as the glucose phase due to the consumption of glucose as the only carbon source (0.13 g/gdw*h, Table 2). In spite of the specific growth rate of 0.13 h^{-1} in the shake flasks experiments, the average μ of the first growth phase in bioreactors attained its maximum of 0.03 h^{-1} and was the highest among all growth phases

observed (Figure 5B). Simultaneous CO_2 production and O_2 consumption were shown in the off-gases profile (Figure 5D). Acetic acid production was consistent with sugar consumption and was accompanied by alkali addition into the bioreactor to maintain a constant pH. A low amount of xylitol (0.21 g/L) was produced at the end of this phase.

The second growth phase consisted of the consumption of the remaining glucose (1.5 g/L) as well as other sugar carbon sources – galactose and mannose – until full depletion (Figure 5A). The acetic acid production continued in this phase. Regarding production and consumption of CO_2 and O_2, respectively, the off-gases profile showed constant composition (Figure 5D). The production of xylitol continued in this phase, reaching the maximum value of 0.44 g/L. Although the parameters mentioned above indicate active metabolism, the average specific growth rate of this phase dropped significantly (Figure 5B). Despite the fact that this phase had the highest rate of substrate uptake (0.21 g/gdw*h), the lowest biomass yield (0.06 gdw/gs) across all phases is consistent with the pronounced shift in metabolism evident from biomass and constant off-gases readings (Table 2, Figure 5A) as the shift indicates an adjustment to the changing environment, in our case change in substrates' availability.

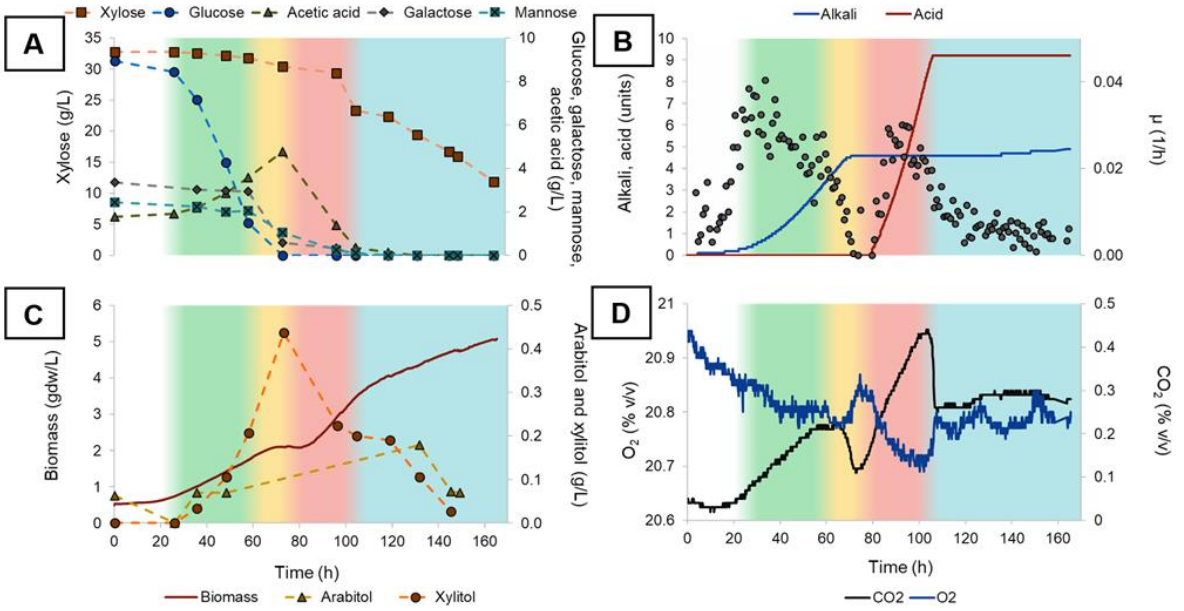

Figure 5. Growth of *K. marxianus* on C5-Birch hydrolysate diluted to 50 g/L of total sugars. (A) Consumption profile of xylose, glucose, galactose, mannose, arabinose (g/L); (B) alkali and acid addition profiles for the constant pH maintenance and average specific growth rate (μ, h^{-1}); (C) profiles of xylitol and arabitol production and consumption (g/L) and biomass growth profile (gdw/L); (D) O_2 consumption and CO_2 production profiles. White is reserved for lag phase; green, yellow, red, and blue correspond to the first, second, third, and fourth growth phases, respectively. Numerical data can be found in S2.

Phase 3 of *K. marxianus* growth included a gradual increase in xylose uptake and acetic acid consumption with the second-lowest biomass yield observed(0.10 gdw/gs). The acetic acid consumption is supported by the inflow of acid for the pH control as the pH increases when the acetic acid is consumed in hydrolysates (Figure 5A, B). CO_2 production and O_2 consumption rapidly increased in this phase together with the average μ (Figure 5B, D), the latter attaining the second-highest phases-wide value of 0.02 h^{-1}. The specific substrate uptake rate for this phase turned out to be the second-highest (0.19 g/gdw*h) across all phases. Less than 0.4 g/L of xylitol was consumed which is likely an indicator of xylose metabolism.

In the fourth phase, xylose consumption noticeably increased and together with xylitol and arabitol utilisation the rate of substrates uptake amounted 0.07 g/gdw*h, the lowest among all phases. Biomass yield on substrates, however, was the second-highest, taking the value of 0.16 gdw/gs. The average specific growth rate decreased to 0.01 h^{-1}. Consumption of O_2 and CO_2 production in the off-gases profile plummeted and almost levelled off.

No ethanol production was detected during the experiment. Up to 0.032 g/L of 2-PE was detected. As most of the substrates were consumed and the biomass slope significantly decreased, the experiment was stopped, and the total cultivation time comprised 165 hours. The final biomass achieved at the end of cultivation was 5.08 gdw/L, resulting in the final biomass yield of 0.14 gdw/gs.

In another experiment of *K. marxianus* in the same diluted hydrolysate, a single pulse of organic nitrogen source (solution of yeast extract and peptone at 10 and 20 g/L, respectively) was supplied to bioreactors at 35h of the cultivation (unpublished). Soon after the pulse, the specific growth rate reached the value of 0.11 h^{-1}, a fold change of 3.6 compared to the experiment with an inorganic nitrogen source (S1). No change in the growth profile was observed for similar a pulse containing ammonium sulphate, indicating that the *K. marxianus* growth seems to have a strong dependence on its nitrogen source.

3.3 DISCUSSION

K. marxianus, being the fastest-growing yeast with the maximum specific growth rate reaching values as high as 0.80 h^{-1} for the selected *K. marxianus* variant as reported by (Groeneveld, Stouthamer and Westerhoff, 2009), did not exhibit the same behaviour when grown on industrial C5-Birch hydrolysate. Surprisingly, the highest μ obtained by the yeast in this experiment was only a fraction of the reported, amounting to 0.03 h^{-1}, a 26-fold decrease compared to the fastest growing *K. marxianus* variant. The specific growth rate of *K. marxianus* was also the lowest among all yeast species tested in the same hydrolysate in the larger study that this thesis was part of.

The formation of inhibitors is a by-product of lignocellulosic biomass pre-treatment, although inhibitor concentration varies depending on the source. In a study by (Goshima *et al.*, 2013), the growth of *K. marxianus* on the hydrolysates of eucalyptus and Japanese cedar was assessed, where the authors concluded that the yeast poor growth on eucalyptus-based hydrolysates could be due to the higher inhibitors content. Such low specific growth rate values demonstrated by *K. marxianus* in C5-Birch hydrolysate could be explained by the higher concentration of inhibitors present in the lignocellulosic hydrolysate.

The presence of a preferred carbon source prevents the expression of genes required for the utilisation of secondary substrates - a universal microbial mechanism known as carbon catabolite repression (CCR). Carbon catabolite repression is one of the main hurdles for the efficient utilisation of the variety of substrates present in lignocellulosic hydrolysates. Glucose-induced catabolite repression was evident in the experiment using *K. marxianus* based on the presence of diauxic profile, where the uptake of other substrates took place only after glucose was considerably depleted in the environment. After the considerable depletion of glucose, galactose and mannose consumption occurred with acetic acid consumption starting halfway to the sugars' full depletion. Interestingly, considerable xylose utilisation happened only after all other sugar carbon sources and acetic acid were consumed. Alleviation of CCR was successfully demonstrated in (Kim *et al.*, 2019), where ethanol yield in a xylose and glucose co-fermenting strain improved by 84% compared to the parental *K. marxianus* strain. (Hua *et al.*, 2019) revealed in real-time PCR analysis of the transcription of the gene related to xylose utilisation that the critical gene for xylose metabolism is a xylitol dehydrogenase gene (*KmXYL2*) which is tightly regulated by glucose repression. In the same study, *K. marxianus* platform strain with enhanced glucose-xylose co-utilisation was constructed, making the yeast more suitable for use as a lignocellulosic biorefinery.

The reason for higher specific growth rate for all concentrations of total sugars in shake flasks experiments than in the bioreactors experiments can be attributed to the uncontrolled nature of cultivation in shake flasks. As the pH of the environment changes due to the external metabolite fluxes, pH can reach more suitable values than initial pH 6.0 for *K. marxianus* cultivation. (Chang *et al.*, 2014) compared *K. marxianus* growth in a range of pH values (2 – 9) and the results showed that at pH 4 and 5 the growth was the highest.

The choice of nitrogen source is one of the key factors to be considered for the successful cultivation of microorganisms. (Hua *et al.*, 2019) compared *K. marxianus* growth on various nitrogen sources for xylitol production from xylose-glucose medium, concluding that the yeast growth performance is indeed correlated to the nature of the nitrogen source used. (Rollero *et al.*, 2019) noted that *K. marxianus* genome sequence suggested one ammonium transporter with low affinity, likely explaining the nitrogen source preference. However, the costs of yeast extract and peptone in industrial cultivations should be considered as it is essential for the assessment of biorefinery economic viability. To account for financial side, defatted soybean meal (DSM), a cheaper organic nitrogen source, can be used for *K. marxianus* cultivation as it was shown to be correlated with efficient xylose consumption (Hua *et al.*, 2019).

The nitrogen source nature could also be linked to 2-PE production in *K. marxianus* as (Gethins *et al.*, 2015) reported 2-phenylethanol dependency on nitrogen source choice. The authors compared ammonia, peptone, and yeast extract for the production of 2-PE in *K. marxianus* and found that the yeast grown on yeast extract produced the most 2-phenylethanol. As was demonstrated by (Signori *et al.*, 2014), anaerobiosis or microaerobiosis are the preferred conditions for ethanol and xylitol production, *K. marxianus* produced no ethanol and only trace amounts of xylitol in the tested C5-Birch hydrolysate due to fully aerobic cultivation conditions.

SUMMARY

Lignocellulosic biomass is a plant-based renewable resource currently used for the sustainable production of bulk and fine chemicals in microbial cell factories. For the lignocellulosic biomass sugar monomers to become available for microbial growth, the pre-treatment process is employed during which depolymerisation of polysaccharides (cellulose and hemicellulose) takes place, resulting in lignocellulosic hydrolysate containing a mix of hexoses and pentoses in varying concentrations. Inhibitory compounds formation can result from pre-treatment process of lignocellulosic biomass.

A successful microbial candidate for growth on lignocellulosic hydrolysate should be able to (i) consume xylose as it is one of the main sugars present in the hydrolysates, (ii) exhibit tolerance to inhibitory compounds, (iii) have a set of well-established genetic manipulation techniques, as well as (iv) preferably possess native pathways for the production of compounds of interest. *Saccharomyces cerevisiae* is the most extensively studied yeast and quite expectedly is the host of choice in many cases of microbial biorefineries. For growth on lignocellulosic hydrolysate, however, *S. cerevisiae* lacks the ability to naturally consume xylose. Unlike *S. cerevisiae*, some non-*Saccharomyces* yeast species, known as non-conventional yeasts, are able to naturally utilise xylose as well as produce several industrially relevant compounds such as ethanol and xylitol. *Kluyveromyces marxianus* is one of the yeasts from non-conventional status.

K. marxianus performance in C5-Birch lignocellulosic hydrolysate diluted to 50 g/L of total sugars was analysed under fully aerobic conditions in bioreactor experiments. The cultivation was divided into four distinct phases based on the substrate metabolite profile and supporting online parameters (O_2 consumption, CO_2 production, biomass, alkali, and acid addition). Considered the fastest growing yeast (0.8 h^{-1}), *K. marxianus* grew surprisingly poorly in the hydrolysate, with the maximum average μ of 0.03 h^{-1}. One of the explanations for such poor growth can be the high concentration of inhibitors in C5-Birch hydrolysate. Another parameter implicated in *K. marxianus* subpar performance is the nitrogen source choice as yeast extract seems to be preferred over ammonium sulphate for their growth. Although the yeast is famous for its native volatile compounds production further used as fragrances, the amount of 2-PE produced was low (0.032 g/L), which also has its roots in the choice of nitrogen source.

K. marxianus potential as a lignocellulosic biorefinery is promising due to the yeast thermotolerance, fast growth, presence of native xylose utilisation pathway and known genetic engineering tools. However, *K. marxianus* may not be the best non-conventional

yeast for growth on C5-Birch hydrolysate due to the high concentration of inhibitors in the hydrolysate and the economic toll of the addition of yeast extract and peptone as a nitrogen source. Nonetheless, as *K. marxianus* exhibits a high level of phenotypic variability, screening of other strains may result in a naturally more suitable variant for growth on this hydrolysate. Another solution to poor performance can be sought in the strain optimisation through adaptive laboratory evolution. In addition, cheaper organic nitrogen sources such as DSM can be used to address the nitrogen costs. Moreover, genetic engineering can be used for addressing single low-affinity ammonium transporter.

ACKNOWLEDGEMENT AND FUNDING

I would like to thank Nemailla Bonturi, Petri-Jaan Lahtvee and Paola Monteiro de Oliveira for supervision, and Graanul Biotech OÜ for providing the lignocellulosic hydrolysate.

This project has received funding from the European Union's Horizon 2020 research and innovation program under Grant Agreement No 668997, and the Estonian Research Council grants PUT1488P and PRG1101.

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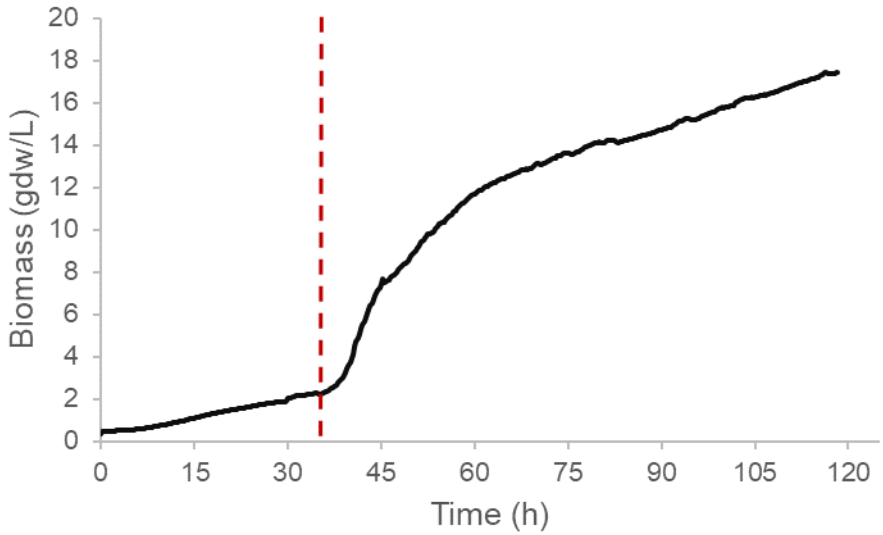
SUPPLEMENTARY



S1. *K. marxianus* cultivation in C5-Birch concentration of 50 g/L of total sugars with a pulse of yeast extract and peptone at 35h (red dashed line).

S2. Can be found in Supplementary Material as Table S3 (de Oliveira *et al.*, 2021).

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