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Co-fermentation of hexose and pentose sugars in spent sulfite liquor with genetically modified *Saccharomyces cerevisiae*

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Abstract

Spent sulfite liquor (SSL) is a by-product of pulp and paper manufacturing, and has called scientific attention as substrate for second generation bioethanol production. In order to enhance ethanol production from lignocellulosic biomass, efficient co-fermentation of hexose and pentose sugars in a robust organism is crucial. The *Saccharomyces cerevisiae* strain IBB10B05 presented herein was enabled to xylose fermentation by metabolic pathway engineering involving the introduction of xylose reductase (XR) and xylulose dehydrogenase (XDH), and laboratory evolution. Further improvement was achieved by balancing co-enzyme utilization between the XR and XDH reaction by genetic modification of XR. The application of such a strain in SSL fermentation is novel. Two SSLs were analyzed in this study; SSL-Thin (14 % (w/v)) and SSL-S2 (30 % (w/v)), and it was possible to ferment 70 % (v/v) of both SSL without detoxification. Ethanol yields varied between 0.31 and 0.39 g ethanol per g total sugar. Besides furfural and HMF, acetic acid is one of the major inhibitors in SSL, and it was found to have a negative impact on xylose fermentation rates in IBB10B05. Fermentation in a bioreactor with controlled pH close to 7 diminished the effect of acetic acid, significantly enhanced xylose conversion rates and increased ethanol yields. Besides ethanol, glycerol and xylitol are the most abundant fermentation by-products, and it was found that there is a correlation between this two redox sink products and the xylose uptake rate. An increase in xylose uptake velocity is accompanied by an increase in xylitol and a decrease in glycerol yields, whilst the sum of molar yields remains constant regardless of the fermentation setup.

Kurzfassung

Sulfit-Ablaugen sind ein Nebenprodukt der Holz- und Zellstoff-Industrie. Bei der Suche nach erneuerbaren Energien haben sie als Substrat für die Bioethanol-Herstellung wissenschaftliches Interesse geweckt. Ausschlaggebend für eine effiziente und rentable Umsetzung von Lignocellulose zu Ethanol ist die Co-Fermentation von Hexosen und Pentosen. Die Xylose-Fermentation in dem hier präsentierten *Saccharomyces cerevisiae*-Stamm IBB10B05 wurde durch „metabolic pathway engineering“ vor allem durch die Einführung der Enzyme Xylose-Reduktase (XR) und Xylulose-Dehydrogenase (XDH) und durch gerichtete Evolution ermöglicht. Weiters wurde das Enzym XR genetisch so verändert, dass die Co-Enzym-Präferenz der von XDH entspricht. Die Verwendung eines solchen Stammes für die Umsetzung von Sulfit-Ablaugen zu Ethanol ist neu. Zwei verschiedene Ablaugen wurden in dieser Studie verwendet, Dünnlauge (14 % (w/v)) und Stufe-2-Lauge (30 % (w/v)). In beiden Fällen war es möglich, 70 % (v/v) der jeweiligen Laugen ohne vorherige Detoxifizierung mit Ethanol-Erträgen zwischen 0.31 und 0.39 g Ethanol/g Gesamt-Zucker umzusetzen. Neben HMF und Fufural, stellte sich Essigsäure als größter Inhibitor heraus, der vor allem die Geschwindigkeit der Xylose-Umsetzung negativ beeinflusst. Eine Verminderung der toxischen Wirkung von Essigsäure wurde durch die Verwendung von Bioreaktoren erzielt. Fermentationen unter kontrollierten Bedingungen mit konstanten pH-Werten um 7 zeigten sowohl gesteigerte Xylose-Umsetzungsraten als auch erhöhte Ethanol-Ausbeuten. Neben Ethanol sind Glyzerin und Xylitol die wichtigsten Nebenprodukte. Unabhängig von dem Versuchsaufbau wurde ein

Zusammenhang zwischen deren Erträgen und der Xylose-Aufnahmerate festgestellt. Je schneller die Xylose aufgenommen wird, desto mehr Xylitol und desto weniger Glyzerin wird produziert, wobei die Summe der molaren Erträge konstant bleibt.

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1. Introduction

Environmental problems and the issue of limited fossil fuel resources have called attention from public, politics and research all over the world. Amongst others, major objectives are containment of climate change and quest a way to fuel the world in future. Therefore a sustainable, environmentally compatible and economically feasible substitute has to be found and research has to aim towards large scale production. One promising renewable energy source is second generation bioethanol, produced from lignocellulosic biomass such as municipal, agricultural or forestry waste. Lignocellulosic materials contain two major fractions, hemicellulose and cellulose, both consisting of polymerized hexose and pentose sugars which can be liberated by hydrolysis and subsequently fermented to ethanol (for a review see Gírio et al., (2010)).

In this study two lignocellulosic hydrolysates are presented. Firstly spent sulfite liquor (SSL), which is a by-product of the chemical pulping process. Secondly wheat straw hydrolysate, which has been already successfully applied for hemicellulose-to-ethanol processes in a previous study (*unpublished results*), and is used as carbohydrate supplementation for SSL fermentation in this study.

As a waste stream, SSL has the advantages of being abundantly available and low-priced. Consequently SSL has been employed for ethanol generation for a long time, and research has been accomplished since the 1980s (Holderby and Moggio, 1960, Björling and Lindman, 1989, Safi et al., 1986, Yu et al., 1987, Helle et al., 2004). The pulping process includes the treatment of wood with sulfurous acid and magnesium bisulfite which solubilises lignin and part of the

hemicellulose fraction, and leaches it out. It is a low yield process but provides high purity of cellulose, which then is further processed to pulp or paper. After recovery of the chemicals the liquor contains a high fraction of organic compounds including sugars which are released during acid treatment. There are several drawbacks in SSL fermentation such as low sugar content and variations in hexose and pentose composition. The portion of pentose sugars present in SSL is dependant on the type of wood used for pulp manufacturing. When hardwood is utilized, the SSL contains a high fraction of xylose because the xylan is degraded quite easily in acid environment. In contrast, softwood is highly acid resistant, and the SSL contains more hexose sugars (Helle et al., 2007, Helle et al., 2004).

In order to facilitate efficient conversion of SSL to ethanol, robust and reliable co-fermentation of xylose and hexoses must be enabled. Naturally pentose fermenting yeast strains utilized for SSL fermentation include the strains *Candida shehatae* (Yu et al., 1987) and *Pichia stipitis* (Björling and Lindman, 1989). However, those yeast strains are neither well adapted to ethanol production nor can they tolerate the harsh conditions present in SSL (Helle et al., 2004). In contrast, *Saccharomyces cerevisiae* combines several desired attributes such as high ethanol tolerance, robustness and operation experience, and is therefore still favoured when it comes to industrial scale ethanol production. Although *S. cerevisiae* is traditionally used for SSL fermentation, the success is restrained by the inability to naturally ferment pentose sugars (Holderby and Moggio, 1960, Helle et al., 2004). To overcome this major

bottleneck and combine the robustness of *S. cerevisiae* with the desired xylose conversion, genetic modification proved to be an adequate method.

The *S. cerevisiae* strain IBB10B05 presented herein is enabled to xylose utilization via the XR/XDH pathway, which is based on the introduction of the enzymes xylose reductase (XR) and xylitol dehydrogenase (XDH). It is a two step process in which xylose is converted to xylulose via the intermediate xylitol. Subsequently, xylulose can enter the pentose-phosphate-pathway via xylulose-5-phosphate (Chu and Lee, 2007, Matsushika et al., 2009). Until recently the success of the XR/XDH pathway was limited by high xylitol formation at the expense of ethanol yields. Reasons for this are found in the unequal co-factor preferences; conventionally XR is NADPH preferring and XDH is NAD^+ depending (Petschacher et al., 2005). This disparity can lead to redox imbalances and result in high xylitol yields (Petschacher and Nidetzky, 2008). In order to diminish this effect, the co-factor preference of XR was altered via structure-guided site-directed mutagenesis towards NADH, and the resulting *S. cerevisiae* strain BP10001 showed excellent co-factor recycling (Klimacek et al., 2010, Krahulec et al., 2010). IBB10B05 was derived from BP10001 via evolutionary engineering which was successfully applied to enhance xylose conversion rate (*unpublished results of the parent strain*). IBB10B05 displays efficient co-fermentation of xylose and glucose at high ethanol yields and lowered by-product formation (*unpublished results*).

Despite of the excellent performance of IBB10B05 in defined glucose and xylose containing substrates, the conditions in SSL fermentations are noteworthy different. SSL contains a high fraction of inhibitors such as furans,

lignosulfonic acids, acetic acid, sulfate and sulfuric dioxide (Björling and Lindman, 1989, Helle et al., 2004, Yu et al., 1987). To facilitate SSL fermentation nonetheless, conventional plants for ethanol production from SSL include a detoxification step (Björling and Lindman, 1989). The concentrations of volatile inhibitors are reduced e.g. by precipitation, overliming or steam stripping (Björling and Lindman, 1989, Yu et al., 1987). Since ethanol is competitive only when sold at a low price, production cost must be kept to a minimum. Thus avoidance of additional steps such as detoxification might contribute to the success of SSL-to-ethanol processes.

In this study, it was possible to efficiently co-ferment xylose and glucose to ethanol utilizing the genetically engineered and laboratory evolved IBB10B05 despite of the harsh conditions present in SSL.

2. Materials and Methods

2.1. Yeast strains

Fermentations were either accomplished with the xylose fermenting *S. cerevisiae* strain IBB10B05, which was previously achieved at the Institute of Biotechnology and Biochemical Engineering, Technical University of Graz, by metabolic and evolutionary engineering (*unpublished results*) or with an industrial wine yeast.

2.2. Materials

SSL with different contents of solids (14 % (w/w) termed SSL-Thin, 30 % termed SSL-S2) was kindly donated from SAPPI, Gratkorn (Austria). Whilst the

pH of SSL-S2 already had the desired starting pH of 6.8, the pH of SSL-Thin was adjusted to 6.8 with magnesium oxide also donated from SAPPI, Gratkorn. All fermentations were carried out anaerobically at 30 °C. The experimental set up was chosen to be 70 % (v/v) SSL, 10 % media supplements, 10 % carbohydrate supplements and 10 % space for inoculation. Three media supplements for fermentations were tested: a) mineral medium as described in Krahulec et al. (2010); b) complex medium (1 % (w/v) yeast extract, 2 % peptone); c) yeast extract (1 % (w/v)). Experiments with supplemented carbohydrates were accomplished with a) 1.4 % (w/v) glucose and 5 % xylose, b) 1.4 % (w/v) glucose, or c) wheat straw hydrolysates. Starter cultures consisted of either a) mineral medium, b) YPG or c) 70 % (v/v) SSL-S2, 2 % (w/v) glucose, 5 % (w/v) xylose and 1 % (w/v) yeast extract termed as “SSL” starter culture. SSL was autoclaved for 10 min to minimise changes in the composition; carbohydrate and media supplementations were autoclaved separately. For the production of hydrolysate, the wheat straw was pre-treated with steam explosion and 15 % dry mass were hydrolyzed by cellulases gained from *Trichoderma reesei* and additional beta-glucosidase. The activity of the enzymes was chosen as follows: 20 FPU and 10 CBU per g dry mass were added at the beginning of the reaction and another 5 FPU/g dry mass after 20 hrs. The pH was 4.8 and 10 mM acetate buffer were added to keep the pH constant during hydrolysis. The straw and the buffer were autoclaved and the enzyme solution filtrated sterile. The reaction was incubated for 48 hrs, 50 °C and 200 rpm in an incubator. Afterwards the hydrolysate was heated to 100 °C for 15 min to stop the reaction. Remaining solids were removed by

centrifugation (DuPont Instruments, Sorvall RC-5B, 4420 g, 10 min, and 4 °C), and the supernatant was concentrated by evaporation to one tenth of its original volume. Anaerobic conversions were carried out in glass bottles tightly sealed with rubber septa (90 ml working volume). To ensure anaerobic conditions, the bottles were sparged with N_2 previous to and shortly after inoculation. After inoculation the bottles were kept in an incubator (Certomat BS-1, Sartorius mechatronics, Germany) at 30 °C and 180 rpm. Bioreactor fermentations were conducted in a Labfors III bioreactor (2000 ml working volume, InforsHT, Bottmingen, Switzerland) equipped with an Innova 1313 acoustic online off-gas analyser for detection of evaporated ethanol and CO_2 (LumaSense Technologies A/S, Ballerup Denmark). The medium was constantly sparged with N_2 (0.26 cm^3/min) and stirred at 200 rpm. The pH was kept constant at 6.8.

2.3. Anaerobic conversions

IBB10B05 was stored at -70 °C in glycerol stocks that were initially plated on YPG-agar (1 % (w/v) yeast extract, 2 % peptone, 2 % glucose, 1.5 % agar). The yeast was aerobically grown in 50 ml starter culture over night at 30 °C and 180 rpm. This culture was used to inoculate a 300 ml seed culture, which was incubated at the same conditions. The cells were harvested in the exponential phase by centrifugation (DuPont Instruments, Sorvall RC-5B, 4420 g, 20 min, and 4 °C). The cell pellet was washed and resuspended with physiological sodium chloride solution (0.9 % NaCl). Bioconversion experiments were inoculated to a starting OD_{600} of approximately 5. Cell growth experiments were

accomplished with SSL-S2 supplemented with glucose, xylose and yeast extract, inoculated with an OD_{600} of 0.5 and 5 from a YPG starter culture.

2.4. Sampling, analysis of metabolites and calculations

Samples of 1.5 ml were frequently removed from the fermentation broth with syringes and centrifuged for 10 min at 15700 g and 4 °C (Eppendorf Centrifuge 5415 R) .The supernatant was stored at -20 °C for HPLC analysis. Extracellular fermentation products (ethanol, xylitol, glycerol, acetate) where quantitatively analysed according to Petschacher and Nidetzky (2008). HMF and furfural were analysed with the same method. An example of a chromatogram can be found in the supplementary information (supplementary information A page 1). The carbohydrates were analysed with the same HPLC system, but equipped with an Aminex HPX-87P column and a de-ashing guard column (both Bio-Rad, Richmond, CA, USA). Operation temperature was 80 °C; the mobile phase was deionised water with a flow rate of 0.4 ml/min. For an example see the supplementary information (supplementary information A page 2). Calculations of yields were accomplished as described previously (Krahulec et al., 2010).

2.5. Cell growth determination

Conventional methods of biomass detection such as photometrical measurement of increase in optical density at 600 nm were not applicable due to changes in colour and content of solids during fermentation. To determine cell growth in SSL nevertheless, the total cell number was defined via a Thoma counting chamber. In order to translate the total cell number into an OD_{600}

value, a correlation between cell number and OD_{600} was established. For this purpose, samples from YPG cultures were simultaneously examined towards the OD_{600} and the total cell number. A linear correlation between an OD_{600} of 0.1 and 12 was found.

3. Results

In this study ethanol production from two different spent sulfite liquors, SSL-Thin and SSL-S2, with the genetically modified *S. cerevisiae* strain IBB10B05 was assessed. When initially analysing both SSLs, the low sugar content (depicted in Table 1) became obvious. Reasons for this are found in process management and in the type of wood used for pulp manufacturing. However, SSL derived from the same pulping process at other locations show higher sugar concentrations (Helle et al., 2004). To mimic these conditions, the SSL fermentation presented in this study was accomplished with carbohydrate supplementation in concentrations similar to those published by Helle et al. (2004).

3.1. Comparison of media supplementations

At first all fermentations were amended with mineral medium which provides optimal conditions for yeast. For economical reasons media supplementation was replaced in two steps, firstly by a combination of yeast extract and peptone, and secondly by yeast extract only. Table 2 shows the influence of the different nutrient supplementations on the yields of fermentation products. In both SSLs there is only a marginal difference between the fermentations supplemented

with mineral medium and with yeast extract and peptone (Table 2). When fermenting with yeast extract only, the glycerol yield in SSL-Thin stays constant whilst the xylitol yield is 20 % higher as in fermentation supplemented with mineral medium. In SSL-S2, replacement of mineral medium by yeast extract resulted in reduced glycerol yields (around 18 %) and enhanced xylitol yields (also around 18 %). Figure 1 shows the influence of altering media supplementation from mineral medium to yeast extract on the course of fermentations of SSL-Thin (panel A/B – 1) and SSL-S2 (panel A/B – 2), respectively. In all fermentations the glucose is depleted within the first 10 hours, and for reasons of clarity the glucose phase is shown separately (supplementary information A page 3). In SSL-Thin fermentation, xylose uptake is unchanged and glucose uptake is slightly slower in yeast extract supplemented fermentation. Fermentation of SSL-S2 supplemented with yeast extract shows reduced velocity of xylose and glucose uptake. The time needed to consume 80 % of the xylose is prolonged form 80 to 145 hours and depletion of glucose takes 2 hours longer. Ethanol yields in SSL-S2 as well as SSL-Thin fermentations supplemented with yeast extract are equal or higher compared to fermentations accomplished with mineral medium or with yeast extract and peptone. Because of the positive effect on ethanol yields and for economical reasons, yeast extract was found to be advantageous for SSL fermentations.

3.2. Comparison of IBB10B05 and an industrial wine yeast

In order to assess the robustness towards the harsh conditions present in SSL, the fermentation performance of IBB10B05 was compared with an industrial

wine yeast strain. In fermentations with xylose and glucose supplemented SSL, IBB10B05 produced up to 75 % more ethanol compared to the industrial yeast strain (*data not shown*). The improvement in ethanol production could be achieved by enabling xylose fermentation in IBB10B05, and shows how important xylose fermentation is for second generation bioethanol production. To have a direct comparison between the fermentation performance of IBB10B05 and the industrial yeast strain, SSL was supplemented with 14 g/L glucose as sole carbohydrate source. The amount of ethanol produced in the first 24 hours of fermentation is depicted in Figure 6. In the first 2 hours of SSL-Thin fermentation, IBB10B05 produced less ethanol than the industrial wine yeast, probably indicating a prolonged lag phase. However, from 4 hours until 24 hours of fermentation the ethanol concentration is similar for both yeast strains. In SSL-S2 fermentation the same effect is shown more clearly. IBB10B05 shows a slower glucose conversion rate and 20 hours are needed to produce an ethanol concentration of approximately 5.5 g/L, which is reached after 4 hours in fermentation with the industrial yeast strain. However, in all fermentations the concentration of ethanol after 25 hours is about 5.8 g/L, except for SSL-S2 fermentation utilizing IBB10B05 where the ethanol concentration after 25 hours is 6.2 g/L. This is probably due to conversion of the higher amount of xylose present in SSL-S2 compared to SSL-Thin.

3.3. Comparison of SSL-Thin and SSL-S2

The two SSLs are derived from the same plant but are taken from different process stages. As depicted in Table 1, SSL-Thin is less concentrated than

SSL-S2, and therefore contains a lower amount of solids and a reduced concentration of toxic compounds such as acetic acid. However, SSL-S2 contains a higher fraction of carbohydrates. Despite of the higher toxicity, ethanol yields in SSL-S2 fermentations are slightly higher compared to SSL-Thin fermentation (Table 2). Comparing the fermentations with the respective SSL, the yields for xylitol and glycerol as well as the xylose uptake velocities are varying (Table 2; Figure 1). Fermentation with SSL-S2 resulted in 0.10 g glycerol/g total sugar on average, which is about 30 % higher than glycerol yields in SSL-Thin fermentations. In contrast, xylitol yields were about 56 % lower (0.08 g/g total sugar). The sum of molar yields of glycerol and xylitol (mol glycerol or xylitol/mol total sugar) is approximately equal for all fermentations regardless of the respective SSL. Xylose is faster consumed in SSL-Thin fermentations, and the time needed for depletion of 80 % of xylose is 75 hours on average. This is significantly faster than in SSL-S2 fermentations where it takes approximately 97 hours. Glucose uptake velocities are slower in fermentations with SSL-S2, but the difference is less significant than it is in xylose uptake velocities (supplementary information A page 3). Due to higher ethanol yields, and since glycerol is advantageous to xylitol formation due to less carbon loss, SSL-S2 was considered to be favourable for SSL fermentation with IBB10B05.

3.4. SSL-S2 fermentation

3.4.1. Influence of starter culture

In order to adapt IBB10B05 to the harsh conditions present in SSL, starter cultures were performed with SSL-S2 in similar concentration as in subsequent fermentations. The influence of the different starter cultures on fermentation is shown in Table 2. Fermentations inoculated with SSL starter cultures resulted in 0.31 g ethanol/g total sugar, which is 9 % less than ethanol yields from fermentations with SSL starter cultures (0.35 g/g total sugar). In contrast, more glycerol (9 %) as well as xylitol (11 %) is formed. In Figure 2, the influence of the different starter cultures on the glucose and xylose phase are shown. In the range of error it can be concluded that the alteration of pre-culture did not have an effect on the yields in the glucose phase. However, in the xylose phase more by-products are formed at the expense of ethanol yield when fermenting with cells grown in SSL containing media. Since the ethanol from glucose yields is unchanged, the loss in ethanol yield (g ethanol/g total sugars) must be caused by the ethanol from xylose yield (g ethanol/g xylose).

3.4.2. Bioreactor fermentations

In Figure 3 the time courses of SSL-S2 fermentation in bioreactor (panel A) and sealed glass bottle (panel B) are compared and the corresponding yields are shown in Table 2. In bioreactor fermentations ethanol yields reached 0.38 g/g total sugar on average, which is approximately 10 % higher compared to similar fermentations carried out in the rubber septa sealed bottles. By-products yields are 0.03 g glycerol/g total sugar and 0.20 g xylitol/g total sugars on average,

which represent the highest xylitol and the lowest glycerol yield of all fermentations presented in this study (Table 2). In contrast to glucose, which is consumed almost at the same velocity (supplementary information A page 4), xylose is significantly faster depleted in bioreactor than in bottle fermentation. After 50 hours more than 90 % of the xylose is consumed, which is the highest xylose uptake velocity observed in this study. Fermentation in bioreactor under pH controlled conditions close to 7 proofed to be an efficient way to enhance ethanol production and accelerate xylose fermentation.

3.5. Influence of xylose uptake on distribution of xylitol and glycerol yields

When comparing the time courses of Figure 1 and 3, a common trend is observed. In both cases faster xylose conversion velocities are accompanied by enhanced xylitol yields and lower glycerol yields. The correlation between the xylose uptake velocity and the glycerol or xylitol yields respectively is shown in Figure 5. Accordingly, the time (T) which is required for consumption of 80 % (w/w) of the xylose is clearly corresponding with the xylitol yield (g xylitol/g xylose $_{\text{consumed until T}}$). The same applies for the glycerol yields (g xylitol/g xylose $_{\text{consumed until T}}$), which also corresponds with the xylose uptake velocity but in an inverse relationship. The correlations depicted in Figure 5 are established throughout all fermentations regardless of the fermentation setup. When transforming the glycerol and xylitol yields into molar yields (mol/mol total sugars), the sum of both is approximately equal for all fermentations (within an error of 10 %).

3.6. Fermentation of SSL supplemented with wheat straw hydrolysate

Enhancing carbohydrate concentration in SSL is considered to be one step towards successful SSL-to-ethanol processes, and one way to achieve this is to merge two or more carbohydrate rich waste streams (Helle et al., 2004, Helle et al., 2007). Wheat straw hydrolysate was regarded to be a promising substrate and was already successfully converted into ethanol by IBB10B05 (unpublished results). In this study, the wheat straw hydrolysate containing 20 g/L glucose and 10 g/L xylose were added to SSL-S2 (Figure 4, Panel A) and SSL-Thin (Figure 4, Panel B). In fermentation with SSL-Thin, the glucose is depleted within ten hours; the xylose is consumed within 50 hours. After 140 hours of fermentation 0.45 g ethanol/g total sugar is produced (Table 2). In comparison to SSL-Thin, glucose is consumed at a significantly slower velocity in fermentation with SSL-S2 (within 50 hours) and xylose is only consumed marginally. The reduced speed of substrate conversion in SSL-S2 in contrast to SSL-Thin fermentations supplemented with wheat straw hydrolysate is an indicator for a higher level of inhibition.

4. Discussion

4.1. Fermentation of undetoxified and carbohydrate supplemented SSL

All fermentations in this study were carried out without detoxification. Compounds known to inhibit fermentation of lignocellulosic substrates are, besides furan derivates, phenolic compounds and weak acids (Palmqvist and Hahn-Hägerdal, 2000). In order to minimize the concentration of toxics in SSL, it is often treated prior to fermentation (Amartey and Jeffries, 1996, Helle et al.,

2008, Martinez et al., 2000, Yu et al., 1987). Procedures include steam stripping (Björling and Lindman, 1989, Yu et al., 1987), overliming (Helle et al., 2008) or selective reduction of acetic acid by fermentation (Schneider, 1996). However, in terms of industrial scale production, pre-treatment may not prove to be feasible. An additional process step and problems such as disposal of the gypsum generated by overliming are contributing to the overall production costs which must be kept to a minimum to make bioethanol a competitive fuel source (Björling and Lindman, 1989, Helle et al., 2008). Thus fermentation without detoxification is advantageous in terms of process costs, but due to the inhibitory nature of SSL, it is a difficult substrate for yeast (Helle et al., 2003). The most abundant compounds present in the SSL utilized in this study are acetic acid, HMF and furfural, and the influence of these inhibitors on fermentation success is discussed in detail later on. Fermentation of 70 % (v/v) undetoxified SSL with 50 g/L xylose and 14 g/L glucose supplementation yielded in 0.31 to 0.39 g ethanol/g total sugar. This represents 61 to 77 % of the theoretical yield which is 0.51 g ethanol/g total sugars (xylose and glucose). In general ethanol yields obtained from co-fermentation of glucose and xylose in SSL is considered to be governed by ethanol on xylose yields. Because unlike glucose, xylose conversion has a higher liability to inhibition by toxics such as furals or acids (Helle et al., 2003). Consistently, xylose conversion velocities in this study are slower in SSL-S2 fermentations because SSL-S2 contains twice as much solids, considerably more acetic acid and more furfural than SSL-Thin. In carbohydrate and yeast extract supplemented SSL-S2 the biomass growth was negligible. The cells grew only while glucose was present and divided just

once independent of the starting OD_{600}. Inhibited cell growth is a commonly observed effect in fermentation of SSL (Safi et al., 1986, Helle et al., 2003, Schneider, 1996) as well as other lignocellulosic biomass (Lindén and Hahn-Hägerdal, 1989, Palmqvist and Hahn-Hägerdal, 2000). Notwithstanding, fermentation of SSL-S2 resulted in higher ethanol yields compared to fermentation of SSL-Thin. Thus the higher content of inhibitory compounds in SSL-S2 is influencing cell growth and xylose uptake velocities, but not ethanol yields. This is consistent with previous studies, where growth rates, biomass yields and substrate conversion rates are more significantly affected by inhibitors than overall ethanol yields (Casey et al., 2010, Helle et al., 2003, Helle et al., 2008, Palmqvist and Hahn-Hägerdal, 2000). In comparison to the industrial wine yeast, IBB10B05 showed a prolonged lag phase, an effect also observed in a previous study (Helle et al., 2004). Wine yeast from industrial plants can tolerate low nutrient conditions and have a high tolerance towards SO_2 (Helle et al., 2004), and are probably more robust towards the inhibitors present in SSL. However, after a short time fermentation of glucose in SSL with IBB10B05 produced equal or slightly higher amount of ethanol.

4.2. Influence of inhibitory compounds on SSL fermentation

4.2.1. Acetic acid: a major inhibitor of xylose conversion

In this study varying xylose uptake velocities are observed when comparing SSL-Thin with SSL-S2 fermentations (Figure 1), glass bottle with bioreactor fermentations (Figure 3) and glucose and xylose supplemented fermentations with wheat straw hydrolysate supplemented fermentations (Figure 4). As

depicted in Figure 5, the time needed to consume 80 % (w/w) of the provided xylose can range from 31 hours to 150 hours.

Since xylose conversion is susceptible to inhibition, the slower fermentation rate of xylose in SSL-S2 indicates that it has a higher inhibitory effect than SSL-Thin. The inhibitory compound which is most abundantly present in both SSL is acetic acid. However, with 9 g/L the concentration is almost twice as high in SSL-S2 as it is in SSL-Thin (4.7 g/L). The inhibitory effect of acetic acid is thought to be based on the undissociated form which can pass the cell membrane causing a drop of internal pH (Casey et al., 2010, Pampulha and Loureiro-Dias, 1989). The negative influence of undissociated acetic acid on xylose consumption rates was previously shown by Casey et al., (2010) who studied mixed glucose-xylose fermentation with XR/XDH containing *S. cerevisiae*. The pK_a of acetic acid is 4.75 and all fermentations presented in this study had an initial pH of 6.8. Thus over 99 % of the acid is present in the less toxic dissociated form, diminishing some of the inhibitory impact. During one week of fermentation the pH fell about one unit and the concentration of acetic acid increased to 12.6 g/L and 7.6 g/L for SSL-S2 and SSL-Thin, respectively. An acetic acid concentration of 12.6 g/L at pH 5.8 represents 1 g/L undissociated acid, which can significantly decrease the xylose consumption rate (Casey et al., 2010, Helle et al., 2003, Helle et al., 2008). Inhibition of xylose uptake velocities at least partly caused by the undissociated form of acetic acid can be also observed in the results depicted in Figures 3 and 4. When fermentations are run in a bioreactor under controlled conditions, the pH is kept constant close to 7 and acetic acid cannot accumulate in its undissociated form. Under these

conditions, the xylose uptake velocities are considerably faster than similar fermentations carried out in glass bottles. This supports the assumption that the undissociated from is responsible for the inhibitory impact of acetic acid (Casey et al., 2010, Pampulha and Loureiro-Dias, 1989), and a positive effect of higher pH on xylose fermentation in SSL was also observed in previous studies (Helle et al., 2008, Helle et al., 2004).

In SSL-Thin fermentation supplemented with xylose and glucose, 40 g/L xylose is depleted within 89 hours. In fermentations of solely wheat straw hydrolysate (from 10 % dry mass, 70 % (v/v)) utilizing IBB10B05, 40 g/L xylose is depleted within approximately 50 hours (unpublished results). When fermenting SSL-Thin (70 % (v/v)) in combination with wheat straw hydrolysate (80 % (v/v)) the same time (50 hours) is needed to consume 8 g/L xylose. The reason for the reduced velocity of xylose conversion might be found in the additional amount of acetic acid (approximately 1 g/L) and the low pH (4.8) of wheat straw hydrolysate, enhancing the concentration of undissociated acetic acid in SSL-Thin. This effect is more significant in combination with SSL-S2, where the total concentration of acetic acid reaches 10 g/L and only a marginal amount of xylose it consumed in one week of fermentation. Comparable results were observed in the study of Helle et al. (2007) where SSL fermentation with XR/XDH containing *S. cerevisiae* was fortified with hydrolyzed knot wood. In solely knot hydrolysate xylose and glucose are rapidly converted to ethanol, but in combination with SSL (16- 20 % (v/v)) the xylose uptake rates are reduced.

4.2.2. By-product formation: the role of furfural, HMF and the content of solids

Another difference between the varying fermentation setups is the unequal pattern of by-product formation. The distribution of xylitol and glycerol yields in SSL fermentations utilizing IBB10B05 is influenced by two factors. Firstly by-product distribution seems to be governed by xylose uptake velocity (this study). The assumption is based on the correlation depicted in Figure 5, and is described in detail later on. Secondly the difference in glycerol and xylitol yields can be ascribed to the different concentrations of inhibitory compounds found in SSL-S2 and SSL-Thin, respectively. In SSL-S2 fermentation glycerol yields were 0.10 g glycerol/g total sugar on average (Table 2) which is comparable to previous studies (Helle et al., 2004, Lindén and Hahn-Hägerdal, 1989, Olsson and Hahn-Hägerdal, 1996) but higher than in SSL-Thin fermentations (0.07 g glycerol/g total sugar). Glycerol is formed to offset redox imbalances (Taherzadeh et al., 1997). It is known to increase at the presence of osmotic stress (Rapin et al., 1994) and decrease at the presence of furfural and HMF (Taherzadeh et al., 1999). Furfural and HMF can be formed from pentoses and hexoses when hemicellulose is degraded at high temperatures and pressure (Dunlop, 1948, Palmqvist and Hahn-Hägerdal, 2000). Both compounds are thought to have a similar mechanism of inhibition (Palmqvist and Hahn-Hägerdal, 2000, Taherzadeh et al., 1999), but whilst furfural has a higher inhibitory impact on fermentation, it is also faster metabolized by *S. cerevisiae* than HMF (Taherzadeh et al., 2000). In *S. cerevisiae*, furfural is reduced to furfuryl alcohol via the enzyme NADH-dependant alcohol dehydrogenase (Palmqvist et al., 1999). Palmqvist et al., (1999) postulated that this reaction is

competing with the reduction of dihydroxyacetone phosphate to glycerol. Thus, in presence of furfural, glycerol yields are lower compared to fermentation without furfural (Palmqvist et al., 1999). SSL-Thin contains more furfural leading to the assumption that this causes a decrease in glycerol formation. However, neither furfural nor HMF do exceed 0.5 g/L, which is beyond the published concentrations of HMF with 1 g/L (Taherzadeh et al., 2000) and 10mM – 30mM for furfural (Liu et al., 2004, Palmqvist et al., 1999). Further, large inoculums as presented here (start OD_{600} about 5) are decreasing the inhibitory impact of furfural and HMF due to faster depletion through metabolism (Helle et al., 2008). The differences in glycerol yields between the fermentations of the respective SSL-S2 might be also caused by the solids present in SSL. SSL-S2 contains twice as much solids as SSL-Thin, which enhances the osmotic stress. This might lead to higher glycerol production in order to ensure maintenance of internal osmotic balance (Rapin et al., 1994). A simultaneous increase in SSL solid content and glycerol production was also observed by Helle et al., (2004). Helle and co-workers observed a glycerol yield of 0.21 g glycerol/g total sugar when fermenting SSL with 30 % (w/w) solids, which is considerably higher than the glycerol formation observed in this study.

Xylitol yields are also varying between SSL-Thin (0.18 +/- 0.02 g xylitol/g total sugar) and SSL-S2 fermentations (0.08 +/- 0.02 g xylitol/g total sugar). Xylitol formation is known to decrease at the presence of electron acceptors (Wahlbom and Hahn-Hagerdal, 2002). In SSL fermentation, acetaldehyde and furfural are the most common electron acceptors, and they can act as redox sink (Wahlbom and Hahn-Hagerdal, 2002). However, sulfite present in its bisulfite form can

bind those electron acceptors and diminish some of its impact (Helle et al., 2004).

4.3. The influence of xylose conversion velocity in IBB10B05 on by-product distribution

A novelty in this study is that the by-product distribution seems to be connected to the rate of xylose uptake. The established correlation is depicted in Figure 5 which shows a clear dependence of xylitol (Panel A) and glycerol (Panel B) yields on xylose uptake rate, which is here circumscribed with the time required for consumption of 80 % of the xylose. Whereas xylitol yields are increasing with faster xylose uptake, glycerol yields are decreasing. The correlation was established with data from all fermentations presented in this study, and is therefore independent from the respective SSL or the fermentation setup. Further, the sum of redox sink products (mol glycerol and xylitol/ mol total sugar) throughout all fermentations was found to be constant. This suggests that the xylose uptake velocity influences the distribution of xylitol and glycerol yields, but has no influence of how much redox sink products are produced in total.

Shift in by-product formation, most importantly towards enhanced xylitol yields, are reported for xylose fermentation with XR/XDH containing *S. cerevisiae*, and are based on redox imbalances caused by the two step transformation of xylose to xylulose (Anderlund et al., 2001, Helle et al., 2004). Enhanced xylitol formation due to redox imbalances in IBB10B05 xylose metabolism is unlikely, since the co-factor preference of XR and XDH are balanced, and co-factor

recycling works well (Krahulec et al., 2010). However, due to the laboratory evolution the activity of XR in IBB10B05 was enhanced significantly (*unpublished results*), and the catalytic efficiency of XR might have been improved in a way that it exceeds the catalytic abilities of XDH. Although this explanation is speculative, it would account for the increased xylitol yields in fermentations where xylose uptake rates are high. The importance of XDH to be over expressed was already highlighted in an earlier publication (Eliasson et al., 2001). But in which way xylitol formation might be linked to the metabolic pathways which produce glycerol as redox sink remains unknown at this time. Fact is that the correlation between xylose uptake velocity and by-product distribution in IBB10B05 must be caused by metabolism since it was established independently from the respective SSL or the fermentation setup.

4.4. Variation in fermentation conditions for improvement of SSL conversion

Although SSL is a cheap substrate, production costs must be kept to a minimum since ethanol only sells at a low price. Based on the low sugar content, SSL conversion results in low ethanol concentration which causes energy intensive recovery at high costs (Björling and Lindman, 1989, Helle et al., 2008). Thus enhancement of ethanol production together with reduction of fermentation costs must be attained.

An important step towards maximizing ethanol yields was already achieved by enabling efficient co-fermentation of glucose and xylose in IBB10B05. Although *S. cerevisiae* already is a robust strain, it was tried to achieve further improvement of ethanol yields by adapting the cells to SSL media by replacing

YPG with SSL starter cultures. SSL is a challenging substrate, and previous studies have shown that *S. cerevisiae* can react positively when adaptation occurred prior to facing the challenges provided by lignocellulosic substrates (Johansson et al., 2011, Alkasrawi et al., 2006, Silva and Roberto, 2001). The study of Johansson and co-workers suggests that pre-cultures performed with the same substrate as in the subsequent fermentation can enhance ethanol production. This does not apply in this study, and a decrease in ethanol production after successive transfers in fresh SSL media was also observed in the study of Helle et al., (2004) as well as for other yeast strains (Lindén and Hahn-Hägerdal, 1989). Reasons might be found in inhibitors and lack of vitamins, nutrients or trace elements, leading to low energy content of the cells and lowered fermentation performance (Johansson et al., 2011).

Another way of boosting ethanol production can be achieved by raising sugar concentration in SSL, thus attaining higher ethanol concentration in solution. This might be either accomplished by enriching the sugars in solution, e.g. with membrane processes (Restolho et al., 2009), or by merging SSL with another cheap but carbohydrate rich substrate as presented in this study and in earlier publications (Helle et al., 2007). Fermentation of SSL-Thin in combination with wheat straw hydrolysate resulted in higher ethanol yields (0.45 g ethanol/g total sugar) than fermentation of SSL only (0.33 g ethanol/g total sugar). In the study of Helle and co-workers (2007), fermentation of wood knot hydrolysate and hardwood SSL resulted in approximately equal ethanol yields as when fermenting SSL alone. With 0.4 g ethanol/g total sugar produced, the ethanol yield is lower than fermentation of SSL and hydrolysate in this study. Direct

comparison of ethanol yields is difficult since the compositions of the respective fermentation media are varying, especially the glucose and xylose concentrations.

Due to the impact on fermentation performance as well as on fermentation cost, a study about nutrient supplementation was accomplished. Mineral medium provides excellent conditions for *S. cerevisiae* (Jeppsson et al., 2006) and was successfully applied for SSL fermentation in this study and in similar composition in other studies (Johansson et al., 2011, Björling and Lindman, 1989). However, it is too expensive for larger scale fermentations, thus it was tried to replace it with complex substrates. Yeast extract also contains essential nutrients such as vitamins and minerals (Kadam and Newman, 1997), and fermentations with yeast extract supplemented SSL are comparable to those with mineral medium (*this study*). Since it is still too expensive for industrial scale production, alternatives such as corn steep liquor have to be evaluated as well (Helle et al., 2008, Kadam and Newman, 1997).

Significant improvement in ethanol yields could be achieved when fermenting in bioreactor. Fermentation under pH controlled condition resulted in the highest ethanol yields (38 g ethanol/g total sugars on average) and the fastest xylose conversion velocity achieved in this study. Although the pH was kept at 6.8 during fermentation, the glycerol yields were below average. This contradicts previous assumptions that fermentation with *S. cerevisiae* at pH close to 7 results in higher glycerol formation (Rapin et al., 1994). In contrast bioreactor fermentation resulted in 0.20 g xylitol/g total sugar, which is higher than xylitol yields in similar fermentation setups in this and in a previously published study

(Helle et al., 2004). As a control the same fermentation was also carried out in bottle fermentation so changes in by-product formation caused by compounds found in the fermentation matrix can be ruled out. Thus the empirically established correlation between xylose conversion velocity and by-product distribution also seems to apply here; comparatively high xylose uptake rates are accompanied with high xylitol and low glycerol yields.

5. Conclusion

In this study it was shown that it is possible to utilize IBB10B05 for fermentation of undetoxified SSL. Simulating carbohydrate compositions from other plants, SSL was supplemented with glucose and xylose, and fermentation resulted in successful conversion to bioethanol. Ethanol yields varied between 0.31 and 0.35 g ethanol/g total sugars for SSL-Thin fermentations and between 0.31 and 0.39 g ethanol/g total sugars for fermentations of SSL-S2. Acetic acid was found to be the major inhibitor of xylose fermentation, and a significant improvement of xylose conversion rate was achieved when fermenting under pH controlled condition with constant pH close to 7. The two main redox sink compounds are glycerol and xylitol, and the yields of both seem to be governed by xylose uptake velocity. Faster xylose conversion rates are accompanied by an increase in xylitol and a decrease in glycerol yields. Further, the sum of molar yields of glycerol and xylitol remains constant throughout all fermentations independently of the respective SSL or the fermentation set up. Over all, IBB10B05 emerged to be an efficient and robust yeast strain for fermentation of lignocellulosic hydrolysates.

6. References

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Tables and Figures

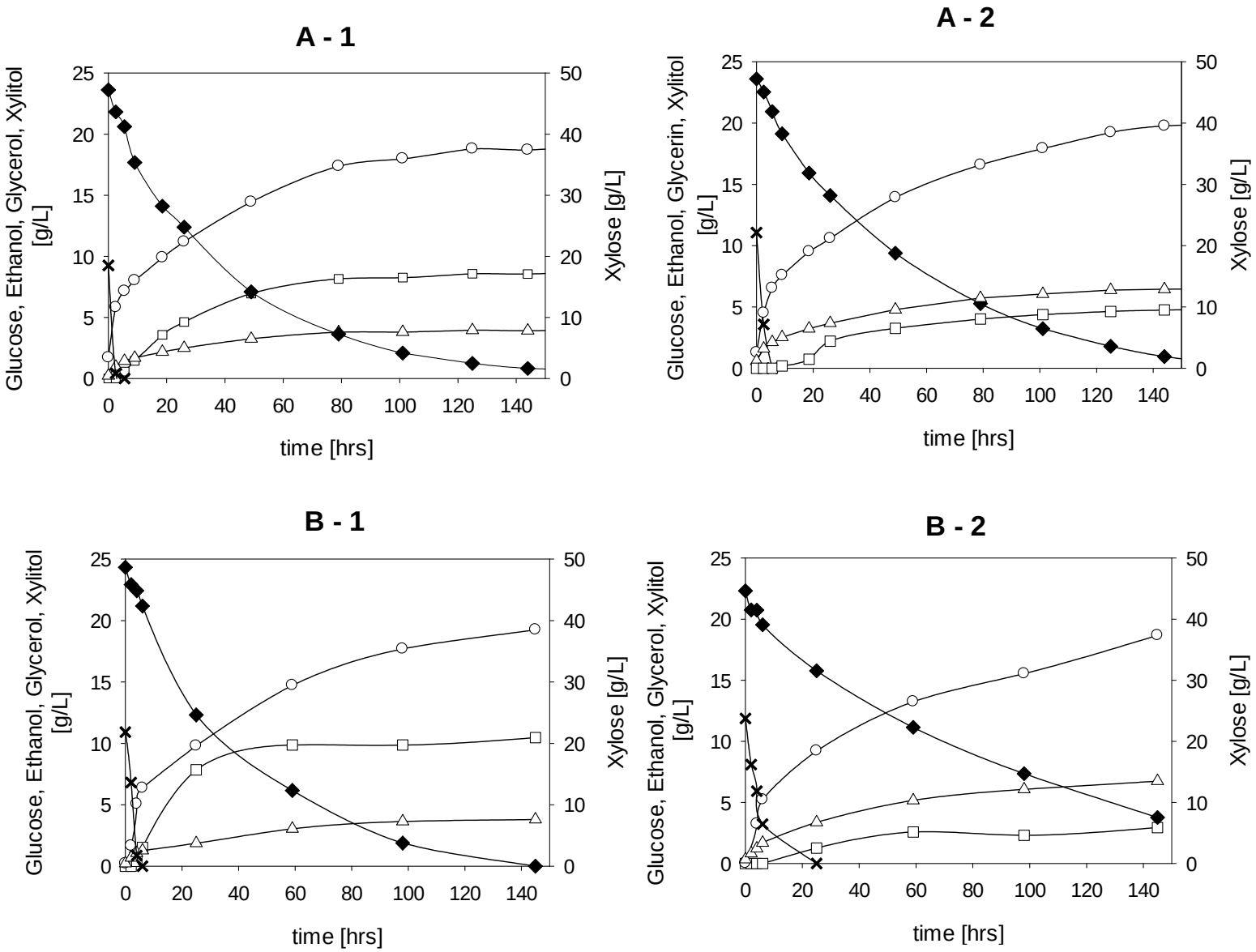


Figure 1: Comparison of SSL-Thin (A/B - 1) and SSL-S2 (A/B - 2) fermentation each carried out with two different medium supplementations; A) mineral medium and B) yeast extract. Fermentations accomplished with 70 % (v/v) SSL-S2/ SSL-Thin, 1.4 % (w/v) glucose, 5 % (w/v) xylose, mineral medium or 1 % (w/v) yeast extract. Symbols: xylose (full diamonds), glucose (crosses), ethanol (empty circles), glycerol (empty triangles), and xylitol (empty squares).

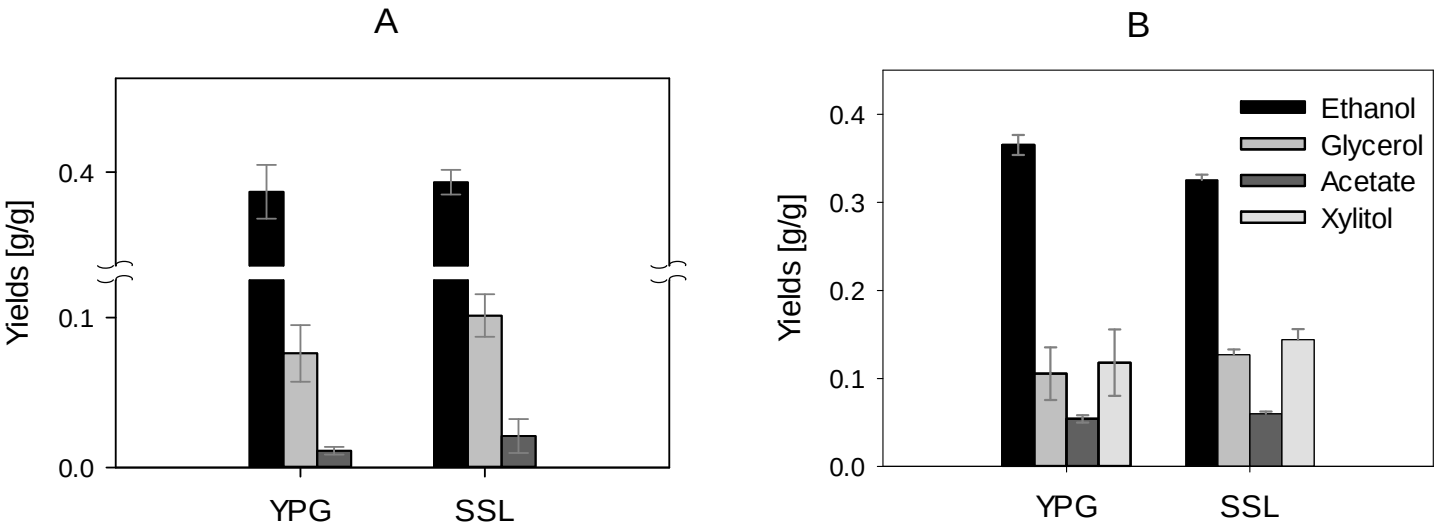


Figure 2: Evaluating the influence of different pre-cultures on glucose phase (A) and xylose phase (B) of SSL-S2 fermentation. SSL: 70 % (v/v) SSL-S2, 2 % (w/v) glucose, 5 % (w/v) xylose, 1 % (w/v) yeast extract; YPG: 2 % (w/v) glucose, 1 % (w/v) yeast extract, 2 % (w/v) peptone

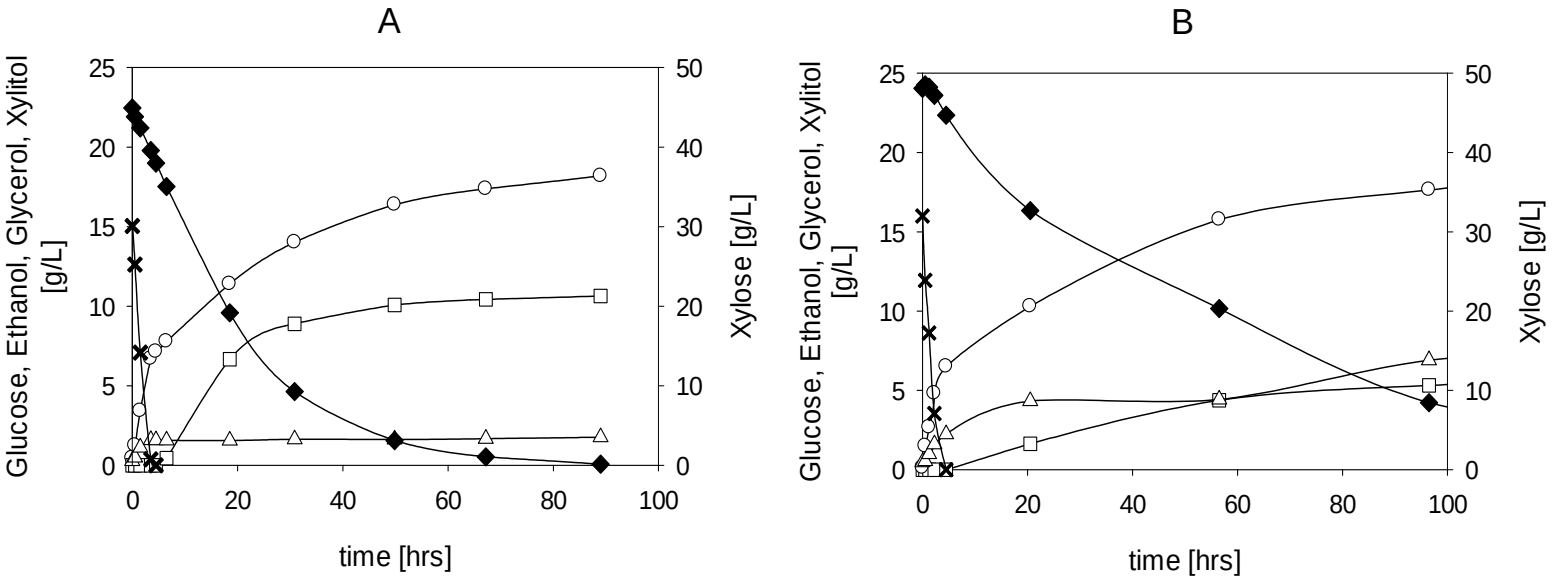


Figure 3: Comparison of fermentations carried out in bioreactor (A) and rubber sealed glass bottle (B). Both fermentations accomplished with 70 % (v/v) SSL-S2, 1.4 % (w/v) glucose, 5 % (w/v) xylose, 1 % (w/v) yeast extract. Symbols: xylose (full diamonds), glucose (crosses), ethanol (empty circles), glycerol (empty triangles) and xylitol (empty squares).

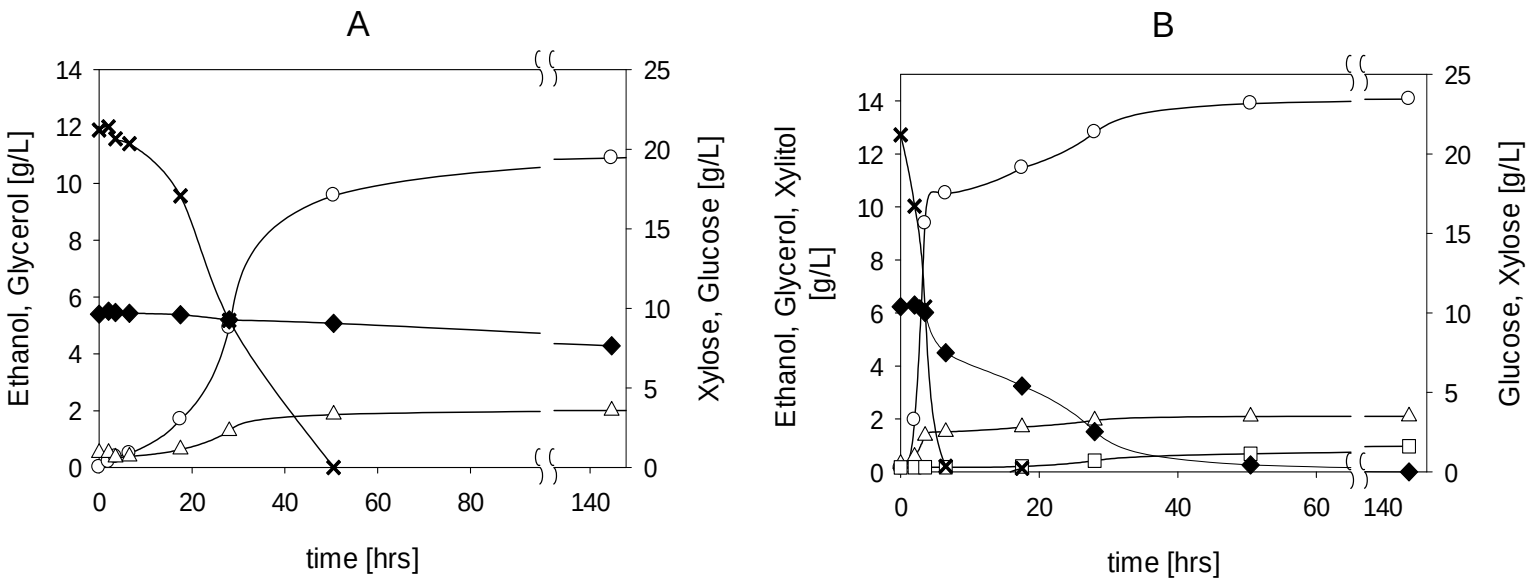


Figure 4: Time courses of SSL-S2 (A) and SSL-Thin (B) fermentations (70 % (v/v) SSL-S2/ SSL-Thin, 1 % (w/v) yeast extract, 2 % peptone) with supplemented wheat straw hydrolysates (80 % (v/v) from 15 % DM). Symbols: xylose (full diamonds), glucose (crosses), ethanol (empty circles), glycerol (empty triangles) and xylitol (empty squares).

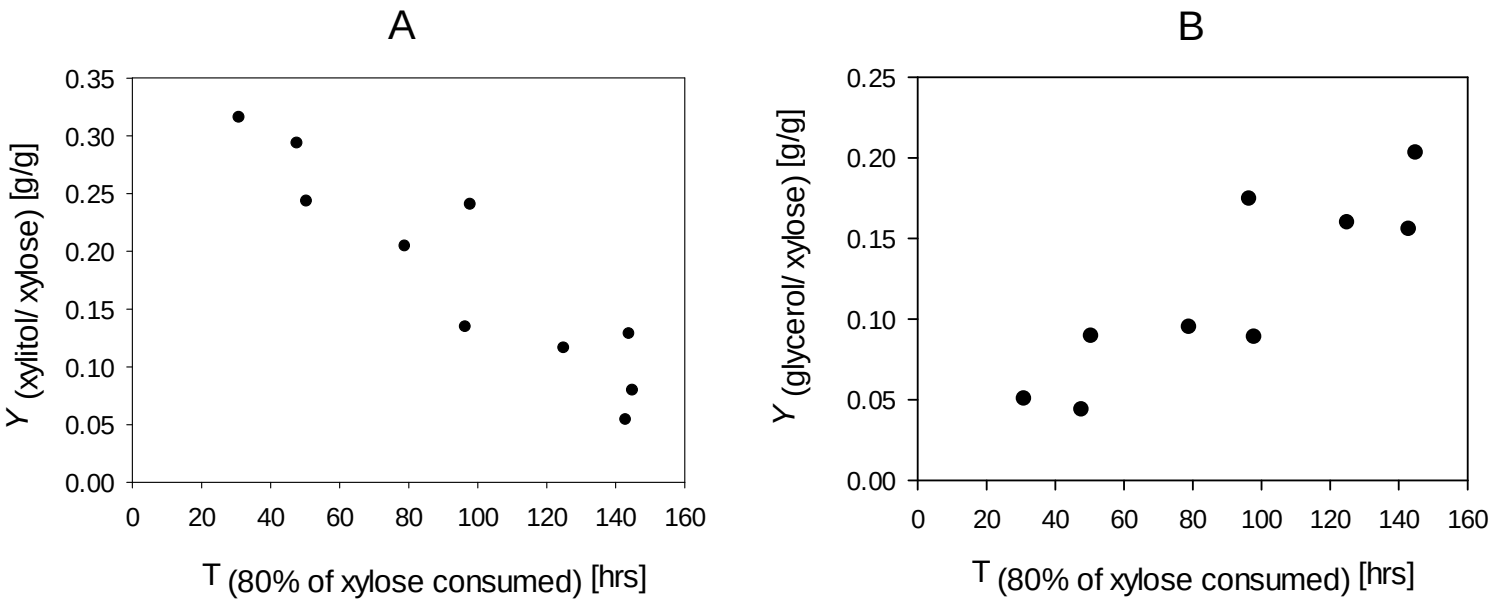


Figure 5: Correlation between time (T) required for the consumption of 80 % (w/w) of xylose and resulting xylitol yields (g xylitol/ g xylose $_{\text{consumed until T}}$) (A) and glycerol yields (g glycerol/g xylose $_{\text{consumed until T}}$) (B).

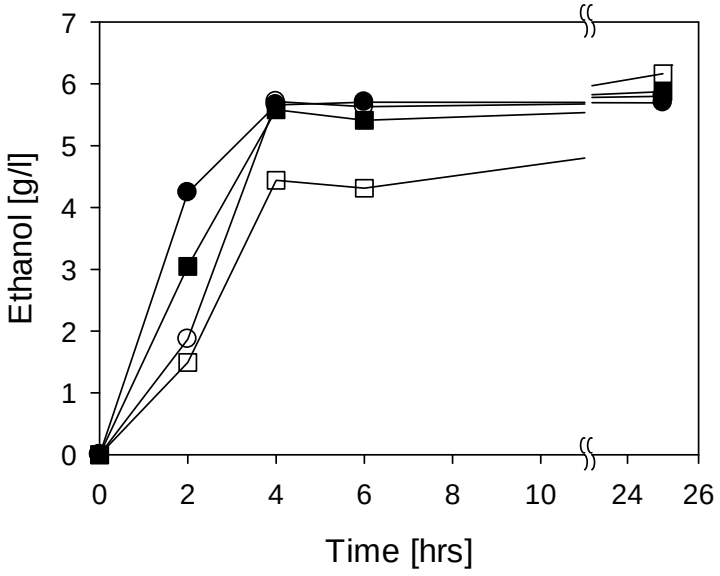


Figure 6: Comparison of ethanol production with IBB10B05 (empty symbols) and industrial wine yeast (full symbols). Fermentation carried out with 1.4 % (w/v) glucose, 1 % (w/v) yeast extract and either 70 % (v/v) SSL-Thin (circles) or 70 % (v/v) SSL-S2 (squares).

Table 1: Composition of SSL (within an error of 10%)

	Dry mass [%]	Xylose [g/L]	Glucose [g/L]	Galactose [g/L]	Mannose [g/L]	Acetic acid [g/L]
SSL-Thin	14	0.8	1.7	0.2	4.0	4.7
SSL-S2	30	0.3	3.6	1.1	7.1	9

Table 2: Yields in g/g total sugars after approximately 144 hours of fermentation

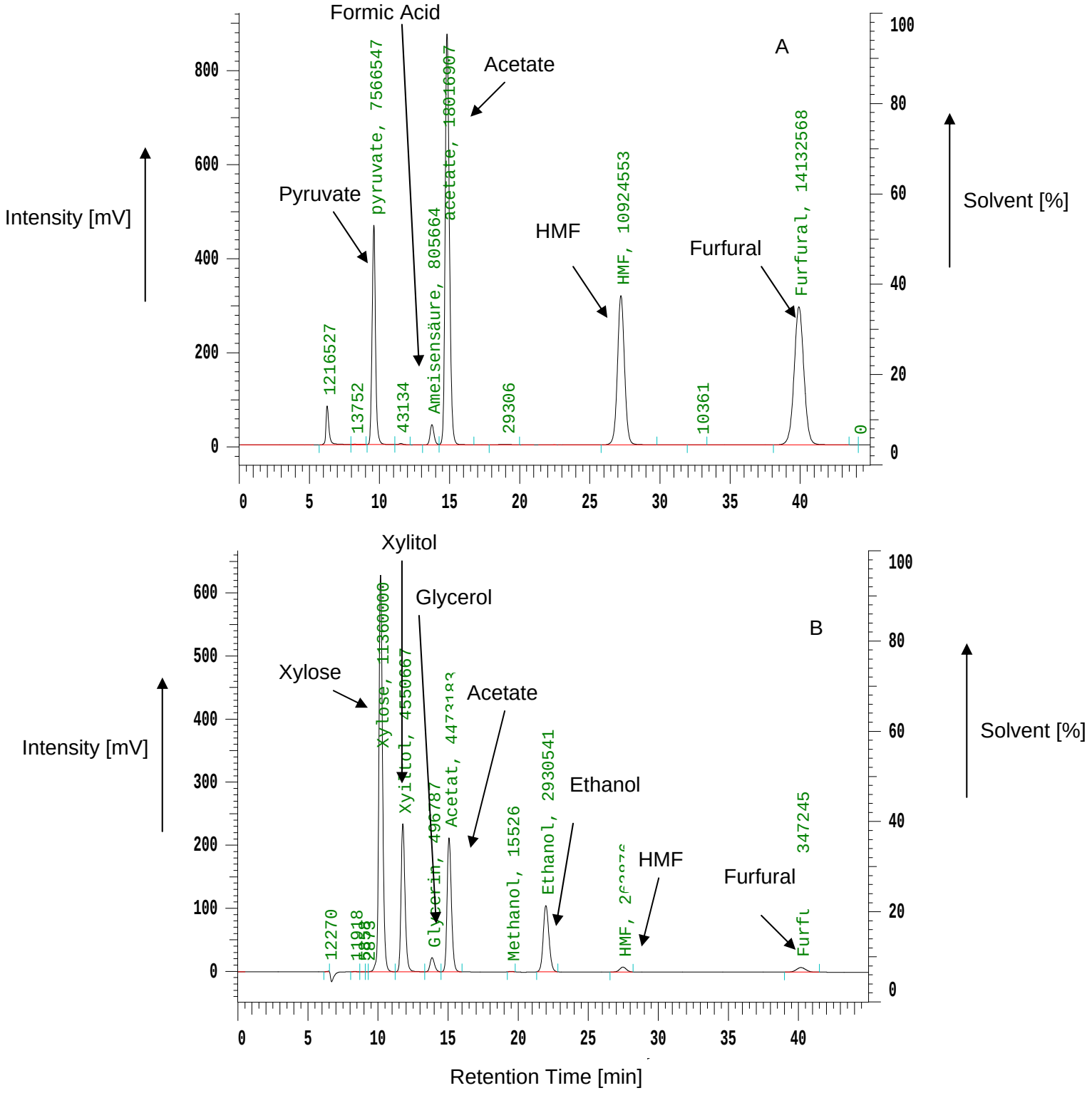
MM= mineral medium; YE= yeast extract; PEP= peptone; WS-H= wheat straw hydrolysate

SSL-Thin					
	MM	YE + Pep	YE [c)]	Mean [a)]	WS-H [g)]
Ethanol	0.31	0.31	0.35	0.33 +/- 0.03	0.45
Glycerol	0.07	0.07	0.07	0.07 +/- 0.00	0.07
Xylitol	0.16	0.18	0.20	0.18 +/- 0.02	0.03
Acetate	0.04	0.03	0.05	0.04 +/- 0.01	0.13
SSL-S2					
	MM	YE + Pep	YE [c)]	Mean [a)]	WS-H [f) + g)]
Ethanol	0.35	0.34	0.34 +/- 0.03	0.34 +/- 0.02	0.41
Glycerol	0.11	0.11	0.09 +/- 0.04	0.10 +/- 0.02	0.08
Xylitol	0.09	0.04	0.11 +/- 0.05	0.08 +/- 0.02	0.00
Acetate	0.05	0.05	0.04 +/- 0.01	0.04 +/- 0.00	0.02
SSL-S2 + YE					
	Pre-cultures				
	SSL	YPG	Bioreactor [d)]		
Ethanol	0.31 +/- 0.01	0.35 +/- 0.01	0.38 +/- 0.01		
Glycerol	0.11 +/- 0.00	0.10 +/- 0.02	0.03 +/- 0.00		
Xylitol	0.09 +/- 0.01	0.08 +/- 0.01	0.20 +/- 0.01		
Acetate	0.05 +/- 0.00	0.04 +/- 0.04	0.05 +/- 0.00		
CO_2 [b)]			0.35 +/- 0.01		

a) Arithmetic middle of fermentations carried out with SSL-Thin and SSL-S2, respectively supplemented with different media amendments.
b) Only for bioreactor fermentations with off gas analysis. Elsewhere with calculation as described in Krahulec et al. (2010).
c) Courses of fermentations are depicted in Figure 1.
d) Courses of fermentations are depicted in Figure 3, panel A.
e) Yields in glucose and xylose phase depicted in Figure 3.
f) Yields are only based on glucose, since xylose uptake was strongly inhibited.
g) Courses of fermentations are depicted in Figure 4.

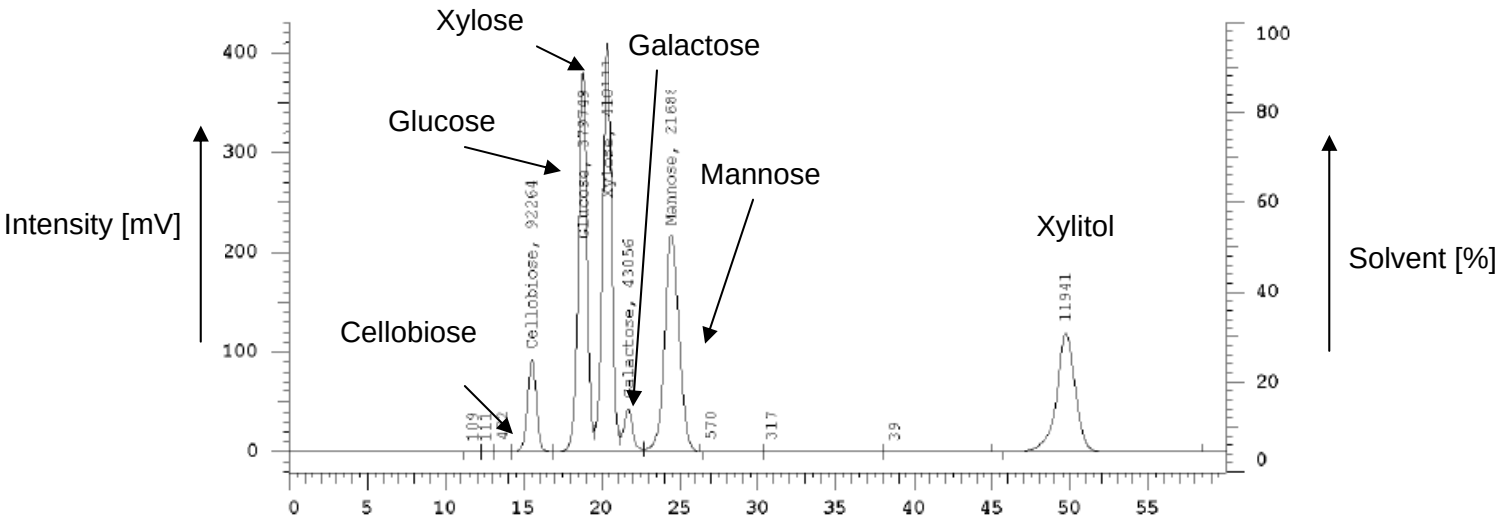
Supplementary information A

Supplementary Information 1: Quantitative analysis of extracellular fermentation products, HMF and furfural. UV (panel A) and RI (panel B) signal for measurement of a standard.



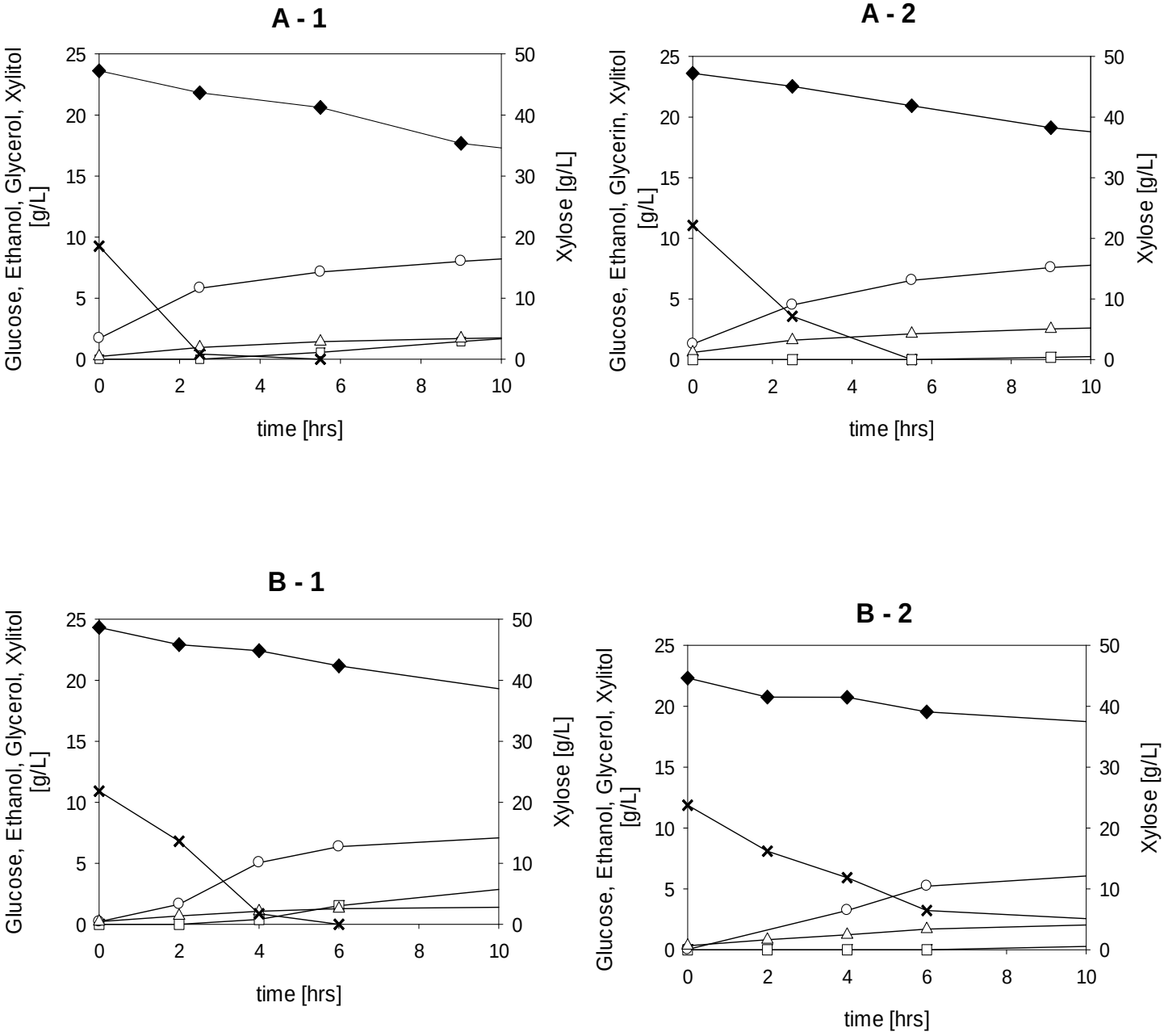
Compounds (concentrations in g/L): Pyruvate (0.51); glycerol (1.04); ethanol (14.99); acetate (20.00); furfural (0.64); HMF (0.42); xylose (25.06); xylitol (10.13); formic acid (0.54)

Supplementary Information 2: Carbohydrate analysis. RI signal for measurement of a standard.



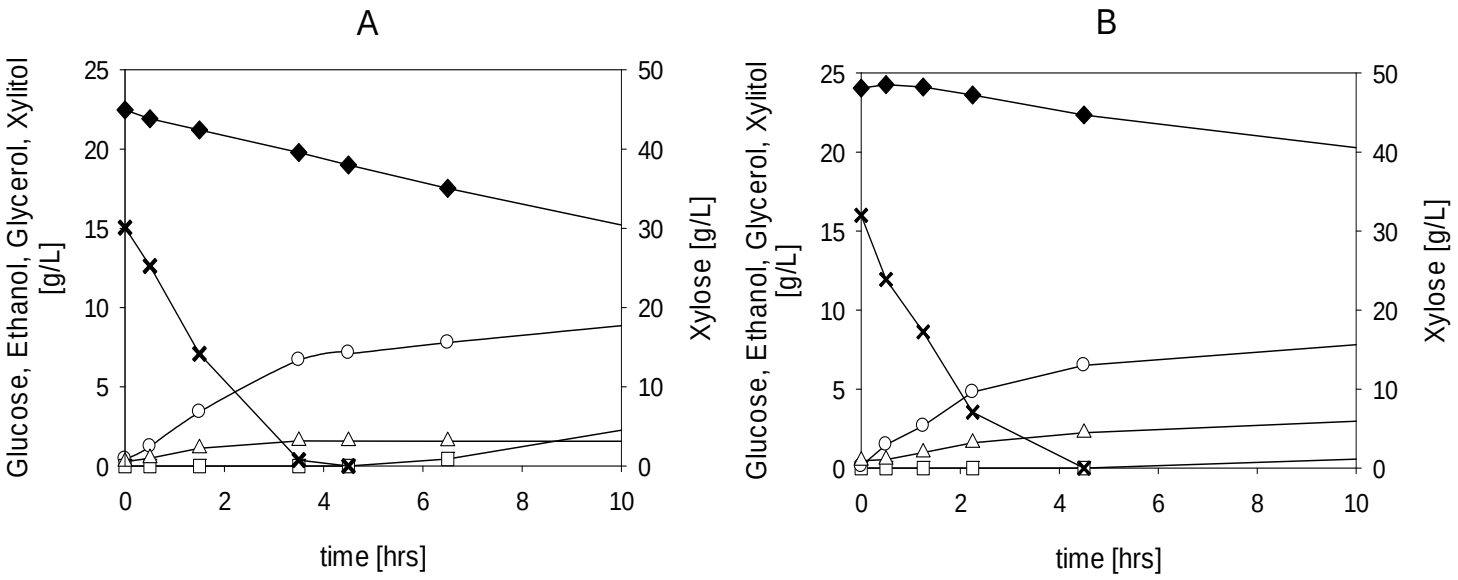
Compounds (concentrations in g/L): Glucose (20.00); xylose (25.00); cellllobiose (5.00); mannose (20.00); galactose (2.50); xylitol (10.00)

Supplementary Information 3: Glucose phase of time courses shown in Figure 1 - comparison of SSL-Thin (A/B - 1) and SSL-S2 (A/B - 2). Fermentation carried out with two different medium supplementations; A) mineral medium and B) yeast extract.



Fermentations: 70 % (v/v) SSL-S2/ SSL-Thin, 1.4 % (w/v) glucose, 5 % (w/v) xylose, mineral medium or 1 % (w/v) yeast extract. Symbols: xylose (full diamonds), glucose (crosses), ethanol (empty circles), glycerol (empty triangles), and xylitol (empty squares).

Supplementary Information 4: Glucose phase of time courses shown in Figure 3 - comparison of fermentations carried out in bioreactor (A) and rubber sealed glass bottle (B).



Fermentations: 70 % (v/v) SSL-S2, 1.4 % (w/v) glucose, 5 % (w/v) xylose, 1 % (w/v) yeast extract. Symbols: xylose (full diamonds), glucose (crosses), ethanol (empty circles), glycerol (empty triangles) and xylitol (empty squares).

Supplementary Information B:

Optimizing Carbohydrate Analysis in SSL

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1. Problems of carbohydrate analysis of SSL with HPLC system

HPLC-system:

Merck-Hitachi LaChrome HPLC system with a Merck-Hitachi LaChrome L-7250 autosampler and a Merck L-7490 RI detector

SA1 for carbohydrate analysis:

Glucose: 20g/L; xylose: 25 g/L; xylitol: 10 g/L; mannose: 20 g/L; galactose: 2.5 g/L; arabinose: 2.5 g/L; cellobiose 5 g/L

1.1. Basic problems

- The best column for carbohydrate analysis is Aminex HPX-87P column (Bio-Rad, Richmond, CA, USA) (Bio-Rad Laboratories, 2011)

- Anions from mineral medium (mainly phosphates and sulfates) and organic/ inorganic salts present in SSL can bind with plumb cations on the Aminex HPX-87P column and build insoluble or hardly soluble salts

- → Reduction of separating effect of column
    - The peaks are not separated by the base line
    - Peaks of arabinose and mannose as well as xylose and galactose are overlapping

- → Reduction of economic life time

1.2. Problems caused by the matrix (SSL)

- High background noise
    - Distort the UV as well as the RI signal (based on lignosulfonic acids and other organic compounds)

- The issue of solids in the SSL (30% for SSL-S2)
    - Destroying the column
    - Distorting the signal

2. Solutions

2.1. Sample application without dilution

→ For distinguished peaks and distinguish them from the background noise

→ Problem: range of the linearity is exceeded for xylitol and glycerol

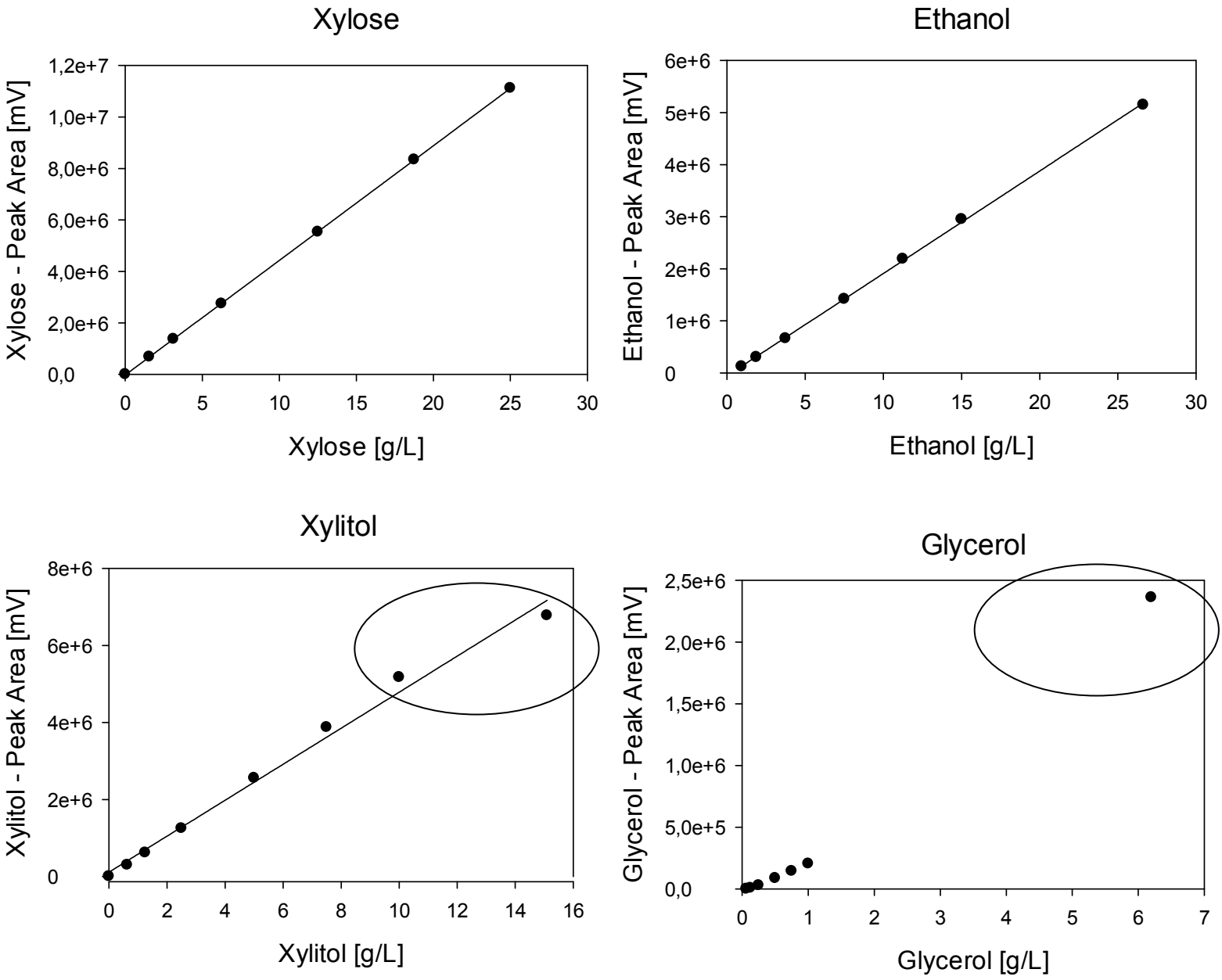

Figure 1: Determination of range of linearity for the analytes xylose, xylitol, glycerol and ethanol (in concentration range of application)

2.2. Elongation of running time

Every second sample runs for two hours to recover the signal and diminish the starting peak

- Running time to long → not suitable for a high number of samples
- Starting peak still high

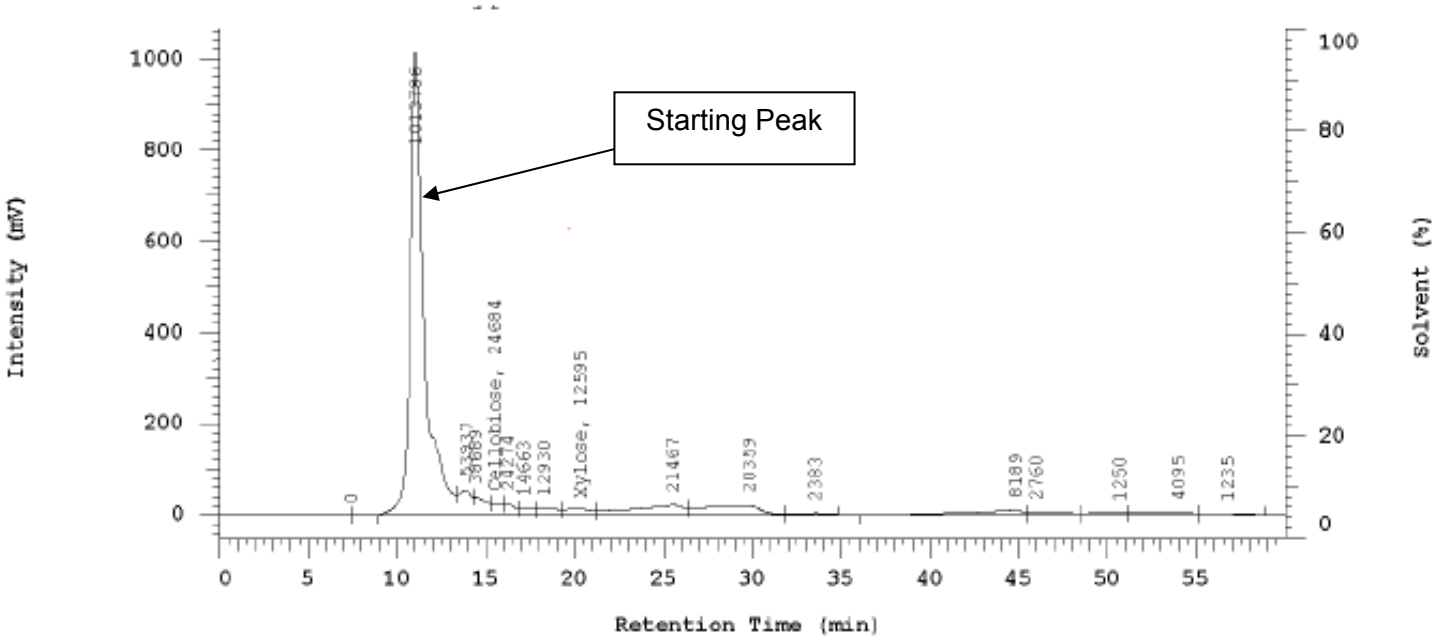

Figure 2: Example for starting peak (Aminex HPX-87P column; Aminex HPX-87C column; T=85 °C; F=0.6 ml/min; 20µl)

2.3. Regeneration of the Aminex HPX-87P column

Regenerating the Aminex HPX-87P column twice according to the Bio Rad manual (Bio-Rad Laboratories, 2011).

→ No improvement in peak separation of the carbohydrates

2.4. Precipitation of phosphate with Fe(III)-nitrate-solution

Precipitation with equimolar Fe(III)-Nitrate solution
→ Peaks are distorted

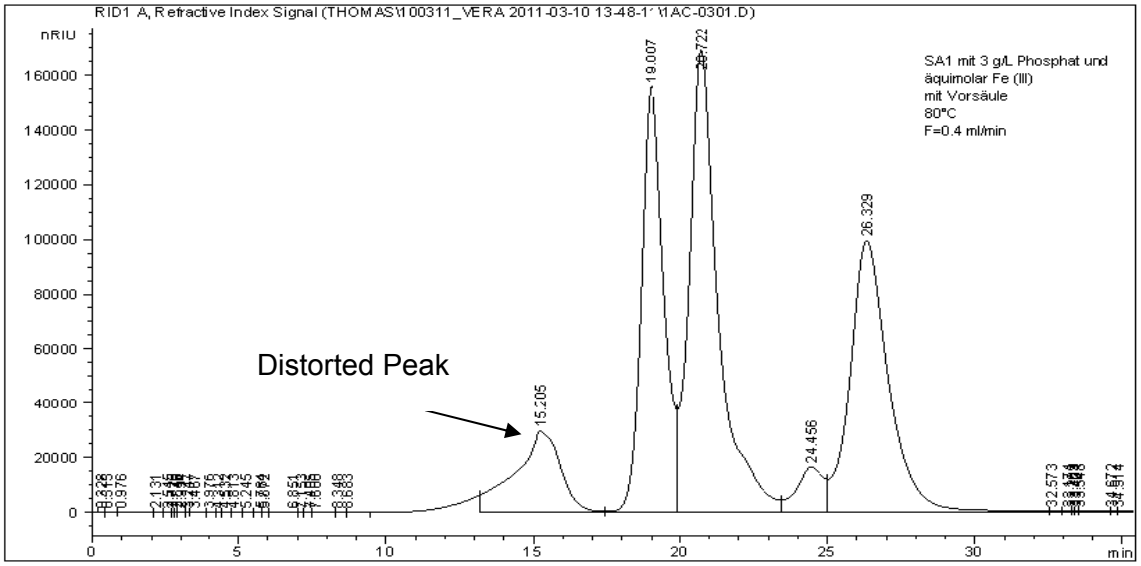

Figure 3: SA1 measured after precipitation with equimolar Fe(III)-nitrate solution Aminex HPX-87P column; Aminex HPX-87C column; T=85 °C; F=0.6 ml/min; 20µl

2.5. Optimization of conditions

2.5.1. Temperature

2.5.1.1. T=85°C

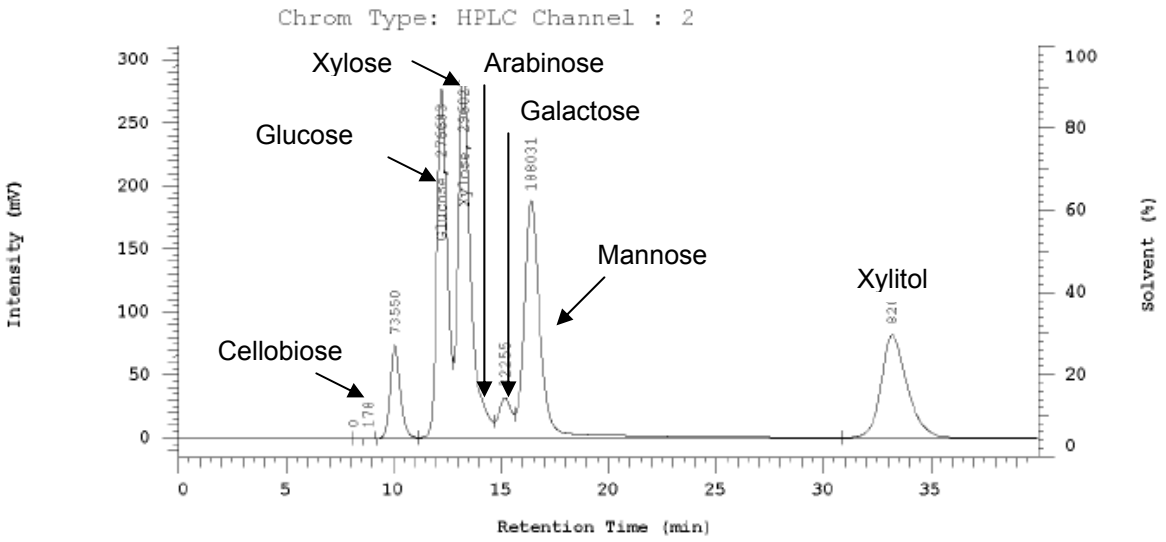

Figure 4: Chromatogram: SA1, Aminex HPX-87P column; without guard column; **T= 85°C** ; F= 0.6 ml/min; 20 µl

2.5.1.2. T=80°C

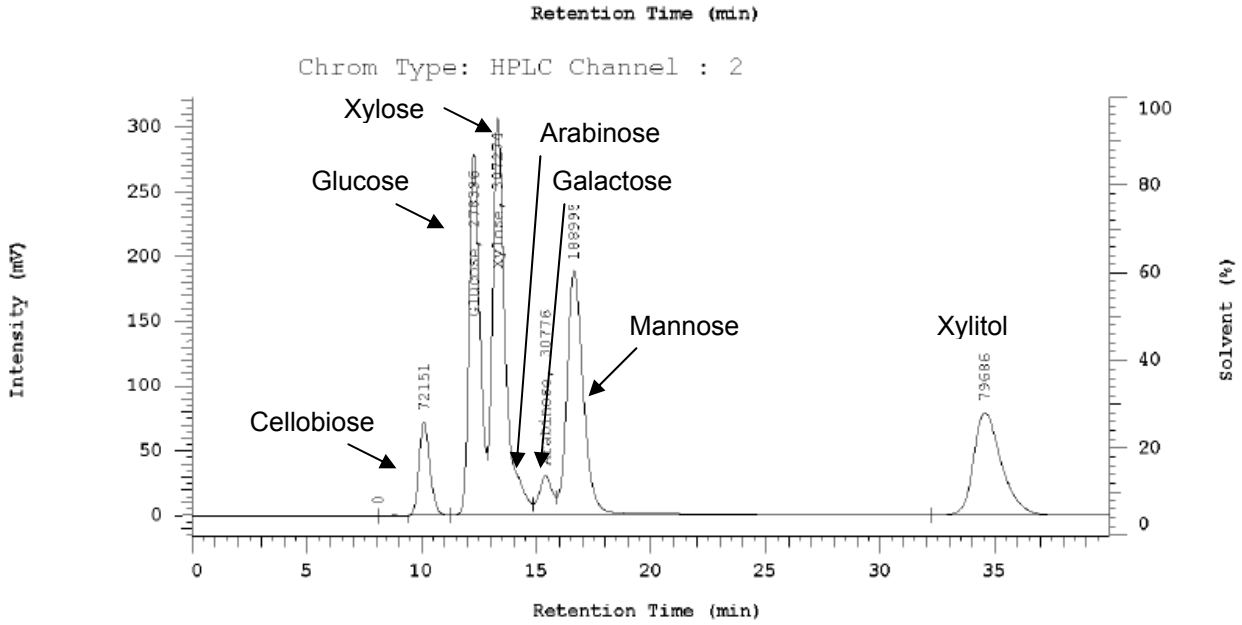

Figure 5: Chromatogram: SA1, Aminex HPX-87P column; without guard column; **T= 80°C**; F= 0.6 ml/min; 20 µl

2.5.1.3. T=70°C

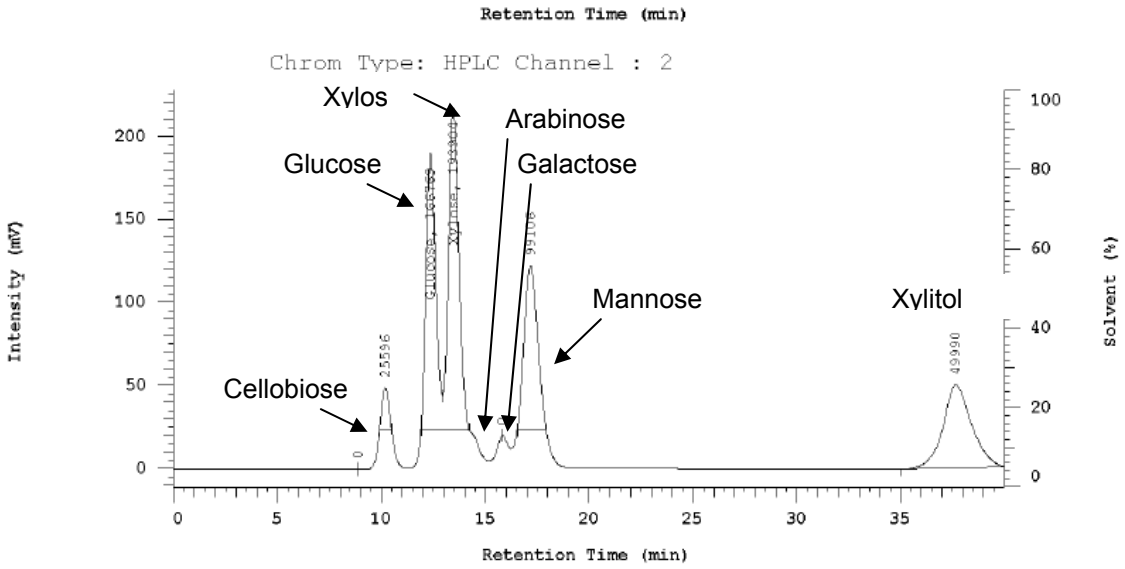

Figure 6: Chromatogram: SA1, Aminex HPX-87P column; without guard column; **T= 70°C**; F= 0.6 ml/min; 20 µl

→ **T=80°C** emerged to be the best

2.5.2. Injection volume (V_I)

2.5.2.1. V_I = 20 µl

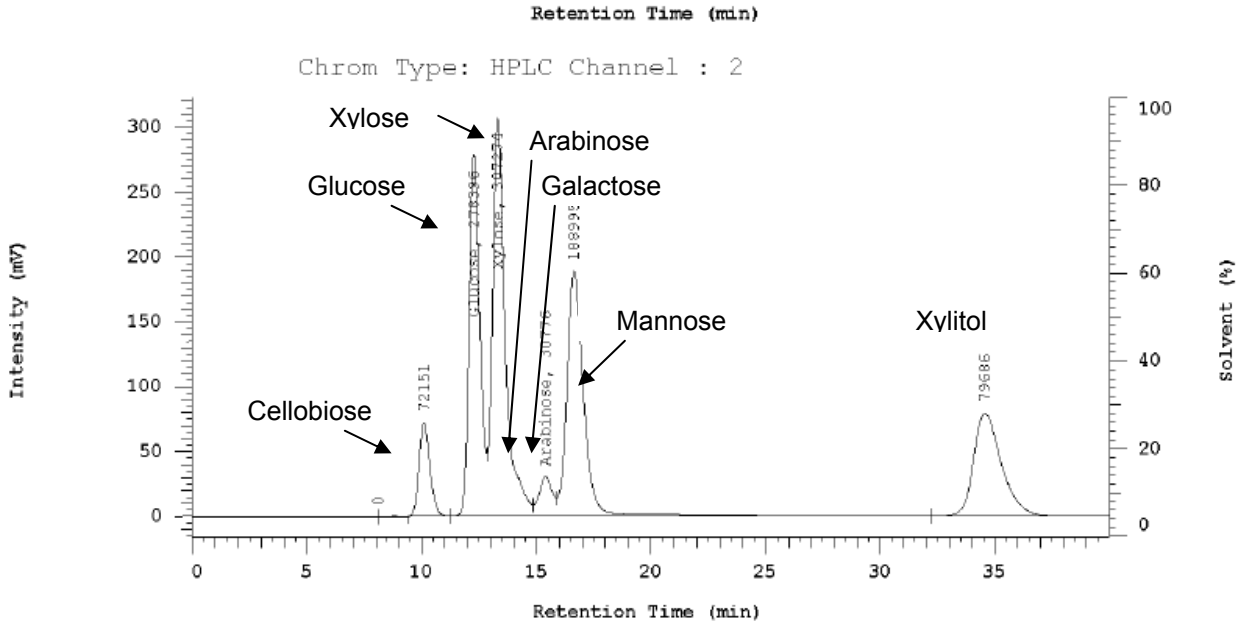

Figure 7: Chromatogram: SA1, Aminex HPX-87P column; without guard column; T= 80°C; F= 0.6 ml/min; **20 µl**

2.5.2.2. V_I = 10 µl

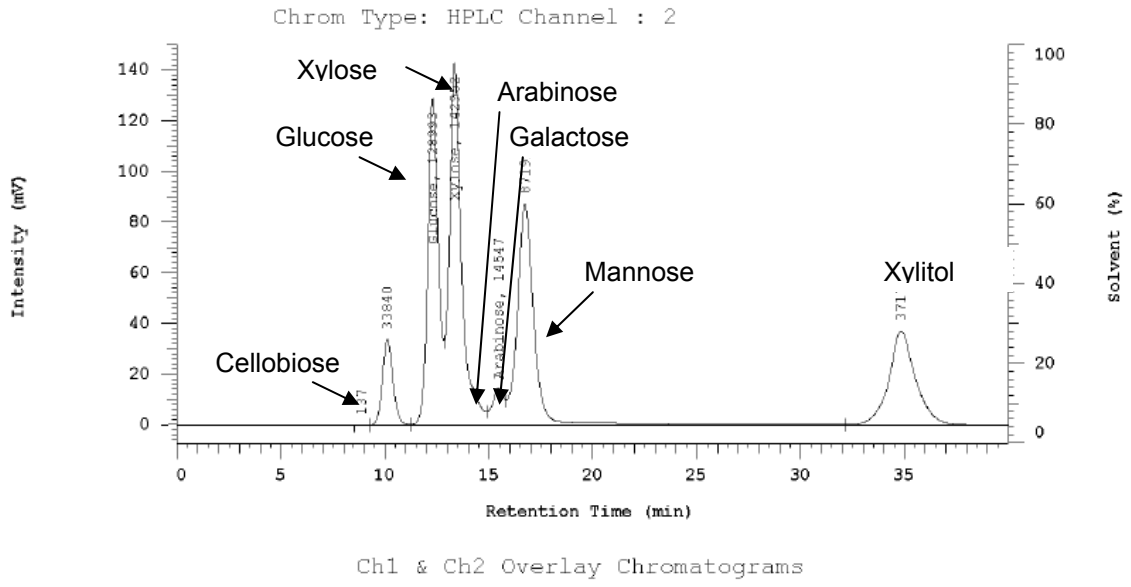

Figure 8: Chromatogram: SA1, Aminex HPX-87P column; without guard column; T= 80°C; F= 0.6 ml/min; **10 µl**

→ **20 µl** emerged to be the best

2.5.3. Flow (F) of running buffer

2.5.3.1. F= 0.6 ml/min

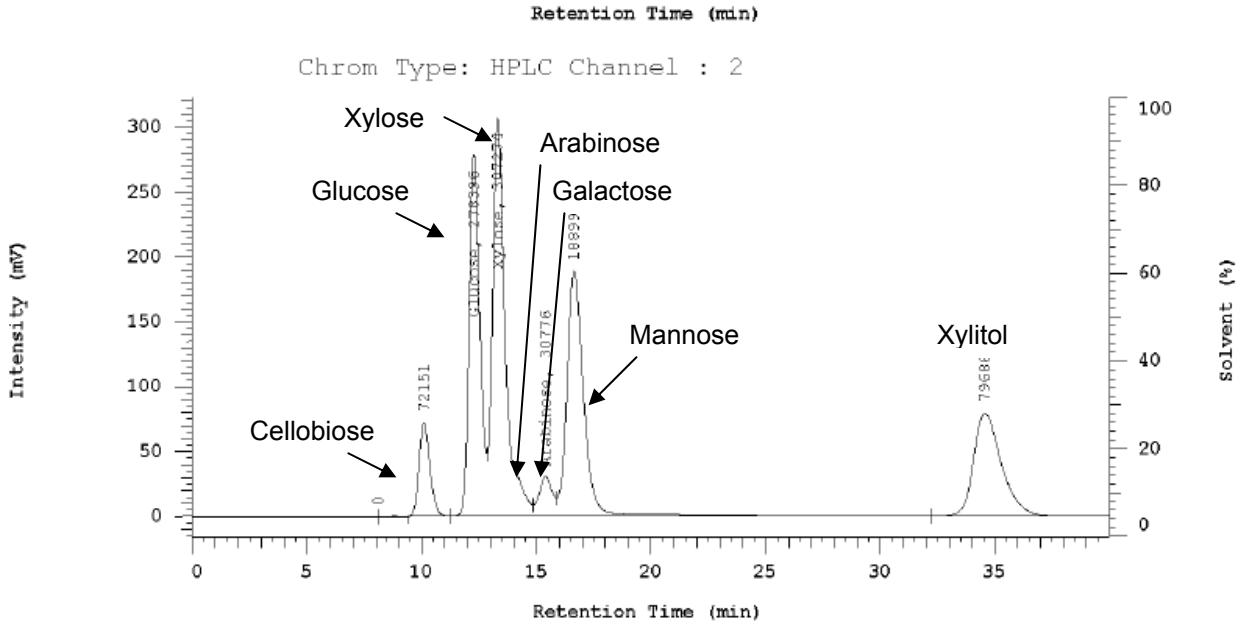

Figure 9: Chromatogram: SA1, Aminex HPX-87P column; without guard column; T= 80°C; **F= 0.6 ml/min**; 20 µl

2.5.3.2. F= 0.4 ml/min

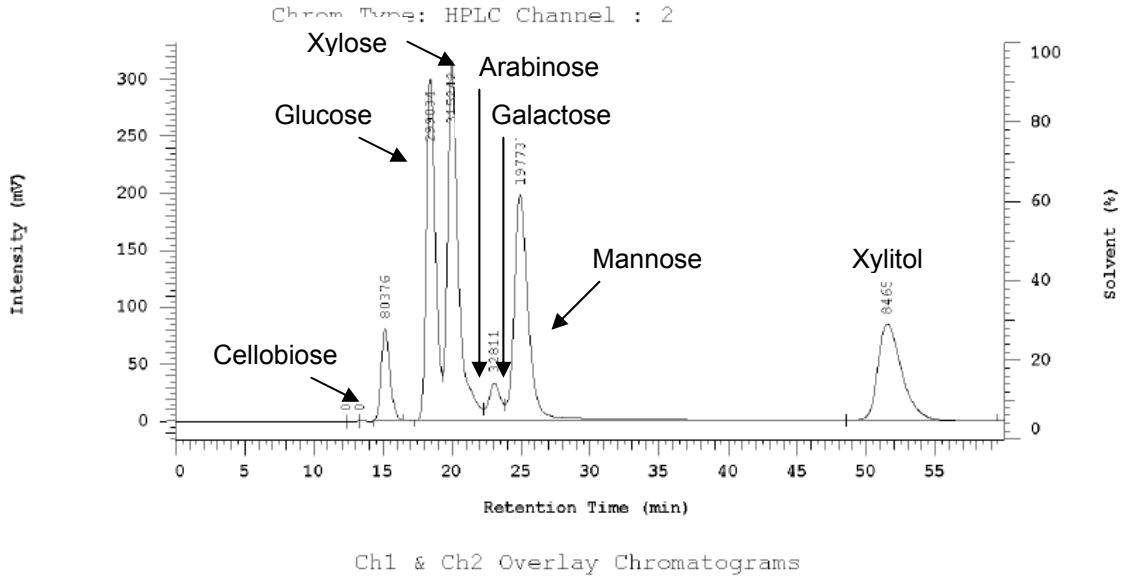

Figure 10: Chromatogram: SA1, Aminex HPX-87P column; without guard column; T= 80°C; **F= 0.4 ml/min**; 20 µl

→ F= 0.4 ml/min emerged to be the best

→ **Best conditions: F=0.4 ml/min; V_l=20µl; T=80°C**

2.6. Precipitation of phosphate with DEAE Support and Hi Trap Q FF columns

- with DEAE support
- 100µl of the support are loaded with 1 ml of sample
- incubate for 5 min, centrifuge at 14750 g, store supernatant

→ Only minimal amount of phosphate removed (from 22mM to 18mM)

- with HiTrap Q FF column (GE Life Sciences)
- wash column with 5 to 10 ml water
- load 0.5 ml of sample
- eluate with 2 ml water
- regenerate column with 10 ml 10M NaCl, wash with water and store in 20 % ethanol

→ Excellent phosphate removal (from 25mM to 0.5 mM)

2.7. New Aminex HPX-87P column and guard column

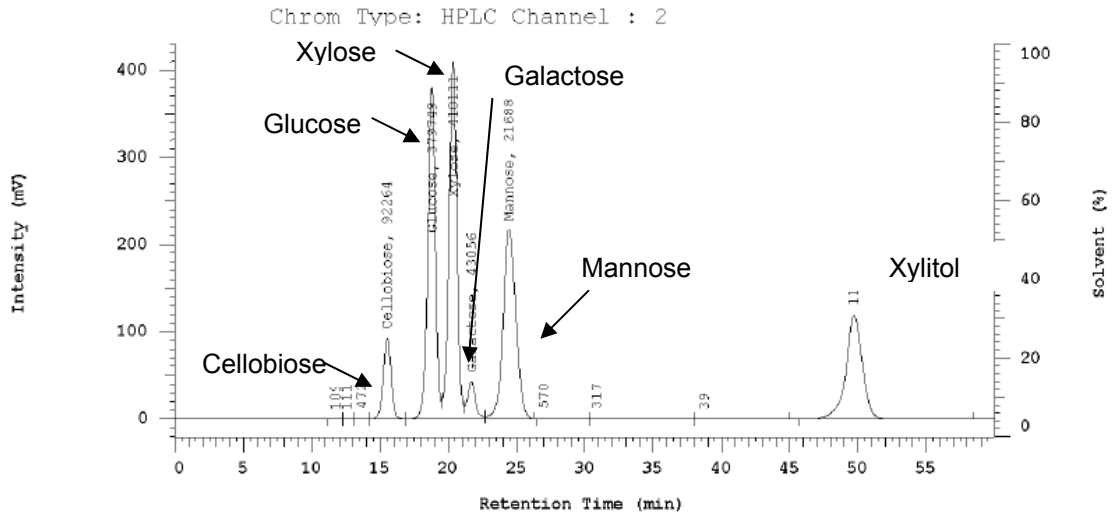

Figure 11: Chromatogram: SA1, Aminex HPX-87P column and guard column; T= 80°C; F= 0.4 ml/min; 20 µl

- From this point the standards for analysis did not contain arabinose, it is hard to separate from mannose and is only present in SSL in marginal concentrations.

→ Peak separation improved

2.8. Analysis of samples without sugar addition: Aminex de-ashing guard column (Bio Rad)

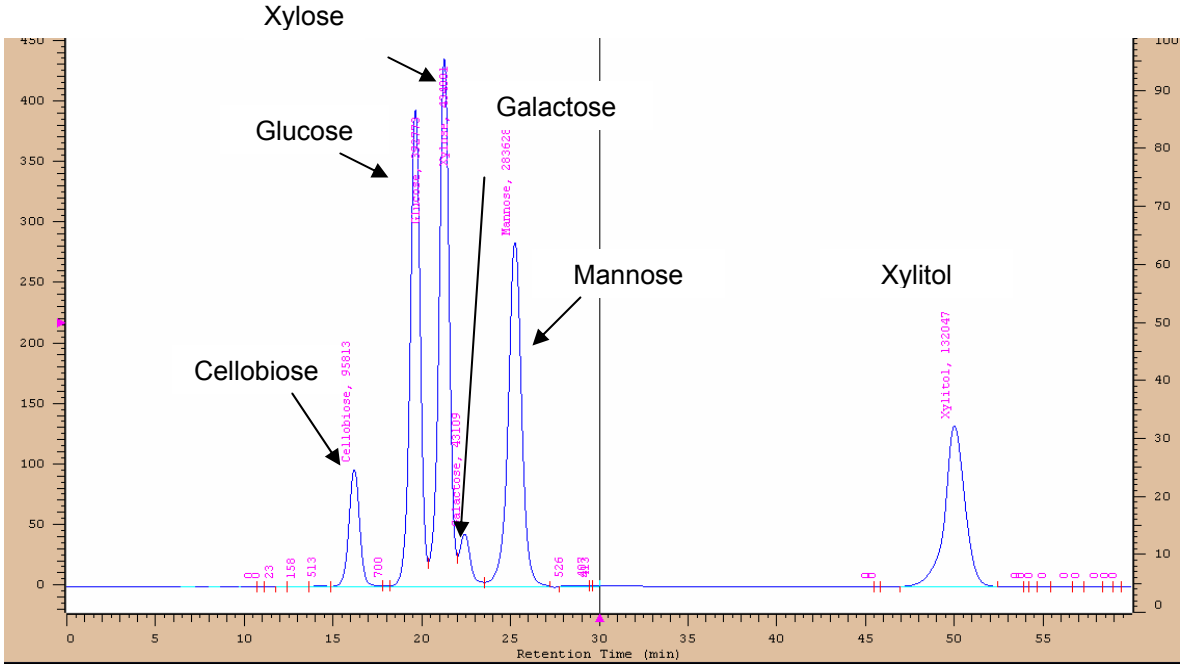

Figure 12: Chromatogram: SA1, Aminex HPX-87P column (new); de-ashing guard column; T= 80°C; F= 0.4 ml/min; 20 µl

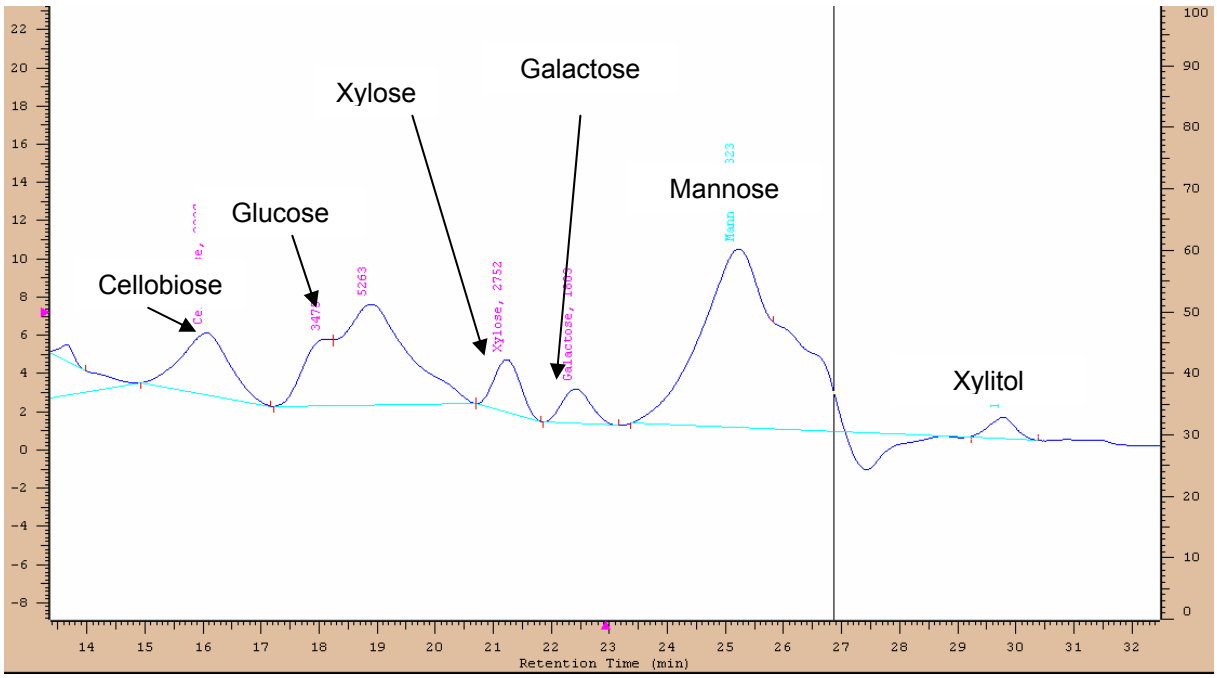

Figure 13: Chromatogram; sample VIII0 (VR1: 70% SSL-S2), Aminex HPX-87P column; de-ashing column; T= 80°C; F= 0.4 ml/min; 20 µl

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4. Reference

Bio-Rad Laboratories, I. 2011. *Aminex Carbohydrate Analysis Columns* [Online]. Wien. [Accessed 05.12.2011 2011].