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LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

Process development for combined pentose and hexose fermentation

FREDRIK NIELSEN

DEPARTMENT OF CHEMICAL ENGINEERING | LUND UNIVERSITY



Process development for combined pentose and hexose fermentation

DOCTORAL DISSERTATION
2016

Fredrik Nielsen

Department of Chemical Engineering
Lund University, Sweden



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Academic thesis which, by due permission of the Faculty of Engineering of Lund University, will be publicly defended on 8 September 2016 at 1.15 pm in lecture hall K:C at the Center for Chemistry and Chemical Engineering, Naturvetarvägen 12, Lund, for the degree of Doctor of Philosophy in Engineering.

The faculty opponent is Professor Svein Jarle Horn, Norwegian University of Life Sciences, Ås, Norway.

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| Abstract <p>Second-generation ethanol from lignocellulose is a sustainable alternative that can partially replace fossil fuels. To be competitive with first generation ethanol from sugar and starch crops and fossil fuels, the conversion efficiency and ethanol yields of second-generation ethanol conversion processes must be improved. Improving the performance of the fermenting microorganism and efficiently convert both glucose and xylose in lignocellulosic biomass is imperative to achieve these targets. This thesis addresses means to improve the performance of the biochemical steps of the lignocellulose-to-ethanol process.</p> <p>The main focus has been on enhancing the xylose utilization of xylose-fermenting <i>Saccharomyces cerevisiae</i> by adapting the yeast to lignocellulosic hydrolysates during propagation and developing novel co-fermentation strategies that promote xylose utilization. Co-fermentation strategies based on separate hydrolysis and co-fermentation (SHCF) and simultaneous saccharification and co-fermentation (SSCF) were investigated. Furthermore, scale-up of co-fermentation strategies and the use of multiple and blended feedstocks in the conversion process were investigated.</p> <p>The findings show that adaptation of the yeast to the conditions in fermentation during propagation provides a broad adaptive response that improves fermentation performance of xylose-fermenting <i>S. cerevisiae</i>. Co-fermentation designs that take the xylose consumption patterns of xylose-fermenting <i>S. cerevisiae</i> into consideration can further enhance the xylose utilization and ethanol yields. Furthermore, feedstocks with similar attributes and blends thereof could be concurrently pretreated and co-fermented, eliciting comparable ethanol yields of the whole range of feedstocks and feedstock blends. This suggests that feedstocks with similar attributes can be used interchangeably to improve supply efficiency and hedge economic and technologic risks. Scale-up experiments show that the advanced co-fermentation strategies can be scaled-up from lab scale to process development and demonstration scale and maintain comparable ethanol yields, thus bringing the lab-scale process improvements closer to implementation at commercial scale.</p> | | |
| Keywords Bioethanol, lignocellulose, xylose fermentation, co-fermentation, process design, agricultural residues, <i>Saccharomyces cerevisiae</i> . | | |
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Date August 2nd, 2016.

Process development for combined pentose and hexose fermentation

Fredrik Nielsen

Department of Chemical Engineering
Lund University, Sweden



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*“Nothing in life is as
important as you think it is
when you are thinking about it”*

Daniel Kahneman in *Thinking, Fast and Slow*

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Appended publications:

- Paper I. Short-term adaptation during propagation improves the performance of xylose-fermenting *Saccharomyces cerevisiae* in simultaneous saccharification and co-fermentation.
- Paper II. Prefermentation improves ethanol yield in separate hydrolysis and cofermentation of steam-pretreated wheat straw.
- Paper III. Sequential targeting of xylose and glucose conversion in fed-batch simultaneous saccharification and cofermentation of steam-pretreated wheat straw for improved xylose conversion to ethanol.
- Paper IV. Simultaneous saccharification and co-fermentation for bioethanol production using corncobs at lab, PDU and demo scales.
- Paper V. Evaluation of the effect of mixed agricultural feedstocks on pretreatment, hydrolysis and cofermentation efficacy.

Abstract

Fermentative conversion of lignocellulosic biomass into ethanol is a sustainable alternative that could partially replace traditional products from petroleum refining. To successfully commercialize lignocellulosic technologies, economic sustainability must be ensured. The key factors for improving the process economics are high ethanol yields from the raw materials and high final ethanol concentrations. To achieve these goals, efficient hydrolysis and fermentation and the utilization of a variety of sugars present in the feedstock are necessary. The main obstacles to efficient fermentation of lignocellulose-derived sugars are the limitations of the microbial physiology, which restricts efficient conversion of various substrates and decreases the ability to cope with inhibitors. The naturally inhibitor-tolerant *Saccharomyces cerevisiae* has been metabolically engineered to be able to assimilate xylose into its central metabolism, thus expanding the scope of potential substrates. However, the conversion of xylose is constrained by the xylose uptake rate and metabolic restrictions.

In this thesis, strategies to improve the utilization of xylose derived from the agricultural residues wheat straw and corn stover and blends thereof are presented. The aim is to enhance xylose utilization by xylose-fermenting *S. cerevisiae*, improve ethanol yields from nondetoxified hydrolysates, and determine whether it is feasible to employ the strategies using feedstock blends and maintain ethanol productivity and yields.

Two process-related approaches were investigated to improve yeast properties and accommodate the patterns of substrate consumption in xylose-fermenting *S. cerevisiae* that harbors exogenous genes for xylose reductase and xylitol dehydrogenase. First, the yeast was short-term adapted to the fermentation conditions by introducing hydrolysate liquor from pretreated agricultural biomass in the propagation. Short-term adaptation improved the tolerance of inhibitor-resistant yeast strains and increased the ethanol yield and xylose-fermenting capacity. The xylose utilization and overall ethanol yields, based on total available glucose and xylose, improved by over 70% and 50%, respectively, in simultaneous saccharification and cofermentation when short-term adaptation was used.

Second, various fermentation designs based on separate hydrolysis and cofermentation and simultaneous saccharification and cofermentation were

investigated to improve xylose utilization. Both strategies have advantages, and the choice of the strategy is strain- and feedstock-dependent. Because the xylose-fermenting capacity of the yeast declines over time but it still converts glucose to ethanol, it was advantageous to target xylose conversion upfront. Xylose-rich hydrolysate liquor was separated from glucose-rich solids and fermented sequentially. Fed-batch feeding strategies were employed to accommodate the substrate consumption patterns of the yeast and maximize ethanol yields. It was found that xylose utilization and ethanol yields could be increased with the customized cofermentation and feeding strategies. Xylose utilizations exceeding 90% of total available xylose and overall ethanol yields exceeding 90% of the theoretical maximum were attained.

It was further shown that the xylose utilizations and ethanol yields attained with single agricultural feedstocks could be maintained with concurrently processed blends of agricultural feedstocks with the devised strategies. Industrially relevant ethanol titers and overall ethanol yields exceeding $50 \text{ g}\cdot\text{L}^{-1}$ and 80% of the theoretical maximum, respectively, were obtained across the full range of steam-pretreated feedstocks, encompassing wheat straw, corn stover, and blends thereof. With feedstock blending, the potential supply of raw materials available to a commercial plant is increased and allows one to hedge the risks associated with changes in feedstock availability and prices. Benefits from economy of scale can be reaped for a commercial plant, and the cost of raw materials can be minimized if a variety of raw materials can be utilized, which will positively impact the process economics.

Populärvetenskaplig sammanfattning

En av de större utmaningarna som samhället står inför idag är omställningen från en ekonomi som är baserad på fossila råvaror till en ekonomi som förlitar sig på förnybara råvaror. Fossila råvaror är våra primära källor för energi, bränslen och råmaterial till tillverkningsindustrin, och de är ändliga resurser. Kostnaden för utvinning av olja och naturgas ökar år för år. Regionala kriser skapar kraftigt varierande energipriser och att trygga energiförsörjningen har blivit en het politisk fråga. En annan aspekt är den ständigt närvarande frågan om miljö- och klimatpåverkan. Kommer vi att kunna försörja en växande befolkning på jorden med energi och livsmedel om vi fortsätter att konsumera ändliga resurser i samma takt som idag? Förnybar energi och förnybara bränslen ger i kombination med energi-effektiviseringar en del till lösningen.

Bioetanol har varit på tapeten länge som en potentiell ersättare till fossila drivmedel och produceras redan i stor skala. Den omfattande produktionen av etanol är dock ifrågasatt. Första generationens bioetanol, den etanol som marknadsförs idag, tillverkas enligt samma principer som jästa drycker. Grödor som innehåller stärkelse eller socker mäskas och jäses till etanol av mikroorganismer. Dessa grödor används även i stor utsträckning som livsmedel och foder, och konkurrensen om råvarorna riskerar öka marknadspriserna på dessa och därmed öka priset för dina dagliga måltider. Det är även kontroversiellt att odlingsbar mark tas i anspråk för produktion av bränslen på bekostnad av livsmedelsproduktion.

Men, en mans skräp kan vara en annan mans skatt. Andra generationens bioetanol använder lignocellulosa som råmaterial istället för stärkelse och socker. Lignocellulosa är det som ger växter dess styvhet och motståndskraft och till skillnad från stärkelse så innehåller dess sockerpolymerer flera olika sockerarter, däribland glukos och xylos. Stora mängder lignocellulosa återfinns i rester från jordbruk, skogsindustri och i avfall från konsumtionssamhället. Plötsligt så kan halmen som lämnas på fälten efter skörden, sågspån från sågverk, spill från massabruk, ja till och med dina gamla avlagda jeans bli råmaterialet för biobränslen. Eftersom det är rester från existerande verksamheter så är de både billiga, tar mindre resurser i anspråk och de konkurrerar inte med livsmedelsproduktion. Men det finns en hake. För att kunna omvandla lignocellulosan till etanol måste socker frigöras från råvaran. Samma egenskaper som gör växterna motståndskraftiga gör det svårare att bryta ner materialet och frigöra

socker. De behandlingar som krävs för att frigöra socker från råvaran bildar inhibitorer. Dessa är ämnen som är skadliga för mikroorganismer, eller till och med dödar dem.

En biokemisk process framstår idag som den mest fördelaktiga metoden för att omvandla biomassa till jäsbära sockerlösningar. Den kombinerar kemisk förbehandling av biomassan vid höga temperaturer med biologisk nedbrytning med hjälp av enzymer. Den föreslagna processen bildar fortfarande inhibitorer under förbehandlingssteget som är skadliga för mikroorganismerna, men ger sockerlösningar som går att jäsa. Enzymerna har isolerats från naturliga miljöer där mikroorganismer bryter ner cellulosa och får biomassa att ruttna. Biokemister arbetar frenetiskt på att karakterisera och förbättra egenskaperna hos enzymerna och göra dem användbara för industriella processer. Samtidigt så arbetar mikrobiologer på att göra mikroorganismerna mer toleranta mot inhibitorer och förbättra dess förmåga att jäsa socker till etanol. Med genteknik och genom att imitera evolutionen har de förbättrat organismerna. Resultatet är mer toleranta mikroorganismer som kan omvandla flertalet olika sockerarter, omvandla dem snabbare och med högre utbyte. Idag får vi ut betydligt mer etanol från våra råvaror än förr och dagens forskning fortsätter att tänja på gränserna för vad som går att uppnå. De gemensamma ansträngningarna har gjort processen möjlig, och nu pusslar ingenjörerna ihop bitarna. Med hjälp av erfarenheterna från första generationens bioetanol designas nu processer som drar nytta av styrkorna hos och samverkan mellan enzymer och mikroorganismer. Målet är att designa processer som gör att andra generationens bioetanol blir konkurrenskraftig.

En av de stora utmaningarna för att omvandla jordbruksrester till etanol är att ta tillvara på sockerarten xylos. Xylos kan utgöra upp emot hälften av den totala sockermängden i vissa växtmaterial. Traditionell bagerijäst, *Saccharomyces cerevisiae*, kan inte omvandla xylos till etanol. Med hjälp av genteknik har förmågan introducerats i jästen, men xylosjäsningsen är fortfarande ofullständig och långsammare än för andra sockerarter. Förmågan att omvandla xylos är kopplad till jästens preferenser för olika sockerarter, hur den omvandlar dem och hur väl den tolererar inhibitorer. Arbetet i den här avhandlingen har syftat till att förbättra utnyttjandet av xylos från olika råvaror och effektivisera jäsningsen. Allt för att få ut mer etanol från biomassan.

Ett sätt att förbättra användningen av xylos är att ändra jästens beteende under jäsningsen. Genteknik ger stabila förändringar i egenskaper som dessutom ärvs av dess avkomma, men det går även att kortsiktigt ge jästen andra egenskaper. Genom att odla jästen delvis på sockerlösningar från förbehandlade jordbruksrester, som innehåller inhibitorer, kan jästen vänjas vid förutsättningarna under jäsningsen och anpassa sig. Det visade sig att jäst som anpassats till förhållandena under jäsningsen blev mer toleranta mot inhibitorer och dess förmåga att använda xylos förbättrades

avsevärt. Förbättringen kombinerades med olika strategier för jäsnings av det förbehandlade växtmaterialet. Strategier där den enzymatiska nedbrytningen och jäsningsen utfördes antingen separat eller samtidigt kombinerades med att xylos och glukos jästes i följd i olika faser av jäsningsen. Genom att först fokusera på att jäsa xylos och sedan glukos kunde xylosjäsningsen framhävas trots jästens preferens för glukos. Som en följd ökade användningen av xylos under jäsningsen och mer etanol kunde produceras från råvaran. Detta är viktigt för att förbättra lönsamheten för jäsningsprocessen.

Kvaliteten och tillgången på olika råvaror, så som olika sorters halm, varierar under året och från år till år. För att trygga försörjningen av råvaror för produktion av bioetanol så är det viktigt att flera olika typer av förbehandlade råvaror kan utnyttjas och det är en fördel om dessa kan blandas och processas samtidigt. På så sätt kan man trygga försörjningen, minska kostnaderna för råvaror och jämna ut kvaliteten på det förbehandlade materialet som enzymer och jäst skall omvandla till bioetanol. Tyvärr så är det inte självklart att alla omvandlingsprocesser fungerar lika bra med olika material och blandningar. Vi kunde visa att med noggrann design av jäsningsprocessen kunde material med liknande egenskaper, såsom vete-halm och majsblast, blandas och ge likvärdiga mängder bioetanol från råvarorna. Genom att visa att förbättringarna består när olika råvaror och råvarublandningar används och att processen går att skala upp från laboratorieskala till produktionsliknande skala så har vi tagit ytterligare ett steg mot mer omfattande kommersiell produktion av andra generationens bioetanol. Ytterligare ett steg på vägen mot att ersätta fossila bränslen med förnybart och hållbart bränsle.

List of publications

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

- I. **Nielsen, F.**, Tomás-Pejó, E., Olsson, L., Wallberg, O., 2015. Short-term adaptation during propagation improves the performance of xylose-fermenting *Saccharomyces cerevisiae* in simultaneous saccharification and co-fermentation. *Biotechnology for Biofuels*. 8 (1), 1-15.
- II. **Nielsen, F.**, Zacchi, G., Galbe, M., Wallberg, O., Prefermentation improves ethanol yield in separate hydrolysis and cofermentation of steam-pretreated wheat straw. *Sustainable Chemical Processes*, 4 (1).
- III. **Nielsen, F.**, Zacchi, G., Galbe, M., Wallberg, O., Sequential targeting of xylose and glucose conversion in fed-batch simultaneous saccharification and cofermentation of steam-pretreated wheat straw for improved xylose conversion to ethanol. (*Manuscript, submitted*)
- IV. Koppram, R., **Nielsen, F.**, Albers, E., Lambert, A., Wännström, S., Welin, L., Zacchi, G., Olsson, L., 2013. Simultaneous saccharification and co-fermentation for bioethanol production using corncobs at lab, PDU and demo scales. *Biotechnology for Biofuels*. 6 (2).
- V. **Nielsen, F.**, Galbe, M., Zacchi, G., Wallberg, O., Evaluation of the effect of mixed agricultural feedstocks on pretreatment, hydrolysis and cofermentation efficacy. (*Manuscript*)

My contributions to the publications

Below are brief descriptions of the contributions to each paper made by the author.

- I. I participated in the conception and design of the study and planned all of the cultivations and fermentation experiments. I performed all cultivations and fermentations and performed most of the analyses. I analyzed the results with my coauthors and wrote the manuscript. I handled the submission process.
- II. I participated in the conception and design of the study. I planned and performed all of the experimental work. I analyzed the results with my coauthors and wrote the manuscript. I handled the submission process.
- III. I participated in the conception and design of the study. I planned and performed all of the experimental work. I analyzed the results with my coauthors and wrote the manuscript.
- IV. I planned and performed the experimental work on the PDU scale, analyzed the results, and commented on and critically reviewed and commented on the manuscript.
- V. I designed and planned the study. I performed all of the experimental work. I analyzed the results with my coauthors and wrote the manuscript.

Abbreviations

| | |
|---------------------|---|
| AFEX | Ammonia fiber/freeze explosion |
| ATP | Adenosine triphosphate |
| DM | Dry matter |
| GRAS | Generally regarded as safe |
| HMF | 5-hydroxymethyl-2-furaldehyde |
| IPCC | The Intergovernmental Panel on Climate Change |
| NAD ⁺ | Nicotinamide adenine dinucleotide (oxidized form) |
| NADH | Nicotinamide adenine dinucleotide (reduced form) |
| NAD(P) ⁺ | Nicotinamide adenine dinucleotide phosphate (oxidized form) |
| NAD(P)H | Nicotinamide adenine dinucleotide phosphate (reduced form) |
| PPP | Pentose phosphate pathway |
| SHCF | Separate hydrolysis and cofermentation |
| SHF | Separate hydrolysis and fermentation |
| SSCF | Simultaneous saccharification and cofermentation |
| SSF | Simultaneous saccharification and fermentation |
| TCA | Tricarboxylic acid cycle |
| WIS | Water-insoluble solids |
| XDH | Xylitol dehydrogenase |
| XI | Xylose isomerase |
| XK | Xylulokinase |
| XR | Xylose reductase |

1. Introduction

1.1 Background

Global economic growth has contributed to a dramatic increase in the demand for energy, which is projected to increase by 32% by 2040 (1). New and alternative sources of energy, such as biofuels, have a role to play in meeting this demand. As of today, fossil resources are a pillar in energy systems and are projected to remain so the coming decades (1). We rely on fossil resources to supply our society with energy, transportation fuels, and raw materials for the manufacturing industry. Annually, 389 EJ of primary energy is consumed (2), 67% of which is of fossil origin (2). But, concerns regarding our dependency on fossil energy have been raised, based on environmental considerations, sustainability, and energy security arguments—even more so because the production costs for fossil energy will increase as reserves approach exhaustion and as more expensive technologies are used to explore and extract less attractive resources (1, 3). This has emphasized the need for non-fossil alternatives. The transition from an economy based largely on fossil resources toward a sustainable economy that relies on renewable energy and fuels constitutes one of the greater challenges that our society is facing today.

The development of renewable energy has been driven by governmental support policies (4, 5), induced by a few key global challenges. The prevailing concern regarding greenhouse gas emissions and environmental pollution is one driver for reducing the dependence on fossil resources. Renewables are less polluting, both in terms of local emissions (e.g., particulates, sulfur, and lead) and anthropogenic greenhouse gases (6, 7). The Intergovernmental Panel on Climate Change (IPCC) recently pointed out that each of the last three decades has been successively warmer at the earth's surface than any preceding decade since 1850, which was attributed to the anthropogenic emissions of greenhouse gases (7). The aim to mitigate climate change has induced fiscal policies supporting a transition toward renewables. The asserted effects of global warming were debated at the United Nations-sponsored COP21 Summit in Paris in 2015, and new global targets and measures for the mitigation of greenhouse gas emissions were agreed on (8). Mitigation options are available in every major sector, and combined measures to reduce energy use and greenhouse gas emissions by end-use sectors, decarbonize the energy supply, reduce

net emissions, and enhance carbon sinks in land-based sectors are likely the most cost-efficient ways to mitigate climate change (7).

Another challenge is the increasing concerns regarding energy security and the ability to provide constant availability and supply of affordable energy for consumers and industries. Most of the supply of oil and natural gas originates from politically unstable regions of the world, which puts the energy security of countries and regions at risk. Risks to energy security include disruptions to the supply of imported fossil fuels, the limited availability of fuel, and increases in energy price volatility (9). The possibility of deriving energy from local resources is attractive for many countries and regions that are dependent on imported fossil fuels. Renewable energy can be produced in many regions of the world that do not have plentiful fossil resources (4), opening up new routes for the production of energy, fuels, and chemicals. Increasing the share of renewable energy in the worldwide energy mix will further help prolong the existence of fossil fuel reserves.

An appealing feature of the local sourcing of energy is that it may aid governments and regions in sustaining economic growth. Investment in local energy production could lead to local economic development and job creation. This is especially true for renewable energy derived from biomass, which is labor-intensive and thus requires more workforce per unit of energy produced as compared with fossil resources (4). New sources of income would be created for farmers, and new jobs would be created in the product valorization chain (10). This would be of particular benefit to developing countries in which a large proportion of the population is employed in agriculture. The potential of renewable energy to address all of the key global challenges makes it an attractive option to policymakers.

1.2 Biofuels and bioethanol

One of the sectors where biomass can be employed to significantly reduce net greenhouse gas emissions is transportation. It is one of the most energy-intensive sectors and a significant contributor to anthropogenic greenhouse gas emissions (2, 7). In 2013, the transportation sector worldwide accounted for 64% of the world's entire oil consumption (2). By introducing biofuels, either blended with fossil fuels or neat, it is projected that significant contributions to the reduction of net greenhouse gas emissions can be achieved.

Biofuels are already established and marketed products. Presently, the two main types of biofuels derived from biomass are biodiesel and bioethanol. Biodiesel is generally produced from raw vegetable oils and fats, usually after conversion into a range of fatty acid methyl (or ethyl) esters. In 2014, 29 billion liters of biodiesel was produced worldwide (11), primarily from rapeseed oil, palm oil, soy beans, and certain

industrial wastes. The potential for biodiesel is more limited than for ethanol, due to limitations in feedstock supply for production on a larger scale (12). Bioethanol from sugar and starch crops, commonly referred to as first-generation bioethanol, is already produced on a large scale. In 2015, 97.7 billion liters of fuel ethanol was produced worldwide—the two biggest producers being Brazil and the United States with production of roughly 27 and 56 billion liters, respectively (13). However, concerns have been raised against the expansion of global biofuel production over the last few decades. Its potential to meet the targets set by governments regarding oil product substitution, climate change mitigation, and economic growth has been questioned and scrutinized.

One source of criticism is the use of arable land for the production of fuel and energy at the expense of the production of food and cattle feed. The criticism encompasses both ethical dimensions and the influence of increased competition on global food price dynamics. The ethical dimension concerns priorities regarding the allocation of arable land. It is a question about whether arable land should be allocated to food production to feed a growing world population or to energy production to meet the increasing energy demand and mitigate climate change. The effect of first-generation biofuels on food prices and supply is complex. It has been asserted that the increased competition has increased the price of food (14, 15), but limited and ambiguous evidence to support the long-term effects of that hypothesis has been provided (16–18). The reports are ambiguous because of the complexity in evaluating the impact of increased competition on food prices. Factors that have an impact on the price and availability of food in the short and long term must be considered and forecasted—e.g., demographics, changes in crop yield, market conditions, and demand changes for both food and products from alternative uses (12). Nevertheless, competition for land and water resources used for food production (19, 20), as well as the *Food vs. Fuel* debate, provides limitations to further expansion of the production of first-generation biofuels, which limits the potential to meet oil substitution targets.

Another aspect concerns the contributions to the climate change mitigation and the sustainability of first-generation biofuels. Assessments of the sustainability and net greenhouse gas reductions of first-generation bioethanol have put the benefits of first-generation bioethanol into question (6, 19, 21). Life cycle assessments generally state that bioethanol production is, to various extents, beneficial with regard to climate protection and fossil fuel conservation; however, impacts on acidification, human toxicity, and ecological toxicity are often unfavorable (6). Further, reductions in resource use and net greenhouse gas emissions might not be enough to meet policy targets (12). Estimates vary greatly between regions, crops, and conversion routes (6, 12) and even more so when land use change is taken into account (12, 19, 22). In particular, the sustainability of the corn-to-ethanol route has been questioned (19, 23, 24). If greenhouse gas mitigation is to be achieved, biofuel technologies must become more efficient than current practices for first-generation bioethanol in terms of net

life cycle greenhouse gas emissions. Ethanol produced from sugar cane is a possible exception that appears to meet most of the sustainability criteria (6).

The cumulative impacts of these concerns have brought about the development of second-generation biofuels produced from lignocellulosic biomass. The emergence of lignocellulosic technologies and second-generation biofuels has thus occurred as a response to concerns in a broader societal context, in relation to the use of both fossil resources and other renewable resources. This development has largely been driven by governmental policies (4, 5). Due to its high potential to meet the required oil substitution targets, existing infrastructure, and access to mature markets, second-generation bioethanol is a prime candidate to bring to market, and it has many advantages compared with first-generation bioethanol.

The use of lignocellulosic feedstocks circumvents the *Food vs. Energy* debate. Low-cost crop and forest residues, wood process wastes, organic municipal wastes, and dedicated energy crops can all be used as feedstocks. These feedstocks are considered to be able to be produced more sustainably (6, 12) and have better land use opportunities (4, 6) than sugar and starch crops used for the production of first-generation bioethanol. The use of waste products is asserted to be especially beneficial in limiting net greenhouse gas emissions (19).

Second-generation bioethanol produced by the biochemical route, widely regarded as the most promising route, has the benefits of biofuels in general and a greater impact on climate change mitigation. Its promotion can help in achieving policy targets regarding energy security and diversification, rural economic development, and greenhouse gas mitigation. It can also assist in the reduction of several other environmental impacts (6), at least relative to the use of other transport fuels. Constraints of the commercialization of second-generation bioethanol on a broad scale include ensuring a feedstock supply to meet oil replacement targets and improving the process economics of the conversion. These factors need to be addressed to successfully expand the production on a commercial scale and to render second-generation bioethanol competitive with first-generation biofuels and fossil fuels.

1.3 Feedstock availability

Many types of lignocellulose may serve as feedstock for second-generation ethanol production. The choice of feedstock will vary regionally and depend on various factors, such as growth conditions, markets, infrastructure, agricultural practice, and political environment (4). A plentiful supply of feedstock is a prerequisite for the implementation of second-generation bioethanol on a commercial scale. Studies on biomass potentials show that assessments of the projected supply vary broadly, from

primary energy equivalents of 33 EJ to 1200 EJ on an annual basis (4, 25-29). The projected supply encompasses dedicated energy crops, as well as primary and secondary residues, and depends on various factors, including sustainability considerations and expected future demand from alternative uses.

The forestry sector is one source of biomass that could contribute considerably to the projected total renewable energy potential. The primary energy potential for forest-derived biomass has been projected to be within a range of 10 EJ to 150 EJ on an annual basis (25-27, 29). The supply would be derived from logging residues, processing residues, and from wood waste. Agricultural residues and wastes are another source of biomass, and their primary energy potential has been projected to be in the range of 10 EJ to 71 EJ on an annual basis (25, 27-29). However, the economic potential of these projections depends on how much of these resources that can be made available in a profitable way. The greatest contributions to the projected supplies come from the intensification and expansion of cropland onto marginal land to cultivate dedicated energy crops. This expansion is likely to be constrained by the *Food vs. Energy* debate. The key factor that influences the potential is the availability of surplus agricultural land, which in turn depends on the level of intensification in the agricultural sector (4). The greatest potential for expansion of cropland for energy crops can be found in Latin America, the Caribbean, and sub-Saharan Africa (4). The variation in projected supply makes it uncertain to what extent biomass for energy production can become available, but even at the lowest projection, enough biomass is available to substitute a significant part of the liquid fossil fuel usage in today's transportation sector worldwide.

1.4 Process economics

Although second-generation bioethanol is recognized as a transportation fuel with important economic, environmental, and strategic attributes and although pioneer commercial-scale production plants have been built, it has not been widely commercialized and has not reached market penetration. The development of second-generation bioethanol has thus far been driven largely by supportive policy actions from governments (4), but to commercialize second-generation bioethanol, its economic competitiveness must be improved. Several technological barriers remain for second-generation biofuel production.

Ethanol is a commodity product. Being a substitute for petroleum-based products and a perfect substitute for first-generation bioethanol, second-generation bioethanol must compete with these products regarding price. In order to do so, cost reductions must be made in the supply chain, the initial investment, and the conversion process (30-33), and alternative revenue sources must be found. On the revenue side, ethanol

production must likely be a part of a broader biorefinery concept, where exported heat, electricity, and other value products enhance revenues (33-35).

In spite of the relatively low cost of lignocellulosic biomass, the price of feedstock is a major factor for the process economics. Its contribution to the total cost has been estimated to be between 30% and 40% (36). A significant portion of this comes from harvesting practices and logistics (30, 36). The considerably lower bulk density of lignocellulosic biomass results in significant storage and logistical challenges (37), which increase with sourcing radius. The development of harvesting practices and supply systems for large-scale production is required to cut costs (38). Another aspect is to ensure steady year-round supply when supply varies from year to year, season to season, and region to region. From an economic perspective, a production plant should preferably be able to efficiently convert any biomass feedstock that is available at the required levels and at affordable prices in order to maintain productivity and profitability. The use of diverse feedstocks provides challenges, because the different properties of feedstocks can make them unequally well suited for a given process.

Cost reductions for the processing step encompass reductions in both capital costs and operational costs. By lowering the initial investment, the amortized capital costs can be reduced. Omitting the emergence of a technology shift that will change the entire scenario, the development of processes with a lower initial investment is the key to reducing capital costs. It can be achieved by simplifying operations, eliminating process steps, and accelerating conversion rates (33).

Operating costs can be reduced by lowering the inputs into the process and improving the conversion efficiency of the process. The cost-saving opportunities vary between different conversion routes. In the biochemical route, the energy and chemical inputs can be reduced by improving the integration of the overall process (33) and limiting the amount of chemicals and nutrients in the process (39-41), respectively.

When it comes to improving the conversion efficiency, there are two major problem areas in the biochemical route: the recalcitrance of the lignocellulosic material and the fermentative conversion efficacy. Sugar yields are low in the processing of native lignocellulosic biomass because of the inherent recalcitrance developed by plants to microbial and physical degradation. The processing operations for overcoming this recalcitrance and providing fermentable sugars are the most expensive steps. Improved and more cost-efficient pretreatment (33, 39, 42-44) and enzyme production (45), as well as improved efficacy of enzymes (45, 46), are needed to reduce the cost of fermentable sugars.

In the fermentative conversion step, the restrictions are based on the physiological limitations of the fermenting microorganism (47, 48). Microorganisms with improved tolerance to inhibitors—present in the raw materials and generated during

pretreatment—that are capable of converting a multitude of sugars more efficiently need to be identified or engineered (49). An alternative approach is to modify the conversion process so as to mitigate the impact of inhibitors and promote fermentation efficiency (48, 50, 51). High ethanol yields and high final ethanol concentrations have been identified as key factors for the economy of the fermentative conversion step (52). High yields make the most out of the employed feedstock and thus minimize the feedstock cost. High ethanol concentrations reduce the energy requirements in downstream processes (53) and thus reduce the operational costs. To achieve these benchmarks, efficient pretreatment, hydrolysis, and fermentation, as well as the utilization of a variety of sugars present in the feedstock, are necessary. The challenges in the biochemical route are to advance the decomposition processes and conversion technologies so that fuels and chemicals from cellulosic resources are competitive without subsidies.

1.5 Scope and outline of the thesis

In this thesis, the cofermentation of xylose and glucose derived from single agricultural feedstocks and feedstock blends is discussed. The aim has been to improve xylose conversion and ethanol yields in non-detoxified hydrolysates and to assess the feasibility of employing feedstock blends with maintained ethanol productivities and yields. The thesis addresses the need for improved conversion efficiency and an expanded feedstock base. The work has focused on improving xylose utilization by improving the yeast phenotype during propagation and by applying novel feeding and fermentation strategies to accommodate microbial substrate consumption patterns during cofermentation. Further, the feasibility of scaling up and employing feedstock blends with the devised strategies is assessed.

The thesis summarizes the findings of **Papers I** through **V** and aims at putting the findings into a broader process context. The first three chapters of the thesis provide a brief background on the research field. Chapter 1 puts the thesis and the publications into a broader societal context, Chapter 2 describes the availability and compositions of the primary lignocellulosic feedstocks, and Chapter 3 outlines the lignocellulose-to-ethanol process. In the following chapters, strategies to improve ethanol yield and the progression toward commercialization are discussed in greater depth. Chapter 4 discusses the improvement in fermenting microorganisms by metabolic engineering and adaptation strategies, and **Paper I** is discussed. In Chapter 5, the main fermentative conversion concepts, their implications, and **Papers II** and **III** are discussed. Chapter 6 delves into commercial aspects, such as mixed feedstock bases and scale-up of fermentation processes, and **Papers IV** and **V** are discussed. Chapter 7 summarizes the most important findings and proposes future work.

2. Structure of lignocellulosic feedstocks

The cell wall of the plant is the source of lignocellulosic biomass. It defines the macroscopic structure of the plant and has been evolved to withstand microbial degradation and environmental conditions. The cell wall of the plant consists primarily of cellulose, hemicellulose, and lignin—collectively known as lignocellulose—but also small fractions of proteins, extractives, and inorganic compounds. The cellulose polysaccharide provides structural support and tensile strength and is embedded in a cross-linking matrix composed of hemicellulose and lignin in the outer secondary cell wall. The ultimate structure depends on the plant's taxon, tissue, cell type, and age (10). Due to its highly recalcitrant and complex structure, the decomposition of lignocellulose is one of the principal challenges for the commercial production of lignocellulosic ethanol.

Lignocellulosic materials are often subdivided into softwoods (gymnosperms), hardwoods (angiosperms), and herbaceous crops. This division is based on their composition and common characteristics, although the variations within each category are broad (Table 1). The relative proportion of cellulose, hemicellulose, and lignin is one of the determining factors in identifying the suitability of plant species as feedstock for second-generation bioethanol production.

Table 1. Composition of various lignocellulosic feedstocks.

Examples of typical compositions of various groups and types of lignocellulosic materials expressed as % of dry weight.

| | Glucan | Mannan | Galactan | Xylan | Arabinan | Lignin | Ash | Reference |
|------------------|------------------|----------------------|----------|-------|----------|--------|---------|-----------|
| Herbaceous crops | Cellulose: 24-50 | Hemicellulose: 12-38 | | | | 6-29 | 0,9-20 | (54, 55) |
| Wheat straw | 32-49 | 0-0.3 | 0.7-0.8 | 18-31 | 2-4 | 23.4 | 4-11 | (56, 57) |
| Corn stover | 36-44 | 0.3-1.8 | 1-3 | 16-25 | 2-6 | 16-22 | 6-8 | (56, 58) |
| Barley straw | 29-40 | 0 | 1-2 | 16-35 | 3-5 | 6-24 | 3-9 | (56, 57) |
| Bagasse | 40 | 0.3 | 0.5 | 21.1 | 1.9 | 25 | 4 | (56) |
| Rice straw | 34 | | | 24.5 | | 12 | 16 | (56) |
| Softwood | Cellulose: 41-50 | Hemicellulose: 11-33 | | | | 19-30 | 0,3-6 | (54-56) |
| | 41-50 | 10-12 | 0-3 | 6-9 | 1-3 | 27-29 | 0,3-0,4 | (54-56) |
| Hardwood | Cellulose: 39-53 | Hemicellulose: 19-36 | | | | 17-24 | 0,5-8 | (54-56) |
| | 42-53 | 0.5-5 | 0-1 | 13-20 | 0.3-13 | 21-29 | 0,5-1 | (54-56) |

2.1 Cellulose

Cellulose is generally the largest fraction of biomass, representing about 30% to 50% dry weight (59). This polysaccharide consists of anhydroglucopyranose units that are joined together by β -1,4-glycosidic bonds, forming a linear condensation polymer (60). The basic repeating unit in cellulose is cellobiose, the β -1,4-linked disaccharide of glucopyranose. The degree of polymerization of β -(1,4)-glucopyranose polysaccharide chains varies between 100 to over 15000, depending on the source of cellulose (60). The cellulose chains are organized into progressively more complex structures. The anhydroglucopyranose units are rotated 180° with respect to each other, forming hydrogen bonds along the chain that maintains and reinforces the flat linear structure of the polysaccharide chain (61). The resulting topology is fundamental for the formation of the crystalline cellulose microfibril. The formation of hydrogen bonds and van der Waal bonds between chains elicits parallel oriented crystalline nanostructures (61). The stacking of hydrophobic faces elicits highly structured crystalline configurations (10, 61). However, the structure is interspersed with less ordered amorphous regions (61). The spatial organization of these polymers makes the structure of cellulose complex. On a macroscopic level, fibrils have a tendency to form microfibril aggregates because of hydrogen bonding between fibrils (10), thus increasing the structural complexity of cellulose.

The crystalline structure of cellulose with stabilizing intra- and intermolecular bonds makes cellulose recalcitrant to not only chemical hydrolysis but also enzymatic degradation (62). Declining hydrolysis rates have been further attributed in part to the crystalline structure of the cellulose and to other factors that are closely related to the crystallinity (63). This has implications with regard to the process, because it causes subsiding hydrolysis rates and often incomplete enzymatic hydrolysis (62).

2.2 Hemicellulose

The tensile strength of cellulose microfibrils is reinforced by the surrounding matrix of hemicelluloses (10). Hemicelluloses are heterogeneous polysaccharides, where most of the components are amorphous and possess branched structures with neutral or acidic side chains (64). Hemicellulose has been attributed with the role of a cross-linker in the cell wall matrix. It is cross-linked or chemically associated with cellulose microfibrils, proteins, lignin, and other hemicelluloses (10) and thus provide rigidity to the macroscopic structure. The degree of polymerization between different types of hemicellulose varies, but it is lower than that of cellulose (64). The composition and features of hemicellulose vary widely across species, growth conditions, and developmental stage of the plant (10). They are usually classified according to the

predominant sugar in the polysaccharide backbone. The polysaccharide backbone is composed of various sugars—both hexoses, such as glucose, mannose, and galactose, and pentoses, such as xylose and arabinose. In hardwoods and herbaceous crops, hemicelluloses with a β -1,4-xylopyranose backbone, commonly referred to as xylan, are the main types of hemicellulose. They generally constitute up to one-third of the total plant biomass (64, 65). In some tissues of grasses and cereals, xylans can account for up to 50% of the biomass (66). Glucuronoxylans are the predominant backbone polysaccharide in hardwood, while arabinoglucuronoxylans are the predominant hemicelluloses in herbaceous crops (65). In contrast, softwoods are typically dominated by galactoglucomannan (15% to 20% dry weight), and xylans only constitute 5% to 10% dry weight (67).

Neutral or acidic substituents frequently occur in the branched hemicellulose structure. Acetyl groups, ferulic acid esters, and glucuronic acid can be attached to the backbone or side chains, generating highly diverse polysaccharides with wide-ranging properties. Side groups in the hemicellulosic structure, especially acetyl substituents, affect the biodegradability of soluble and matrix-bound hemicelluloses (10). The prevalence of acetyl substituents in hemicellulose varies between species, but they are prominent in hardwoods and herbaceous crops (65).

2.3 Lignin

Lignin is the third major constituent in lignocellulosic biomass. It is a generic term for a diverse group of aromatic polymers, resulting from the combinatorial coupling of 4-hydroxyphenylpropanoids (68). The structure is formed mainly by the monolignol precursors coniferyl alcohol, sinapyl alcohol, and minor amounts of *p*-coumaryl alcohol (69). When incorporated into the lignin polymer, the moieties resulting from the monolignol precursors are denoted guaiacyl, syringyl, and *p*-hydroxyphenyl, respectively. A variety of less abundant units have been identified from diverse species, and they may be incorporated into the polymer to varying extents (68). The moieties are linked together in a complex heterogenic polymer, which exhibits considerable diversity in composition across taxa, species, and cell types (69). Lignins in softwoods are composed principally of guaiacyl moieties, with minor amounts of *p*-hydroxyphenyl moieties, whereas hardwood lignins are composed of guaiacyl and syringyl moieties (69). In contrast, lignins of herbaceous plants contain all three moieties (guaiacyl, syringyl, and *p*-hydroxyphenyl) in significant amounts (70, 71), although at different ratios, depending on species (71). Lignins in herbaceous crops can be further naturally acetylated, coumarylated, and *p*-hydroxybenzoylated, which has consequences on the structure and processing of the material (71).

The lignin in plant tissues is bonded with cellulose and hemicellulose, forming lignin-carbohydrate complexes. The chemical nature of the carbohydrate matrix and the orientation of cellulose microfibrils influence the deposition of lignin (70) and thus the ultimate structure of the lignin-carbohydrate complexes. The structure of the lignin-carbohydrate complexes differs between softwoods, hardwoods, and herbaceous crops, and the ultimate structure is a key determinant of the availability of carbohydrates (71). The lignin-carbohydrate complexes contribute to the rigidity of plants and their resistance to microbial degradation and thus to the recalcitrance of lignocellulosic material. The presence of lignin provides a major obstacle to the decomposition of lignocellulosic materials for the production of biochemicals and biofuels (72).

2.4 Extractives and inorganic compounds

Biomass also contains low amounts of non-structural components, often referred to as extractives. They consist of extracellular and low-molecular-weight compounds that are soluble in neutral organic solvents and water (67). In woody materials, extractives generally encompass compounds, such as terpenoids, steroids, fats, waxes, and phenolics (67). The content of extractives in wood and woody material is usually less than 10% of the biomass but can exist at trace amounts up to 40%, depending on species and cell type (67). A major function of extractives in woody plants is to protect the living tree from pathogens; therefore, they may also be toxic to ethanol-producing organisms (67, 73). Herbaceous biomass is more enriched in extractives than wood and woody biomass (74). Water and ethanol extractives from native herbaceous crops include non-structural sugars, organic acids, inorganic material, nitrogenous material, chlorophyll, waxes, and other minor components (74).

Inorganic materials are present in both unprocessed and extracted biomass. Structural inorganic compounds are bound in the physical structure of the biomass, whereas extractable inorganic compounds can be removed by washing or extraction (74). Wood and woody materials generally contain approximately 1% inorganic compounds (67), while there can be considerably more in herbaceous materials (55). It has been found that annual and fast-growing crops have the highest content of inorganic compounds (55). From a process perspective, the inorganic material that is present in the biomass may have buffering capacity and thus interfere with acid hydrolysis during pretreatment (74). The selection of agricultural feedstocks, and anatomical parts thereof, with low ash content can reduce the uncertainties regarding pretreatment efficiency and reduce the need for acid catalyst in the pretreatment (75).

3. Ethanol production from agricultural residues

Cellulose and hemicellulose are polysaccharides that can be depolymerized to monomeric sugars and fermented or chemically altered to value-added fuels and chemicals. There are numerous routes, based on combinations of mechanical, thermal, chemical, and biological processes, for the conversion of biomass to ethanol (39, 76, 77). Biochemical decomposition and conversion of lignocellulose to ethanol, via a sugar platform, is regarded as one of the most promising alternatives for large-scale production (77). The decomposition of biomass to fermentable sugars can be performed in one step, as demonstrated by concentrated acid and dilute acid hydrolysis (78). However, these methods encounter problems, given their high usage of chemicals, corrosive environments, and substantial sugar degradation (78). Economic and environmental considerations have driven the development toward two-step processes, where a pretreatment step increases the accessibility of the lignocellulosic structure and the subsequent enzymatic hydrolysis step liberates fermentable sugars. Enzymatic hydrolysis offers the advantages of low energy demand, less sugar degradation, and less corrosive environments (77, 78). The sugars are subsequently fermented by microorganisms to ethanol, and the product is recovered. The biochemical conversion process from feedstock to product largely comprises five steps: mechanical size reduction, pretreatment, enzymatic hydrolysis, fermentative conversion of the sugars to ethanol, and product recovery (Figure 1).

3.1 Size reduction

Size reduction can be applied either as a mechanical pretreatment step or as a preconditioning step for pretreatment methods. Because the milling of biomass to small particles is energy-intensive and costly, pretreatment technologies that require limited size reduction are desirable (44). Size reduction of the feedstock that enters the process is needed for most processes and pretreatment technologies. Agricultural residues, like wheat straw and corn stover, which were used in **Papers I-III and V**, can be expected to require the use of a bale-based feedstock supply system (30). The bales need to be shredded and size reduced to ease the handling of the feedstock.

The complex structure of lignocellulosic biomass makes it recalcitrant to degradation. The factors that contribute to the recalcitrance of lignocellulosic biomass include the crystallinity and degree of polymerization of cellulose, pore size and size distribution, accessible surface area, and shielding of cellulose by lignin and hemicellulose (39, 85). In native biomass, the accessibility of enzymes is limited (39, 78, 86), which results in slow and low enzymatic degradation of biomass (39). The primary task of the pretreatment is to reduce the size of the solids in the biomass and alter the structure of the biomass to make it more susceptible to enzymatic degradation (39). This is achieved by applying treatments that increase the accessible surface area by fragmentation and size reduction; hydrolysis and solubilization of hemicellulose, which reduces the shielding effects of cellulose; and making structural changes to the lignin and delignification (85, 86).

Pretreatment is usually carried out with combinations of physical, thermal, chemical and biological methods (39). Various pretreatment technologies are available, such as acid-catalyzed hydrolysis, alkaline hydrolysis, steam explosion, liquid hot water, biological pretreatment, ionic liquid extraction, and ammonia fiber explosion, which are reviewed elsewhere (39, 42, 77, 87-89). The appropriate choice of pretreatment method is determined by the properties of the biomass and economic considerations (44, 89). However, the autocatalyzed and dilute sulfuric acid-catalyzed steam pretreatments methods are widely regarded as the most economic and efficient pretreatments to pursue commercially in the short term (18, 77, 90) and are currently implemented in pioneer commercial production plants (91). Dilute sulfuric acid-catalyzed pretreatments have been shown to be applicable to a wide range of biomasses (39), including agricultural residues. Autocatalysis is often enough to overcome the recalcitrance of agricultural residues due to the high degree of acetylation in herbaceous crops (65, 92). However, acid catalysts enable the use of lower reaction temperatures and shorter holdup times, which can reduce dilution and inhibitor formation (92-94). Dilute sulfuric acid-catalyzed steam pretreatment on the bench and demonstration scale was used in **Papers I through V** to pretreat various agricultural residues. The primary mechanism for improving the susceptibility of the biomass to enzymatic hydrolysis is the hydrolysis and solubilization of hemicellulose, although cellulose may be partly hydrolyzed and although lignin is to some extent solubilized, structurally altered, and redistributed in the material (39, 95).

Inhibitors

A drawback of many pretreatment methods is the generation of inhibitory compounds by secondary decomposition processes (Figure 2). The degradation products that are formed by pretreatment of lignocellulosic biomass depend on both the biomass and the pretreatment conditions—e.g., temperature, time, pressure, pH, and catalysts (54). The most prominent inhibitors are commonly divided into three

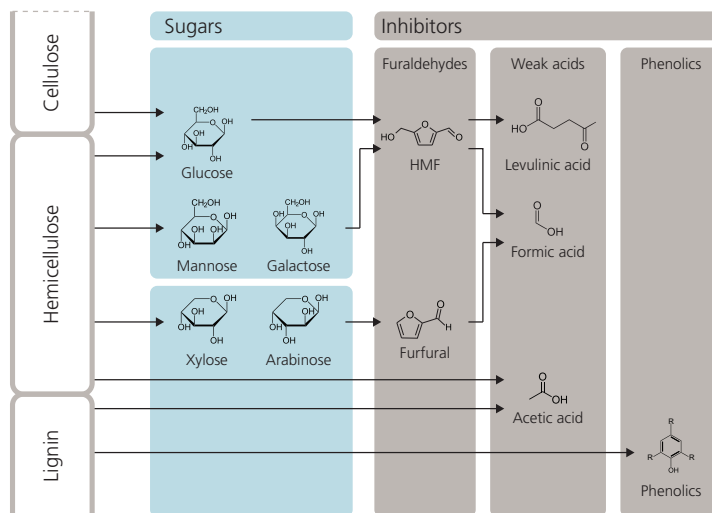


Figure 2. Common inhibitors in pretreated lignocellulosic biomass.

Overview of the origin and formation of principal inhibitors in the lignocellulose-to-ethanol process. Adapted from Palmqvist and Hahn-Hägerdal (48).

groups: furaldehydes, weak acids, and phenolics (Figure 2). These groups have been demonstrated to have various inhibitory impacts on the fermenting microorganisms (48, 50) and enzymes (96) and impose demands on the fermenting microorganism and fermentation design to successfully convert lignocellulose to ethanol.

The decomposition of hemicellulose liberates various hexoses (glucose, mannose, and galactose) and pentoses (xylose and galactose), whereas cellulose is decomposed to glucose (Figure 2). At high temperatures and pressures, these sugars can be degraded further to furaldehydes. 2-furaldehyde (furfural) is formed by the degradation of pentoses, and similarly, hexoses can be degraded to 5-hydroxymethyl-2-furaldehyde (HMF) (50). In addition, secondary degradation of lignocellulosic sugars can generate weak acids. Formic acid is derived from sugar and lignin degradation (48, 97), and levulinic acid is formed by the degradation of HMF (48). Both are formed at elevated temperatures (97), although the extent to which this occurs varies with the pretreatment method and conditions (54, 98). Acetic acid is liberated from structural carbohydrates during pretreatment, mainly by the deacetylation of hemicellulose (48) but also to some extent from lignin (54, 71). The last group, phenolic compounds, are products of the structural change and degradation of lignin (54). Formation of phenolic compounds has also been reported to occur during carbohydrate degradation (48). The amount and type of phenolic compound depend on the composition and structure of the lignocellulose in the biomass source and the pretreatment conditions (54, 99).

Furaldehydes typically have inhibitory effects on both cell growth and specific ethanol productivity of the fermenting microorganism (50), and synergistic effects between different furaldehydes have been demonstrated (100). Cell growth is regarded as being more sensitive to inhibition by furaldehydes than ethanol production (101). In general, microorganisms have the ability to convert HMF and furfural to their corresponding, less inhibitory alcohols (101, 102), although HMF has been reported to be converted at a lower rate than furfural (103). The inhibitory effects can thus be overcome as long as viability of the microorganism can be preserved.

Weak acids have inhibitory effects on the cell growth of the fermenting microorganism (48). The underlying mechanism of inhibition has been suggested to be acidification of the cytosol caused by influx of liposoluble undissociated weak acids (48). The weak acids have different toxicities at the same concentration of undissociated acid (48), which has been attributed to differences in membrane permeability and the toxicity of the anionic form in the cytoplasm (103). The concentrations of undissociated weak acids in lignocellulosic hydrolysates are dependent on pH and the pKa values of the acids; thus, increasing pH during fermentation can counteract the inhibition by weak acids.

The inhibitory effect of phenolic compounds decreases both the growth rate and ethanol productivity of the fermenting microorganism (98), even though the growth rate is more hampered than ethanol productivity (98). Various correlations between the degree of inhibition and the structural features of phenolic compounds have been found (98), but the inhibition mechanisms of phenolic compounds have not yet been completely elucidated. This is in part because of the heterogeneity of the group. Various mechanisms have been proposed and are reviewed elsewhere (48, 54). Phenolic and lignin degradation compounds have also been shown to have an inhibitory effect on cellulolytic and hemicellulolytic enzymes (96). The strength of the inhibitory effect depends on the type of enzyme, the microorganism from which the enzyme is derived, and the type of phenolic compounds present (96). Various cellulases differ in their inhibition by lignin, while xylanases and glucosidases are less affected by lignin and lignin derivatives (104).

Despite the inhibitory nature of furaldehydes and acetic acid, these compounds can have beneficial effects. Low concentrations of furfural can improve the fermentation of lignocellulosic hydrolysates by acting as external electron acceptors for the recycling of co-factors needed in cellular metabolism (105). Further, low concentrations of weak acids have been shown to have a stimulating effect on ethanol production by the microorganism in the fermentation of lignocellulosic hydrolysate (103). Increased demand for ATP to maintain intracellular pH increases ethanol production at the expense of biomass formation under anaerobic conditions (48).

3.3 Enzymatic hydrolysis

After pretreatment, most of the cellulose is recovered as fragmented and chemically altered polymers. Depending on the pretreatment method and applied conditions, various amounts of hemicellulose are recovered as oligomers and polymers, either solubilized or as part of the lignocellulosic solids. Enzymatic hydrolysis provides a second step of hydrolysis, where cellulose is converted into glucose and hemicellulose is converted into various monomeric sugars. The second hydrolysis step is needed, because the most commonly utilized microorganisms for fermentative conversion only take up and metabolize monomeric sugars. In contrast to acid-catalyzed hydrolysis, the high specificity and mild conditions of the enzymatic degradation of cellulose can potentially result in high sugar yields and relatively nontoxic hydrolysates (39, 77). Because of the heterogeneity and complexity of lignocellulosic substrates, enzymatic hydrolysis requires the use of enzyme systems that efficiently degrade both cellulosic and hemicellulosic structures. These enzyme systems can either be produced by the fermenting microorganism (consolidated bio-processing) or added from external sources. The enzymes that were used in **Papers I** through **V** were commercial enzyme preparations that were supplied from external sources. The components of the enzyme systems are reviewed below.

Enzymatic hydrolysis is usually performed at a moderate pH—pH 4 to 6 for most commercial enzyme systems (106)—and at temperatures below 70°C (106). Whereas the hydrolysis rate is favored by higher temperatures, the stability of the enzymes is not (45). Thus, enzymatic hydrolysis is performed at temperatures that combine high activity with sustained enzyme integrity (45). However, in integrated process designs where hydrolysis and fermentation are performed simultaneously, hydrolysis is usually performed at lower temperatures, which is discussed further in Chapter 5. The enzyme load that is needed to digest the pretreated biomass is dependent on the composition of the biomass, pretreatment method, and the properties of the enzyme system (95).

The main factors influencing the performance of enzymatic hydrolysis relates to either the nature of the enzyme system or the properties of the pretreated biomass, and many of these factors are intertwined. Typically, enzymatic hydrolysis is limited by the degree of crystallinity and polymerization; the size of the solids and accessible surface area; porosity; and shielding effects by hemicellulose and lignin (95). These factors govern the amount of accessible reactive sites.

Furthermore, declining hydrolysis rates and stalled enzymatic hydrolysis are common features linked to the limiting factors (62). A contributing factor is the subsiding degree of polymerization, which is correlated to the increasing recalcitrance of residual crystalline cellulose (62). Additionally, enzymes can be physically trapped in the pores

(60), bind nonproductively to lignin and substrate (104), or be inhibited by the end-products (62), thus contributing further to the declining hydrolysis rates.

Another factor that influences the efficiency of enzymatic hydrolysis is the solids loading. It has been shown for several lignocellulosic substrates that increased solids loadings decrease the corresponding hydrolytic yield (107). The effect has been attributed to increased product inhibition (108, 109), inhibition by sugar-derived inhibitors and lignin (110, 111), and mass transfer limitations and other effects that are related to increased solids loadings (112). However, the increased inhibition primarily affects the hydrolysis rate and not the maximum conversion or yield—given sufficient time.

Cellulolytic enzymes

The most widespread commercial cellulolytic products currently available for biomass hydrolysis are produced by *Trichoderma reesei* strains (45, 106). The main group of cellulolytic enzymes comprises cellulases, a broad category of enzymes that can hydrolytically cleave β -1,4-glycosidic bonds (10). These enzymes are the primary actors in enzymatic systems that hydrolyze cellulose. These enzymes are usually active at a pH of 4 to 6, with an optimal pH of around 5, and at temperatures below 70°C, optimally around 50°C (106).

The general classification is based on the different types of activity, and cellulolytic enzymes are broadly categorized into: endoglucanases, exoglucanases, and β -glucosidases. Endoglucanases (EC 3.2.1.4) liberate glucose from soluble and insoluble 1,4- β -glucan structures (10). The hydrolytic action of endoglucanases acts randomly in 1,4- β -glucan structures to reduce the degree of polymerization, preferably in the amorphous regions of cellulose (113). Exoglucanases include both β -1,4-glucan glucohydrolases (EC 3.2.1.74) and β -1,4-glucan cellobiohydrolases (EC 3.2.1.91). Both enzyme groups bind and act on the reducing and nonreducing ends of β -1,4-glucan chains. However, whereas β -1,4-glucan glucohydrolases split off glucose units from the end of the cellulose chain and slowly hydrolyze cellobiose, β -1,4-glucan cellobiohydrolases cleave cellobiose from the β -1,4-glucan chain (10). Exoglucanases are the only group that efficiently degrades crystalline cellulose (113). The last group, the β -glucosidases (EC 3.2.1.21), act on the β -1,4-glycosidic bonds in cellobiose and cellodextrins to liberate glucose (10). The actions of different types of cellulolytic enzymes work cooperatively to solubilize high-molecular-weight cellulose molecules (113). The correct combination of the activities and production level of each cellulase enzyme is critical for efficient lignocellulosic biomass utilization (114).

Hemicellulolytic enzymes

A major group of accessory enzymes that complement the cellulolytic enzyme system and enhance the enzymatic hydrolysis of lignocellulosic materials is the hemicellulolytic enzymes. This diverse group of enzymes degrades hemicellulose to mainly monomeric sugars and acetic acid. Most hemicellulase systems are produced by genetically modified *Trichoderma* and *Aspergillus* strains (10). Most enzymes that are expressed in these host organisms are active at a pH of between 4 and 6 and at temperatures below 70°C (10).

The diversity and complexity of hemicellulosic structures require a broad range of enzymes with different specificities (113), as well as a high degree of coordination between the involved enzymes (10), to degrade hemicellulose. Enzymes that degrade hemicellulose can be divided into depolymerizing enzymes, which hydrolytically cleave the hemicellulosic backbone, and accessory enzymes that remove substituents, which may pose steric hindrances to the depolymerizing enzymes (115). The core enzymes for the degradation of xylan backbones are endoxylanases and β -xylosidase (10). Endoxylanases catalyze the random hydrolysis of the xylan backbone into shorter oligosaccharides, and β -xylosidase cleaves shorter xylan fragments into xylose. Similarly, the core enzymes for the degradation of mannan are endomannanase and β -mannosidase (10). The substituents that are linked to the xylan and mannan backbones require an assortment of accessory enzymes for their removal. The accessory enzymes give the core enzymes access to the backbone and liberate substituents, such as sugars and acetyl groups (10). The role and nature of these accessory enzymes are reviewed elsewhere (10, 65, 113, 115).

Synergism, coordinated action, and accessory proteins

The synergistic and cooperative actions of cellulolytic enzymes are central for the enzymatic hydrolysis of cellulose. The synergistic action between exoglucanases and endoglucanases is particularly important to degrade cellulose and to solubilize high-molecular-weight cellulose molecules (113). This exo-endo synergism is based on the formation of new sites for the action of exoglucanases by random cleavage of the cellulose chain by endoglucanases (10). Cooperative effects have been observed between different exoglucanases; between different endoglucanases; between exoglucanase or endoglucanase and β -glucosidase, which reduces inhibition by cellobiose; and by proximity synergism due to the formation of cellulase complexes (60). Additionally, the synergism and cooperative effects have been reported to be greater under conditions that minimize inhibition by soluble hydrolysis products (60).

The cooperative interaction of the cellulolytic and hemicellulolytic systems has been shown to enhance enzymatic hydrolysis by improving the accessibility of cellulose,

through increasing fiber swelling and porosity, and enhancing enzyme accessibility (116). Coordination between hemicellulolytic enzymes is generally observed by the improved action of endoglucanases and accessory proteins (10). The actions of hemicellulolytic enzymes improve the accessibility to cellulosic structures. Further, cooperative actions between xylanases, β -xylosidase, and α -glucuronidase have been observed, where xylanolytic activity improves the hydrolysis of substituted xylans (10). Another important cooperative action takes place between esterases and hemicellulolytic enzymes. Little or no liberation of sugars occurs from acetylated xylans without the addition of acetyl esterase or acetyl xylan esterase, which is attributed to the disruptive effect of acetyl groups on the hydrolysis of xylan (10). The cooperative action of esterases and hemicellulases is thus central for accessing hemicellulosic sugars.

Enhanced enzymatic hydrolysis by synergetic actions can also be obtained by the introduction of additional accessory enzymes. The introduction of lytic polysaccharide monoxygenases (LPMOs) has improved the performance of commercial enzyme cocktails (117). LPMOs oxidatively cleave the cellulose chains and act cooperatively with hydrolytic enzymes (117). In addition to enzymes that act directly on the covalent bonds in structural carbohydrates, enzymes and proteins with indirect actions on hydrolysis might be of importance to the breakdown of cellulose and hemicellulose. These enzymes fulfill tasks, such as loosening up the structure of the lignocellulose (e.g., swollenins and expansins) (117, 118), degrading nonglycosidic wall components (e.g., ligninolytic enzymes) (46, 119), and degrading small molecules that inhibit the degrading enzymes (46).

3.4 Fermentation

At the core of the biochemical conversion route is the fermentative conversion of liberated sugars into products (Figure 1). The objective of fermentative conversion is to biologically catalyze the conversion of sugars into suitable end-products through cellular metabolism, either through naturally occurring or engineered pathways. Fermentative conversion is proven technology that, if appropriately performed, can convert sugars into suitable end-products with high specificity and high yields. Fermentative conversion is typically performed at moderate temperatures, pH levels, and pressures, although the optimal conditions are dependent on strain and fermentation configuration.

Fermentative conversion of biomass for first-generation bioethanol targets glucose fermentation. The available sugars in lignocellulosic biomass differ greatly from first-generation substrates. While efficient glucose fermentation is still a central aspect, the lignocellulosic material also contains considerable amounts of pentoses, mainly xylose

but also arabinose. Given the importance of realizing high ethanol yields and high ethanol concentrations after fermentation, it is important to access and convert the abundant amount of xylose present in agricultural residues (52). By adding xylose as a fermentation substrate, we move into the realm of cofermentation, where a new dimension of complexity is added to fermentative conversion. Even though xylose can replace glucose as a primary carbon source in many ways (64), it is not a natural substrate for all microorganisms. The extended range of substrates puts demands upon the selection of the fermenting microorganism or consortium. In addition, the omission of detoxification of lignocellulosic hydrolysates can reduce the need for chemical inputs but further places constraints on fermentative conversion, which needs to be addressed (120). The selection of an appropriate fermenting microorganism or consortium and the fermentation strategy is central to elicit efficient conversion of lignocellulose-derived sugars to ethanol.

Fermenting microorganisms

The ideal microorganism or system of microorganisms for the conversion of biomass-derived sugars to ethanol would simultaneously ferment and grow on sugars derived from both cellulose and hemicellulose; be able to tolerate or detoxify inhibitors *in situ*; and produce ethanol at high selectivity, high rates, and high concentrations (49). Most wild-type microorganisms and combinations of microorganisms fall short of this. The inability to utilize the range of carbohydrates present in biomass while producing ethanol at high yields and different oxygen requirements between organisms often restrict their application (77, 121). Addressing these limitations is a key factor in choosing fermenting microorganisms and for improving and developing microorganisms for ethanol production.

Several candidates that are natural xylose converters or that have been genetically modified to do so are being considered as fermenting microorganisms for ethanol production. They include among others the yeasts *Saccharomyces cerevisiae* (49, 122), *Scheffersomyces stipitis* (123), and *Dekkera bruxellensis* (124) and the bacteria *Zymomonas mobilis* (49, 122) and *Escherichia coli* (125). All potential candidates have advantages and disadvantages with respect to their ability to convert a range of sugars, tolerance of inhibitors, productivity, ethanol yield, growth, and ability to sustain viability under anaerobic conditions. The properties of these microorganisms with regard to ethanol production have been reviewed elsewhere (49, 122-125).

One of the prime candidates for the production of ethanol from biomass-derived sugars is *S. cerevisiae*. Its use is well documented in the first-generation bioethanol industry, it is generally regarded as safe (GRAS), and the process technology for large-scale production is well established (49). It has high ethanol productivity and tolerance, is tolerant to many inhibitors that are generated by thermochemical pretreatment (126), and is a facultative anaerobe—i.e., it can grow under both

aerobic and anaerobic conditions (127). However, wild-type *S. cerevisiae* is largely unable to convert pentoses into ethanol without genetic modification (128). Being one of the most well-characterized microorganisms, there is a range of genetic engineering tools available for *S. cerevisiae* (122), which, together with the inherent benefits of the strain, makes it a suitable host for genetic engineering. Exogenous genes that encode for xylose reductase and xylitol dehydrogenase (129, 130), as well as xylose isomerase (131), have been introduced into the *S. cerevisiae* genome to enable xylose to be assimilated, which is discussed further in Chapter 4. In **Papers I through V**, xylose-fermenting *S. cerevisiae* harboring exogenous xylose reductase and xylitol dehydrogenase and overexpressing endogenous xylulokinase is used.

However, the fermentation of xylose to ethanol by engineered *S. cerevisiae* is slower and generally results in lower ethanol yields than glucose fermentation (132). Considerable effort has also been made to improve both the genotype and phenotype of recombinant *S. cerevisiae* strains to improve their tolerance of inhibitors, xylose utilization, and conversion rates and yields, which is discussed further in Chapter 4. The design of the fermentation step can also alleviate the effects of many of the constraints imposed by the physiology of the selected fermenting microorganism, which is discussed below and in Chapter 5.

Process configurations

The appropriate process configuration to maximize sugar conversion rates and ethanol yields is highly intertwined with the characteristics of the fermenting microorganism and the properties of the pretreated material. Restrictions in the capabilities of the fermenting microorganisms early on brought about separate fermentation steps for different sugars (77). However, improved capabilities of the fermenting microorganisms have increased the opportunities for process integration, enabling efficient conversion of multiple sugars by a single microorganism, as well as the integration of enzymatic hydrolysis and fermentation (77).

The two fundamental process configurations for fermentative conversion of biomass-derived sugars are separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF), also referred to as SHCF and SSCF when multiple lignocellulose-derived sugars are cofermented. Both strategies have inherent benefits and limitations. Whereas SHCF benefits from the opportunity for independent optimization of the sequential processing steps, SSCF benefits from synergies between the integrated processes. Opting for either strategy is generally a trade-off between optimal temperatures and inhibitory glucose concentrations during hydrolysis (SHCF) and suboptimal temperatures and ethanol-inhibited cellulolysis (SSCF). Ethanol yields have been improved with both process frameworks by process modifications that generate hybrid fermentation schemes. Typical modifications are the integration of different process streams (133, 134), which is also addressed in

Papers II and III; the use of fed-batch strategies in which both enzymes and substrate are fed to the bioreactor (135-137), which is addressed in **Papers II-IV**; segmented fermentations—e.g., prefermentation (138), which is addressed in **Papers II-IV**; and combinations thereof (139, 140), which is addressed in **Papers II-IV**. The use of hybrid fermentation strategies provides a tool to improve xylose utilization and conversion efficiency by catering to the preferences of the fermenting microorganism and overcoming limitations in hydrolysis. The benefits, limitations, and opportunities of different hybrid designs to facilitate the cofermentation of biomass-derived glucose and xylose are discussed further in Chapter 5.

3.5 Product recovery

The standard ethanol purification train involves two distillation steps and a subsequent dehydration step by molecular sieve adsorption (35, 141). First, the fermentation product is sent to a stripper column. The recovered top product is further purified by a rectifier distillation column and subsequently dehydrated to 99.5 wt% by vapor-phase molecular sieve adsorption (141). One of the main benefits of using distillation for product recovery is its high ethanol recovery—i.e., only 0.1% of the ethanol from fermentation is lost in the bottoms of the distillation columns (36). However, the energy demand of the distillation train is highly dependent on the ethanol concentration in the fermentation broth. At low ethanol concentrations in the fermentation broth, the energy requirements for distillation increase significantly (53). Therefore, a high ethanol concentration after fermentation is a key factor for cost-efficient product recovery (52).

Today's lignocellulose-to-ethanol process typically elicits ethanol concentrations of 3% to 6%, based on lab-scale and pilot-scale studies (35, 36, 53). This is on par with a threshold of 4–5 wt%, which is generally regarded as the lower limit for cost-effective recovery of ethanol (53, 142). To further increase the ethanol concentration and reduce the energy demand in the product recovery step, greater substrate loadings during fermentation are needed (143). Moreover, increased substrate loadings reduce the water requirement, thus lowering the energy demand in the product recovery and reducing the capacity that is needed for waste treatment (35, 53). The energy demand of the product recovery train thus imposes targets for the fermentation in order to improve the overall process economics.

Heat integration of the product recovery process is critical for reducing overall biomass-to-alcohol energy usage (53). A vital part of the process integration is that lignin and other components that are not converted into useful products can be recovered. By fractionation of the residues after product recovery, various by-products can be produced (Figure 1). The recovered solids can be pelletized or burned to

provide the heat and electricity that are needed for the process (53, 144), and the excess can be sold. The liquid fraction can be used for biogas production, for example, thus providing a valorized by-product and reducing the cost and capacity of waste treatment (44). Depending on the feedstock and process design, the bioethanol plant can be self-sustaining on process heat (36, 144).

4. Improving the performance of *Saccharomyces cerevisiae*

One of the means to improve the conversion of biomass-derived sugars to ethanol and improve the cost-efficiency is to enhance the inherent properties of the fermenting microorganism. Wild-type microorganisms have a number of inherent bottlenecks that prevent efficient conversion, such as low conversion rates, the inability to utilize certain substrates, and tolerance to inhibitors that are present in lignocellulosic substrates. Fortunately, biotechnology disciplines have a toolbox to engineer and evolve microorganisms to overcome some of these bottlenecks. From process and economic perspectives, high ethanol yields and high ethanol productivity are the key features that are desirable in a fermenting microorganism (145). Considering the inhibitory nature of most lignocellulosic hydrolysates, inhibitor tolerance is a crucial trait for efficient cofermentation (126). It has been suggested that the engineering of metabolic capacities—e.g., novel pathways—into robust microorganisms may be easier than engineering and evolving tolerance to inhibitors and robustness (146). The inherently high robustness and tolerance of *S. cerevisiae* to various inhibitors makes it a suitable host for the introduction of novel metabolic capacities (126). Below, the fundamental metabolism of *S. cerevisiae*, the fermenting microorganism that is used in **Papers I through V** for the conversion of lignocellulose-derived sugars to ethanol and in approaches to improve its properties, is discussed.

4.1 Metabolism of *Saccharomyces cerevisiae*

S. cerevisiae has evolved to efficiently take up and metabolize hexose sugars under both aerobic and anaerobic conditions (147), while it is largely unable to metabolize pentoses (126, 148). The sugar assimilation pathways and connected central steps of yeast metabolism (Figure 3) are outlined briefly below, focusing on the phenomena that are central to the propagation of yeast and the fermentative conversion of lignocellulose-derived pentoses and hexoses to ethanol.

S. cerevisiae natively harbors genes encoding for several transporters for the uptake of sugars, such as Hxt1-17, which primarily facilitates glucose and fructose uptake, and

Gal2, which facilitates galactose uptake (149, 150). In practice, glucose is mainly taken up by the Hxt1-4, 6, and 7 transporters (150).

These transporters have different characteristics and affinities, and the expression of each gene is tuned by the extracellular glucose concentration (151). The high-affinity transporters are expressed at low extracellular glucose concentrations, and at high glucose concentrations, the low-affinity transporters are expressed (152). *S. cerevisiae* does not harbor specific transporters for xylose, but the Hxt2,4-7 and Gal2 transporters are able to facilitate the uptake of xylose (149, 153). Nonspecific hexose transport mechanisms have an affinity for xylose that is several-fold lower than for hexoses, which limits xylose uptake in the presence of hexoses (153, 154). This forms a bottleneck for efficient cofermentation of xylose and glucose and is therefore a target for strain improvement.

The assimilated glucose, mannose, and galactose are metabolized through glycolysis, although they are introduced through different routes. Glucose and mannose are phosphorylated by hexokinase and isomerized by their respective phosphate isomerases to fructose-6-phosphate (147). Galactose is introduced through the Leloir pathway and enters glycolysis as glucose-6-phosphate (155). In glycolysis, glucose-6-phosphate and fructose-6-phosphate are converted to pyruvate, eliciting a net formation of ATP and NADH (147). Pyruvate constitutes a branch point in metabolism (Figure 3). At the pyruvate branch point, the fluxes are channeled toward ethanol formation (fermentation pathway) or the tricarboxylic acid (TCA) cycle (respiratory pathway), depending on the availability of oxygen and the concentration of glucose in the surrounding media (156).

In respiratory metabolism, pyruvate is converted to acetyl-CoA and becomes subsequently fully oxidized to carbon dioxide in the TCA cycle, yielding redox cofactors that are used for the generation of ATP in the oxidative phosphorylation pathway (147). In the fermentation pathway, pyruvate is decarboxylated to acetaldehyde and further reduced to ethanol while consuming NADH (147). The net effect of the ethanol branch is the redox-neutral formation of ethanol from glucose (147). The fermentation pathways can also be active under aerobic conditions, resulting in aerobic alcoholic fermentation (156). This is known as respiro-fermentative metabolism, or the Crabtree effect. The Crabtree effect has implications during the aerobic propagation of yeast, where ethanol formation diverts carbons away from biomass formation.

Pentose metabolism in *S. cerevisiae* is not as well evolved as hexose metabolism. *S. cerevisiae* harbors genes for xylose utilization, but they are expressed at miniscule levels that are insufficient to support growth (148). It does not harbor arabinose-assimilating pathways, either. However, *S. cerevisiae* grows on the pentose xylulose and ferments it (126). Xylulose is metabolized through the pentose phosphate pathway (PPP), which is connected to central metabolism (126). The PPP is needed

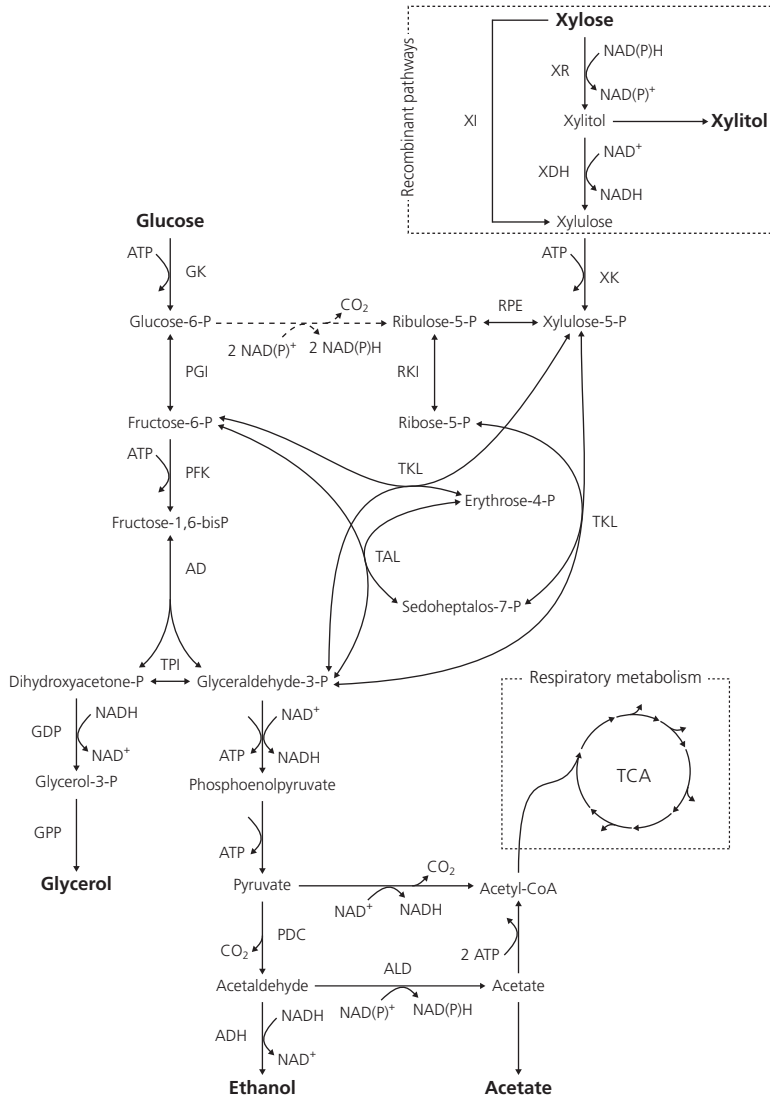


Figure 3. The central carbon metabolism of xylose-fermenting *S. cerevisiae* during fermentation.

Catabolic pathways involved in the conversion of glucose and xylose to ethanol. The following abbreviations are used: AD, aldolase; ADH, alcohol dehydrogenase; ALD, acetaldehyde dehydrogenase; GK, glucokinase; GPD, glycerol-3-phosphate dehydrogenase; GPP, glycerol-3-phosphatase; PDC, pyruvate decarboxylase; PFK, phosphofructokinase; PGI, phosphoglucose isomerase; RKI, ribulose-5-phosphate keto-isomerase; RPE, ribulose-5-phosphate epimerase; TAL, transaldolase; TCA, tricarboxylic acid cycle; TKL, transketolase; TPI, triose phosphate isomerase; XDH, xylitol dehydrogenase; XK, xylulokinase; XI, xylose isomerase; and XR, xylose reductase.

to supply precursors and NAD(P)H for anabolic reactions (157). In addition, the PPP is central in allowing novel pentose-assimilating pathways to be introduced into *S. cerevisiae*.

S. cerevisiae is a facultative anaerobe—i.e., it can grow under both aerobic and anaerobic conditions (127). Its growth is based on assimilatory processes that lead to the formation of biomass and dissimilatory processes that supply the energy required for assimilation and maintenance (158). The biomass yield is higher when energy is generated through the TCA cycle and oxidative phosphorylation than through the fermentation pathways, because of higher energy yields and efficient co-factor recycling (158). The assimilatory processes give rise to a surplus of NADH, due to the co-factor requirements in the biosynthesis of amino acids (159). This implies that assimilatory processes lead to a surplus of reducing equivalents, since the formation of ethanol from glucose is a redox-neutral process (147). Another factor that must be taken into account is the NAD(P)H requirement for biomass formation. A flow of carbon is directed toward the generation of NAD(P)H, which is used as a reductant in biomass anabolism (158). Maintaining redox neutrality in the cell is essential for sustaining its viability. The redox balance can be maintained by the split of glucose metabolism toward glycerol, which enables the re-oxidation of NADH (160). Furthermore, glycerol can be produced as a response to osmotic stress (160). Although growth and glycerol formation are necessary to sustain viability, they divert carbon away from ethanol production. This has made the repression of biomass and glycerol formation, as well as redox balancing, in metabolism targets in strain development (159).

The fundamental metabolism of *S. cerevisiae*, outlined above, sets the stage for improving strains by metabolic engineering and process-related adaptation strategies. The strategies aim at introducing novel capabilities and improving the performance of *S. cerevisiae* in the cofermentation of lignocellulosic hydrolysates.

4.2 Rational metabolic engineering

Rational metabolic engineering is the targeted improvement of cellular activities through the manipulation of the enzymatic, transport, and regulatory functions of a cell. The approach requires preliminary knowledge of the genomic setup and the role of the constituents in the metabolic routes. Rational metabolic engineering of *S. cerevisiae* for ethanol production from lignocellulosic feedstocks has long focused on extending the range of sugars for the conversion to ethanol to encompass xylose and arabinose—two abundant pentose sugars in herbaceous crops. This development has been reviewed extensively elsewhere (49, 131, 132, 161, 162).

The upregulation of the endogenous xylose-utilizing pathway in *S. cerevisiae* has failed to produce strains with sufficient growth and ethanol productivity (126, 163). Instead, two exogenous pathways have been introduced to assimilate xylose into central metabolism (Figure 3): a two-step oxido-reductive pathway catalyzed by xylose reductase (XR, EC 1.1.1.21) and xylitol dehydrogenase (XDH, EC 1.1.1.9) (129, 130) and one-step isomerization catalyzed by xylose isomerase (XI, EC 5.3.1.5) (131). Both pathways facilitate the conversion of xylose to xylulose (Figure 3). Xylulose is subsequently phosphorylated to xylulose-5-phosphate by endogenous xylulokinase (XK) and metabolized via the nonoxidative part of the pentose phosphate pathway before entering glycolysis. Assimilation of arabinose has, in several cases, been combined with xylose-fermenting capabilities to extend the range of substrates (164-166). The strain employed in **Papers I-V**, *S. cerevisiae* KE6-12, harbors an engineered exogenous XR/XDH pathway and overexpresses endogenous XK.

The oxido-reductive XR/XDH pathway encompasses the sequential reduction of xylose to xylitol by NAD(P)H-preferring XR, followed by the oxidation of xylitol to xylulose by NADH-producing XDH (131). In contrast to the XR/XDH pathway, co-factor-independent XI catalyzes the direct isomerization of xylose to xylulose (131). The introduction of either pathway has conferred xylose-fermenting capabilities. However, the introduction of only the pathways for the conversion of xylose to xylulose has been shown to be insufficient for efficient xylose fermentation by *S. cerevisiae*, due to low yields, ethanol productivities, and growth (126). Improvements in ethanol productivity and yield, with both types of xylose assimilation pathways, have been achieved with the overexpression of endogenous xylulokinase (167) and the overexpression of enzymes in the PPP (168). The PPP needs to be upregulated to obtain similar productivities with pentoses as with hexoses, because it is constrained by less favorable thermodynamic kinetics, as compared with glycolysis (162).

The dual co-factor-dependence of engineered XR/XDH pathways has been shown to be a constraining factor for efficient xylose fermentation (154, 169, 170). The different co-factor dependencies between the catalyzing enzymes have been shown to give rise to a co-factor imbalance that restricts flux through the engineered pathway and causes xylitol production (169, 170). Xylitol production is an undesirable trait in ethanol fermentation, since it reduces the ethanol yield. Further, improvements have been made by altering the co-factor specificity of XR toward NADH in order to balance co-factor usage (171, 172). The advantage with XI-catalyzed conversion of xylose to xylulose is that the conversion does not involve pyridine nucleotide co-factors, and thus, the issues with co-factor recycling that are associated with XR/XDH-catalyzed conversions are circumvented (131).

Although the issues with co-factor imbalances are avoided, challenges with strains expressing the XI pathway remain. Initially, the challenge was to express a functional XI that worked effectively at the low temperatures typically used in yeast

fermentations (173). To date, the xylose utilization rates of strains harboring XI are still generally lower than in strains that harbor the XR/XDH pathway (146, 174). Furthermore, the xylose utilization strains harboring XI are inhibited by xylitol that is produced by unspecific aldose reductases in yeast (146).

The integration of pentose-fermenting capabilities in *S. cerevisiae* requires careful optimization of the expression levels of individual enzymes in the engineered pathways to reduce redox and co-factor imbalances and maximize ethanol yield and productivity (162). However, despite advances, the fermentation of xylose to ethanol by engineered *S. cerevisiae* is slower and generally results in lower ethanol yields than glucose fermentation (132). In addition, xylose seems to have limited capacity in supporting anaerobic growth in both wild-type and recombinant xylose-utilizing *S. cerevisiae* (175).

Apart from the introduction of genes conferring pentose-fermenting capabilities, considerable effort has been made to improve the processes upstream and downstream of xylose-assimilating pathways that constrain ethanol productivity and yield. Upstream of the xylose-assimilating pathways, most effort has been directed toward the engineering of transporter systems for pentoses by insertion and upregulation (176-178). The goal has been to overcome the competitive inhibition of pentose transport in the presence of glucose (179). Overexpression of pre-existing transporters (153) and the introduction of specific transporters with favorable kinetics for pentoses with inertness toward glucose (176, 178) could alleviate the restrictions on uptake. Even though improved uptake rates have been demonstrated, little improvement in ethanol yields has been reported for strains with introduced exogenous transporters (176, 179).

Several modifications have been proposed downstream of the xylose-assimilating pathways to eliminate undesirable by-products. One undesirable by-product is glycerol. Improved ethanol yields have been obtained by genetic modification of glycerol anabolism in *S. cerevisiae* (180). However, it might be undesirable to interrupt glycerol synthesis, since it has important physiological roles in metabolism in yeast, including osmoregulation, maintenance of intracellular redox balance under anaerobic conditions, and its function as a precursor for cellular membrane synthesis (180). The importance of glycerol in maintaining the redox balance under anaerobic conditions is of particular interest for cofermentation with *S. cerevisiae* that harbors the XR/XDH pathways, since it provides means of alleviating the issue of insufficient co-factor recycling. Attempts have also been made to eliminate glycerol production by engineering *S. cerevisiae* to re-oxidize NADH through the reduction of acetic acid to ethanol (181). However, growth and ethanol productivity were lower in the engineered strain (181). Increased inhibitor tolerance has also been obtained by rational metabolic engineering (162, 181) but is more frequently addressed by nontargeted metabolic engineering (179).

4.3 Nontargeted metabolic engineering

Nontargeted metabolic engineering encompasses methods of strain improvement by genetic diversification, followed by selection of cells with the desired phenotype. This can be achieved, for example, by natural and induced mutagenesis and recombination and shuffling of genes and pathways (122). Through iterative genetic diversification and selection, natural selection can be mimicked, and cells with advantageous phenotypes are generated. The aim is to establish inheritable characteristics by evolving stable changes in the genome that are linked to a certain phenotype. The advantage of nontargeted methods is that no preliminary knowledge of the pathways and enzymes behind the phenotype is needed. In addition, several traits can be addressed simultaneously, provided that there are suitable ways to screen for the improvement (182). Rational metabolic engineering, combined with nontargeted methods, can elicit faster improvements and further optimize the strain (183). After introduction of the genes and pathways that provide capabilities by rational metabolic engineering, nontargeted strategies have proven to be beneficial in tuning regulatory functions and optimizing strains (184, 185).

In the context of fermentative conversion of lignocellulosic substrates, nontargeted metabolic engineering provides the means to address several intertwined challenges. It has been used to improve the kinetics of pentose fermentation by recombinant *S. cerevisiae* (186-188). It has also been proven to be a useful tool in improving tolerance to multiple stressors and inhibitors, which has led to increased growth and ethanol productivity (122, 182, 189-191). Improving tolerance to inhibitors that are generated during pretreatment is generally difficult to perform systematically, because the different feedstocks and pretreatments generate different sets of inhibitors and because the identity of inhibitory compounds, their mechanisms, and synergisms are poorly understood (122). Particularly, lignin residues and derivatives are multiform. Further, yeast is unlikely to have evolved specific resistance mechanisms toward pretreatment-generated inhibitors formed under nonphysiological conditions, such as furfural and HMF. Thus, the resistant phenotypes that have evolved are more likely to be based on expressed mutations than pre-existing specific mechanisms (122). Nontargeted methods can elicit broad adaptive responses to a variety of inhibitory compounds in the hydrolysate in a combined process, and it has been shown that inhibitor-tolerant phenotypes can be retained in the absence of inhibitors during cultivation (192). Ideally, the microorganisms should be adapted with the same type of pretreated raw material as used in the subsequent fermentation step to elicit an appropriate adaptive response.

The main challenge with nontargeted metabolic engineering is developing screening strategies that effectively select cells with desirable phenotypes. Improving the utilization of multiple substrates, in particular, is challenging, as it is complicated to

select for multiple mutations in different metabolic pathways that require different kinds of selective pressure, which can be in conflict with each other (186). The xylose-fermenting *S. cerevisiae* strain KE6-12, used in **Paper I** through **V**, is a progeny of *S. cerevisiae* TMB3400, which was generated by rational metabolic engineering to introduce and tune the XR/XDH pathway and further subjected to random mutagenesis (193). KE6-12 was evolved from TMB3400 in a two-step process (194). First, xylose utilization and temperature tolerance were improved by evolution schemes, with xylose as the sole carbon and energy source in a turbidostat culture at elevated temperature. Second, xylose utilization and inhibitor tolerance were improved in evolution schemes with bagasse inhibitors in a turbidostat at elevated temperatures.

4.4 Short-term adaptation

Various combinations of metabolic engineering strategies have generated xylose-fermenting *S. cerevisiae* strains with novel and improved capabilities that are tolerant to inhibitors in lignocellulosic hydrolysates. However, despite extensive strain development, pentose fermentation is still slower than hexose fermentation and typically elicits lower ethanol yields (132). Pentose fermentation is also affected to a greater extent by the inhibitory action of compounds generated during pretreatment than hexose fermentation (191, 195, 196). Short-term adaptation provides a means to further improve the performance of genetically engineered xylose-fermenting *S. cerevisiae*.

It has been shown that the propagation strategy is important for obtaining robust cells with improved fermentation properties (197, **Paper I**). By exposing the cells to inhibitors in adaptation media (191, 198) or during the propagation of cells (197, 199, 200, **Paper I**), selective pressure is applied that selects for desirable phenotypes for the fermentation of specific lignocellulosic hydrolysates among cells with phenotypic heterogeneity. The aim of the adaptation strategy is to prime the microorganism to function in the presence of specific environmental factors. The benefits of short-term adaptation have been established for hexose fermentation (198-201) and, to a lesser extent, for cofermentation of lignocellulose-derived xylose and glucose (191, 197, **Paper I**).

Preadapted cells exhibit increased expression of genes that are linked to inhibitor tolerance (197, 199, **Paper I**) and a greater ability to detoxify or tolerate inhibitors, which were observed in both hexose fermentation (199, 200) and cofermentation of glucose and xylose (191, 197, **Paper I**). Further, adapted cells exhibit shorter lag phases and higher specific growth rates than unadapted cells (197) and greater viability and vitality before pitching (**Paper I**). The improvement in inhibitor

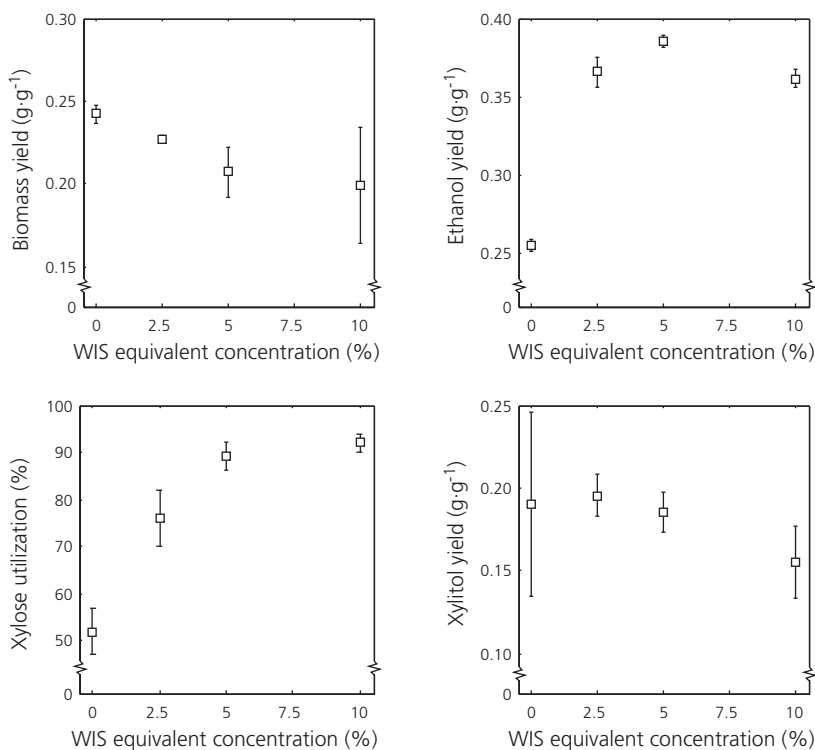


Figure 4. Effect of short-term adaptation on biomass yield and cofermentation results.

The panels show the biomass yield of the propagation and ethanol yield, based on total incoming glucose and xylose; xylose utilization, based on liberated xylose; and xylitol yield, based on consumed xylose, in SSCF by recombinant *S. cerevisiae* KE6-12 pre-adapted with increasing amounts of hydrolysate in the feed during fed-batch propagation.

tolerance was accompanied by improved hexose fermentation (198-200) and cofermentation of xylose and glucose (191, 197, **Paper I**). In the cofermentation of lignocellulose-derived glucose and xylose, short-term-adapted cells exhibited higher xylose consumption rates, xylose utilization, ethanol yields, and ethanol productivity, as well as lower xylitol production (197, **Paper I**), which is illustrated in Figure 4. Considering the benefits of short-term adaptation, it is an attractive option to combine the use of inhibitor-tolerant strains that are generated by metabolic engineering with short-term adaptation strategies to adapt yeast to specific hydrolysates and further improve fermentation performance.

From a process perspective, the improved properties of yeast that is obtained by metabolic engineering and short-term adaptation offer several opportunities apart from improved ethanol yields. Increased tolerance of inhibitors by the fermenting microorganism reduces the need for detoxification of the hydrolysate to realize high

ethanol yields in the fermentation, which avoids additional costs for detoxification (52). The increased ethanol productivity potentially offers shorter fermentation times (199, 200), increased substrate loads, and lower yeast pitch (200). All of these features are desirable. A shorter fermentation period decreases the required fermentor capacity and thus decreases the investment cost. Lower yeast pitch decreases the cost of buying and propagating yeast. Increased substrate loadings reduce the water requirement and enable higher final ethanol concentrations, which is beneficial for the energy demand of downstream processing. However, these gains, and the shorter fermentation times in particular, are likely to be more influential in glucose fermentation than in the cofermentation of xylose and glucose. The underlying reason is the preferential consumption of glucose, which typically causes xylose to be converted only when glucose approaches exhaustion (179, 202) and at lower rates than glucose (132).

Another aspect is the robustness that is gained in the process. By adapting the yeast to specific hydrolysates, consistent production at high yield and productivity can potentially be achieved in the conversion step, regardless of the varying composition in the incoming pretreated feedstock. This is particularly important with biomass, because it is known to vary in composition, depending on source, environmental conditions, season, and year. Reducing the technological risk is regarded as important in facilitating the commercialization of second-generation bioethanol (31). A robust propagation method that can adapt the yeast to variable inputs and deliver consistent fermentation results could also enable the use of feedstock blends at varying ratios in the process, which is discussed further in Chapter 6 and **Paper V**.

Many studies have focused on the technological benefits of preadaptation strategies for hexose and pentose fermentation (191, 199, 200), but these benefits come at a cost. It has been shown that the propagation of yeast in the presence of inhibitors decreases the biomass yield (197, **Paper I**). Several compounds that are generated in the pretreatment step inhibit the growth of *S. cerevisiae* (48, 54) and subsequently decrease the growth rate and biomass yield. The consequences of this are two-fold. First, the reduced biomass yield implies a higher cost of propagating yeast for a specific fermentor capacity, because more sugar is needed to propagate the desired cell mass. Second, the decreased growth rate prolongs the period that is needed to propagate the desired cell mass, since a lower feed rate of sugars during propagation is required to avoid overflow metabolism and the associated decrease in biomass yield (158). Prolonged propagation can increase the need for fermentor volume for propagation and thus negatively impacts the investment and capital costs of the plant.

From an economic perspective, the trade-off between increased fermentation performance and the increased cost of propagation must be considered in order to improve the overall economics of the process. The added cost of short-term adaptation during propagation must at least be offset by the improved fermentation performance of the preadapted yeast.

In **Paper I**, it was found that drastic improvements in xylose utilization and ethanol yield in SSCF already occurred with preadaptation using low amounts of hydrolysate—i.e., low inhibitor concentrations—during the propagation of xylose-fermenting *S. cerevisiae*. Furthermore, the increase in xylose utilization and ethanol yield stagnated with increasing hydrolysate amounts during propagation after a certain point (Figure 4). At the same time, the biomass and xylitol yields continuously decreased with increasing hydrolysate amounts in the propagation (Figure 4a). The inverse trends between ethanol and biomass yields suggest that the most economic preadaptation method entails low amounts of hydrolysate in the propagation. In addition, **Paper I** suggests that the xylose-fermenting capacity is much more sensitive to the propagation procedure than glucose fermentation. Furthermore, the increased variability in cultivation and fermentation outcomes with increasing amounts of hydrolysate in the propagation (**Paper I**) jeopardizes the consistency in fermentation outcomes and thus the robustness of the process, constituting a technological risk.

5. Designing conversion processes for improved xylose utilization

The major technological challenges for achieving efficient conversion of lignocellulose to ethanol are linked to the biology and chemistry of the processing steps; these steps are highly intertwined. The conversion process has to maximize sugar yields in the pretreatment and enzymatic hydrolysis and the ethanol yields, on available glucose and xylose, in the cofermentation step in order to be commercially viable (36, 145). However, the properties of the pretreated lignocellulosic biomass and the fermenting microorganisms present challenges in obtaining efficient conversion.

In the enzymatic hydrolysis step, the rate- and yield-limiting factors need to be addressed (107), as do the limitations in the cofermentation step. The overall efficiency of the fermentation step depends largely on the fermenting microorganism's tolerance to inhibitors and its ability to efficiently convert a variety of substrates to ethanol (126). Although significant strain improvement has been achieved, there are still restrictions that need to be overcome and preferences of the fermenting microorganism that need to be catered to in order to maximize the ethanol yield. Many of these challenges can be resolved, or at least alleviated, and ethanol yield and productivity can be improved by various degrees of integration between different process steps and the design of the conversion process.

In this chapter, different cofermentation designs that are proposed to improve xylose utilization and ethanol yields will be discussed. Emphasis will be placed on process modifications to the two major frameworks, separate hydrolysis and cofermentation (SHCF) and simultaneous saccharification and cofermentation (SSCF). The modifications are introduced to address process-related challenges and optimize the process with regard to ethanol yield. The modifications generate hybrid conversion strategies that benefit from strengths associated with either of the main conversion strategies (Figure 6).

5.1 Typical process design frameworks

Two principal conversion strategies have been pursued to optimize the conversion of glucose and xylose derived from pretreated biomass to ethanol: SHCF and SSCF (51, 203). SHCF is a sequential process, where enzymatic hydrolysis and cofermentation are carried out separately under their respective optimal conditions. In contrast, SSCF is an integrated approach, where enzymatic hydrolysis and cofermentation are combined into one step. In doing so, the benefits from the synergies between processes are reaped, although trade-offs are needed to accommodate both processes. Both strategies have advantages and address constraints to the conversion in different ways.

From an application point of view, there are several aspects to consider when selecting which strategy to implement. The properties of the pretreated biomass, the enzyme system, and the fermenting microorganism all influence this choice.

The rheology of the pretreated lignocellulosic biomass can impose challenges, especially because higher solids loading in the process is imperative to reduce operational costs (204). High viscosity can make mixing difficult and increase the power demand (205). Sufficient mixing is important to disseminate enzymes and homogenize conditions throughout the conversion process and thus facilitate efficient enzymatic hydrolysis and suitable fermentation conditions to attain high process ethanol yields (107, 206). The rheological issues are resolved by the liquefaction that occurs at an early stage during enzymatic hydrolysis. The optimal conditions for enzymatic hydrolysis in SHCF promote liquefaction, whereas the lower temperatures in SSCF, mandated by the thermo-tolerance of the fermenting microorganism, can impose constraints through a lower liquefaction rate. The challenge with high viscosities is augmented by increased solids loadings (107) and has to be resolved by technological improvements (206) or feeding strategies (207, 208), as discussed below.

In SHCF, the optimal conditions in the hydrolysis step, particularly with regard to temperature, favor enzyme performance and elicit high hydrolysis rates (45). However, even though enzymatic hydrolysis is operated at the optimal temperature and pH in SHCF, the hydrolytic yield is affected by end-product inhibition (115), especially by glucose and cellobiose (209, 210). End-product inhibition may result in a subsiding hydrolysis rate and lower yields. This effect is amplified by increased solids loadings (107). In contrast, the hydrolysis rate in SSCF is constrained by the mandated lower temperature, but the integration of enzymatic hydrolysis and fermentation can offset the effects of glucose and cellobiose inhibition (51). By continuous removal of hydrolysis end-products by fermentation, inhibition of the cellulase system is circumvented, which contributes to the improvement in efficacy of hydrolysis. Fermentation has also been shown to convert compounds in

lignocellulosic hydrolysates that are inhibitory to the enzyme system, and thus, SSCF mitigates the inhibitory effect and improves the hydrolysis performance (211). However, ethanol is also known to inhibit the cellulolytic activity of cellulases (212), although not to the same extent as cellobiose, which adversely affects enzymatic hydrolysis during SSCF.

In the fermentation step, SHCF benefits from the possibility of tailoring the physical conditions to the characteristics of the fermenting microorganism. In an SHCF-based design, fermentation can be performed at temperatures close to the optimum of the fermenting microorganism to balance the fermentation kinetics and sustained viability. In the case of *S. cerevisiae*, the optimal fermentation temperature is around 30°C (213, 214), although it is highly strain-dependent (214). In contrast to SHCF, SSCF has to be performed at elevated temperatures, typically 34-37°C, to accommodate enzymatic hydrolysis with reasonable kinetics. This can have detrimental effects on yeast viability and, thus, on fermentation efficiency. However, SSCF does not have to be performed isothermally at elevated temperatures. The temperature can be varied throughout SSCF to balance the fermentation rate against the hydrolysis rate in order to maximize ethanol yields (215). The lower temperature in the fermentation step in SHCF helps maintain the viability of *S. cerevisiae* (213), especially in response to the detrimental effects of the synergy between elevated temperatures and ethanol concentrations on viability (214, 216). Increased susceptibility to acids (214, 217) and hydrolysate-derived inhibitors (215, 218) at elevated temperatures has also been observed.

In addition, cofermentation has to address the preferential consumption of glucose by xylose-utilizing *S. cerevisiae*, which typically causes pentoses to be converted only when glucose approaches exhaustion (179, 202, 219). In principle, it does not matter whether glucose and xylose are consumed sequentially or simultaneously, as long as the sugars are converted rapidly, but xylose consumption rates typically decline over time after the exhaustion of glucose (131, 132). In SHCF, the high glucose concentrations in many hydrolysates after enzymatic hydrolysis competitively inhibit transporters and prevent efficient uptake of xylose (202, 220). These suboptimal kinetics prolong the fermentation time and increase the sensitivity to inhibitors (221). In contrast, the continuous release of glucose during enzymatic hydrolysis of cellulose in SSCF can aid in keeping glucose uptake levels below transporter saturation during cofermentation, thus promoting xylose uptake (220). This has been shown to have beneficial effects on xylose utilization and thus overall ethanol yields (222). Further, it has been shown that a continuous supply of low concentrations of glucose promotes xylose uptake and enhances xylose metabolism in xylose-fermenting *S. cerevisiae* (220, 223). The enhanced xylose uptake rate has been attributed to improved cofactor recycling (224) and the induction of genes for transporter systems (224) and glycolytic enzymes (225). Thus, SSCF is a suitable cofermentation strategy to attain high xylose utilization by xylose-fermenting *S. cerevisiae*.

Studies have shown that SSCF typically exhibits higher ethanol productivity (226) and process ethanol yield than SHCF (203, 227). Process ethanol yields in SSCF have historically benefited from lower end-product inhibition of cellulolytic enzymes, but with improved cellulase systems and accessory enzymes, the impact of end-product inhibition has been reduced (228). This provides a better outlook for SHCF-based designs. The improvement in enzyme systems also suggests that not only the choice of strain and feedstock but also the choice of enzyme system is important for the appropriate selection of the conversion strategy.

5.2 Feeding strategies

Substrate and enzyme feeding strategies have been frequently utilized tools to improve the efficiency of the conversion steps in both major strategies. In many cases, fed-batch strategies have improved the results as compared with batch configurations (134, 139, 140, 222, 229, 230). Substrate feeding strategies have been employed to alleviate issues associated with rheology and inhibitor concentrations, as well as to improve cofermentation performance by providing beneficial sugar ratios and concentrations.

In the enzymatic hydrolysis of agricultural residues, such as wheat straw and corn stover, initial high viscosities can pose a problem, because they make mixing difficult. A fed-batch approach in SSCF and the enzymatic hydrolysis step of SHCF would allow for the gradual addition of solids that are to be continuously liquefied, and thus, sufficient mixing at high solids loadings could be achieved (229). Although agricultural residues typically liquefy rapidly and provide good mixing conditions, sufficient initial mixing at high viscosity is important to disseminate enzymes and homogenize conditions, such as pH, temperature, and concentrations (107, 206). It has been argued that a lack of mixing is not a yield-limiting factor in enzymatic hydrolysis, given sufficient time (107, 206), but initial heterogeneous dispersion of the enzymes and neutralizing agent can create hysteretic effects that lower sugar yields in time-limited enzymatic hydrolysis. Insufficient mixing can form zones with unfavorable conditions, such as high or low pH and zonal overheating, which can have detrimental effects on the stability and productivity of the enzymes (231). Enzymatic hydrolysis experiments that were performed in batch configuration with dilute acid-catalyzed steam-pretreated wheat straw showed that initial heterogeneous dispersion of enzymes and neutralizing agent elicited lower hydrolytic yields, as compared with initial homogeneous dispersion (Figure 5). Although the pretreated wheat straw liquefied rapidly in both instances and provided good mixing regimens during the enzymatic hydrolysis, the initial conditions were reflected in the sugar yields. The effect was increasingly pronounced with greater solids loads (Figure 5), which can be attributed in part to increased initial mixing problems and in part to

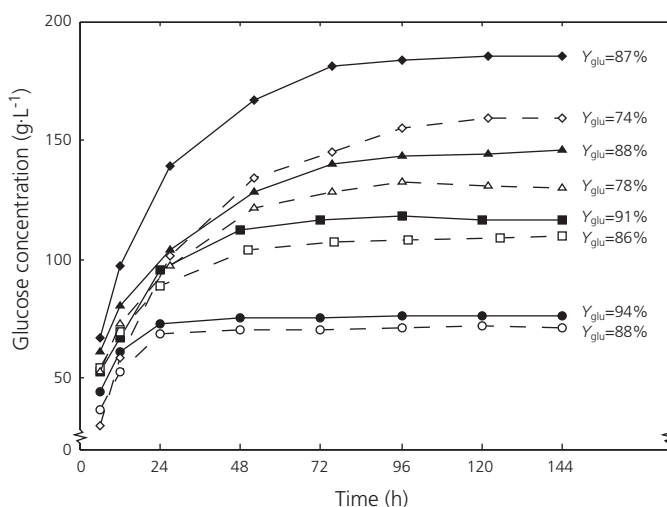


Figure 5. Effect of initial heterogeneous dispersion of enzymes and neutralizing agent in enzymatic hydrolysis. Time courses of enzymatic hydrolysis of steam-pretreated wheat straw with homogenous (—) and heterogeneous (---) dispersion of enzymes and neutralizing agent at solids loadings of 13% (●), 20% (■), 25% (▲), and 30% WIS (◆). Glucose yields are displayed on the right hand side.

other factors associated with higher solids loadings, such as increased inhibition and nonproductive adsorption of enzymes (107). The experiments were performed in conventional stirred-tank reactors. Fed-batch approaches or alternative reactor designs, applying other mixing principles, could improve the outcome (206, 232).

Fed-batch strategies have also been applied to alleviate problems with high concentrations of inhibitors in both cofermentation steps of SHCF and SSCF. Certain inhibitors that impair growth and ethanol productivity, such as furaldehydes and phenolics, can be converted enzymatically by *S. cerevisiae* into less inhibitory compounds (48, 233). By feeding substrate that contains inhibitors at a rate below the yeast's *in vivo* detoxification rate, the inhibitor concentrations in the fermentation broth can be kept low, which enhances fermentability and ethanol productivity compared with batch fermentation (233). To maximize the productivity, the feed rate is ideally equal to the detoxification capacity of the cells. The importance of fed-batch designs in relieving the inhibition increases when less inhibitor-tolerant strains are used (234), low yeast pitch is used (233), and at the elevated inhibitor concentrations that are implied by high solids loadings in the fermentation of lignocellulosic feedstocks (232).

In addition, fed-batch strategies can alleviate the effects of substrate inhibition in the cofermentation step of SHCF (235). This influence can be expected to be more influential at high solids loadings, because higher sugar concentrations and potentially

higher ethanol concentrations are elicited. Substrate feeding strategies can also improve xylose utilization in cofermentation by helping sustain low glucose concentrations in the cofermentation steps of SHCF and SSCF. Significant improvements in both xylose utilization and ethanol yields have been obtained in SHCF of steam-pretreated wheat straw by applying substrate feeding strategies (230). Although part of the improvement can be attributed to alleviated inhibitory action, it has been shown that a distributed supply of substrate can improve xylose utilization (230). Feeding glucose-rich enzymatic hydrolysate from washed solids to the xylose-rich liquid fraction of steam-pretreated wheat straw in SHCF improved xylose utilization by xylose-utilizing *S. cerevisiae* TMB3400 and its progenies (230). In addition, fermentation times were shortened as compared with batch strategies. However, only moderate gains in process ethanol yields were realized, primarily because of extensive by-product formation (230). Since most of the inhibitors were present in the xylose-rich liquid fraction of the pretreated biomass, the improvement was likely due to more beneficial glucose-to-xylose ratios during the fermentation. This implies that the separation of glucose and xylose, in combination with feeding strategies, is a tool that can be used to cater to the substrate preferences of the fermenting microorganism.

Substrate feeding has also been shown to improve process ethanol yields in SSCF of steam-pretreated wheat straw with xylose-fermenting *S. cerevisiae* (222). Through gradual feeding of pretreated material, the glucose concentrations can be kept low (220, 223). However, the feeding needs to be coordinated with the fermentation temperature to balance the fermentation and hydrolysis kinetics (222). In combination with the feeding of enzymes, the control of glucose and xylose concentrations in the bioreactor can be refined, and ethanol yields can improve even further (139). Apart from providing beneficial ratios between xylose and glucose for xylose utilization, substrate feeding schemes have mitigated rheological issues and alleviated the inhibitory burden on yeast, which can explain in part the improved ethanol yields. However, fed-batch SSCF designs have suffered from diminishing xylose utilization rates, leaving substantial residual xylose (136, 139, 222). This shortcoming is addressed in **Paper III** and is discussed further below.

Another substrate feeding strategy is to integrate first-generation bioethanol process streams with the second-generation cofermentation strategy to enhance xylose utilization. Wheat-starch hydrolysates from first-generation bioethanol processes have been successfully integrated into fed-batch SHCF of steam-pretreated wheat straw to improve xylose consumption and to obtain higher final ethanol concentrations than what can be achieved with lignocellulosic biomass alone (134). First-generation starch-based process streams have also been integrated with SSCF of steam-pretreated wheat straw. Although this has resulted in higher final ethanol concentrations, only moderate gains in xylose utilization and ethanol yield have been observed (133). Nevertheless, this demonstrates the feasibility of integrating hexose-rich process

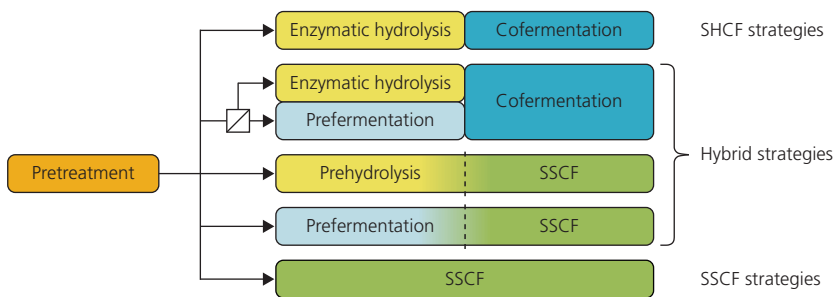


Figure 6. Overview of the principal conversion strategies and their hybrid derivatives.

Schematic representation of hybrid process concepts, where prehydrolysis and prefermentation are coupled with the two principal conversion strategies, separate hydrolysis and cofermentation (SHCF) and simultaneous saccharification and cofermentation (SSCF). Adapted from Palmqvist (84).

streams, external or internal, into second-generation cofermentation processes to enhance xylose utilization.

5.3 Hybrid conversion processes

Different hybrid processes derived from the two main strategies have been developed (Figure 6), combining different features from both strategies to overcome common constraints. The modifications that have been introduced are the coupling of prehydrolysis to SSCF and the coupling of prefermentation to SHCF and SSCF. They share a common trait, in that the modifications to some extent can be customized independently of the rest of the conversion process and can be coupled to various substrate and enzyme feeding strategies.

Prehydrolysis

Prehydrolysis is the introduction of a high-temperature hydrolysis step prior to the yeast pitch and conventional SSCF of whole slurry. The higher temperature that is used in the prehydrolysis step promotes favorable enzyme kinetics (45), before the mandated lower temperature is applied in SSCF to accommodate the fermenting microorganism. Prehydrolysis that is followed by SSCF positions itself between SHCF and SSCF (Figure 6). With variable prehydrolysis periods at elevated temperatures, the fermentation strategy can emphasize the characteristics of either SHCF or SSCF. With shorter prehydrolysis periods, the pretreated biomass is liquefied, and limited amounts of monomeric sugars are liberated prior to pitching of the yeast, and the hybrid strategy takes on many of the cofermentation characteristics

of SSCF. A prehydrolysis step with this aim is often referred to as viscosity reduction (206) and provides an alternative, or complement, to feeding strategies to address rheological issues in SSCF. Employing longer prehydrolysis periods, the hybrid strategy increasingly takes on the hydrolysis and cofermentation characteristics of SHCF, except that lignocellulosic solids are retained in the fermentation broth. With long prehydrolysis periods, the process is essentially SHCF performed in one vessel.

Prehydrolysis provides the means not only to resolve rheological issues (206) but also to alleviate the limitations on conversion rate that are imposed by the hydrolysis rate in SSCF (236). Whether it improves ethanol yields or not is ambiguous (206, 228, 236, 237) and depends largely on the type of pretreated biomass, fermenting microorganism, and fermentation design and whether hexose fermentation or cofermentation is targeted. It has been argued that with new and improved enzyme systems with improved temperature stability and decreased end-product inhibition, the hybrid design is preferred over pure SSF designs (228). This approach is applied in **Paper V** to counter the limitations in hydrolysis rate that occur because of variable composition and characteristics between different feedstock blends. It is discussed further in **Paper V** and Chapter 6.

Along with the process-related benefits, this hybrid strategy is also affected by the drawbacks associated with elevated glucose concentrations in SHCF. However, the extent is governed by the composition of the pretreated lignocellulosic biomass and the duration and temperature during prehydrolysis. This hybrid strategy has been adopted for the fermentation of various lignocellulosic materials, especially for applications with high solids loadings (206, 228, 236, 237); the effects of duration and temperature during the prehydrolysis of steam-pretreated wheat straw have been further elucidated by Gladis et al. (238).

Prefermentation

Prefermentation has been implemented in conjunction with fundamental strategies to improve substrate utilization in cofermentation (134, 138, 140) and is addressed in **Paper II** through **IV**. It provides an additional fermentation step that can be optimized in part independently of the subsequent fermentation strategy. It has been applied for various purposes in cofermentation. In several contexts, it has been applied to deplete the initial hexose content in the liquid fraction of the slurry and thus complements feeding strategies and SSCF to maintain low glucose concentrations during cofermentation, which promotes xylose utilization (223). Depletion of glucose from the liquid fraction of whole spruce slurry, prior to enzyme addition in SSCF, has been shown to alleviate the competitive inhibition of sugar transporters, improve xylose uptake, and increase ethanol yields (138). The authors presupposed that the significance of this process would be even greater with xylose-rich feedstocks.

Prefermentation has also been applied in various cofermentation designs for the conversion of pretreated xylose-rich agricultural residues to ethanol. Generally, the aim has been to deplete glucose in the liquid fraction to condition the hydrolysate for substrate and enzyme feeding strategies that promote xylose utilization (134, 140, **Paper IV**), as described above for steam-pretreated spruce. In contrast, **Papers II and III** have an alternative take on prefermentation. The prefermentation was extended to not only deplete glucose in the liquid fraction but also convert significant parts of the xylose prior to feeding of the solid fraction. The composition of the liquid fraction of steam-pretreated xylose-rich feedstock was such that prefermentation could be employed as a cofermentation step that is customized for xylose conversion. This approach brings about sequential targeting and optimization of xylose and glucose conversion, which can improve ethanol yields. This is discussed further below.

5.4 Sequential targeting of xylose and glucose conversion

The pattern of substrate utilization is important for the overall process design, as coconsumption of all sugars is likely to lead to a shorter and more productive process. With limited coconsumption by xylose-fermenting *S. cerevisiae* strains, the sugars are consumed sequentially during the batch and fed-batch fermentations, and nonpreferred sugars accumulate in the media until the preferred sugars approach exhaustion. This brings about a xylose conversion phase late in the fermentation. Since xylose fermentation is typically slower and elicits lower ethanol yields than glucose fermentation (132), this hampers the overall ethanol productivity and yield. In addition, high concentrations of inhibitory fermentation end-products, such as acetate and ethanol, decrease the xylose utilization rate further (219). Further, the increased inhibition by fermentation end-products, in combination with the inability of xylose to efficiently support growth in xylose-fermenting *S. cerevisiae* (175), has detrimental effects on yeast viability (48). The selective and sequential utilization of sugars typically reduces ethanol yields and productivity (239) and has been shown to lead to incomplete xylose utilization (135, 139, **Paper IV**).

The cofermentation process can be designed to cater to the sugar consumption patterns of xylose-fermenting *S. cerevisiae*. SSCF designs address the issue of selective consumption of different sugars, as discussed above, and have been developed further to sequentially target xylose and glucose. The differences between hemicellulose and cellulose in their susceptibility to degradation by various pretreatments and the selective use of hemicellulolytic and cellulolytic enzymes enable the controlled release of hemicellulosic sugars separately from cellulosic sugars. This enables hybrid designs that initially target the conversion of sugars in the xylose-rich hemicellulosic fraction. Because the fermenting microorganism seemingly loses its xylose-fermenting capacity over time (139, **Paper IV**) but still converts glucose to ethanol, it can be

advantageous for overall xylose utilization to target xylose conversion first and then glucose conversion.

This concept has been demonstrated in a two-step batch SSCF process with prehydrolysis and enzyme feeding (140). AFEX-pretreated switchgrass was prehydrolyzed with hemicellulolytic enzymes to liberate hemicellulosic sugars, mainly xylose. In a first SSCF step, xylose-fermenting *S. cerevisiae* was pitched and small amounts of cellulases were added, and the hemicellulosic sugars liberated during prehydrolysis were cofermented in the presence of low glucose concentrations. In the second SSCF step, more cellulases were added to liberate the remaining glucose from the cellulosic structures, and the remaining pentoses were cofermented with the resulting glucose. The design created advantageous conditions for xylose utilization and considerably improved ethanol yields, as compared with a more conventional batch SSCF strategy with 8 h of prehydrolysis with both hemicellulolytic and cellulolytic enzymes (140).

In **Papers II** and **III**, hybrid SHCF and SSCF strategies, respectively, are presented, which target xylose and glucose conversion in succession using xylose-fermenting *S. cerevisiae* KE6-12 (194). The applied dilute acid-catalyzed steam pretreatments of wheat straw feedstock solubilized the hemicellulosic sugars and generated xylose-rich hydrolysate liquors and cellulose-rich solids. Separation of the xylose-rich hydrolysate liquor from the solid fraction enabled hybrid designs, where xylose and glucose conversion could be targeted in succession in two-step designs. In the first step, the conditions and feeding strategies were customized to enhance the conversion of sugars in the xylose-rich hydrolysate liquor to ethanol during prefermentation. In the second step, cellulose-rich solids, or prehydrolyzed solids, were added. The conditions and feed strategies in this stage were adapted to improve the xylose utilization and ethanol yield in the combined sequence. In the case of xylose-fermenting *S. cerevisiae* that harbors an engineered XR/XDH pathway this encompasses promoting the coconsumption of glucose and xylose and minimizing the formation of undesirable by-products, such as xylitol, glycerol, and biomass.

Sequential targeting in hybrid SHCF strategies

In **Paper II**, hybrid SHCF designs with prefermentation were investigated. The proposed strategy benefited from the use of optimal temperatures in the process steps: higher temperatures during enzymatic hydrolysis and lower temperatures for efficient cofermentation in the two fermentation steps. Prefermentation of the hydrolysate liquor improved overall ethanol yields, yet batch and fed-batch prefermentation had different impacts on the fermentation. Under the influence of low inhibitor concentrations, fed-batch prefermentation resulted in lower xylitol excretion during all phases of the fermentation and prompted higher final ethanol yields compared with the corresponding fermentations with batch prefermentation. Overall ethanol

yields exceeding 80% of the theoretical maximum, based on the available glucose and xylose, were obtained. Further, xylitol production could be kept below $0.04 \text{ g}\cdot\text{g}^{-1}$, based on the consumed xylose, with a strategy that employed fed-batch prefermentation, and more than 95% of total available xylose was consumed. This indicates that the fermentation design allows adequate co-factor recycling to maintain the cellular redox balance. Under the influence of higher inhibitor concentrations, the fermentation design was paramount in sustaining fermentation capacity. *S. cerevisiae* KE6-12 was resilient to high inhibitor concentrations in batch configurations but succumbed to continuous exposure to inhibitors in the fed-batch configurations (Figure 7). This behavior is highly strain-dependent, because the opposite behavior was observed with *S. cerevisiae* KE6-13i, a mutant strain with the same progenitor as KE6-12 (194). In contrast to KE6-12, KE6-13i coped with a continuous feed of lignocellulosic hydrolysate but succumbed to the high inhibitor concentrations in the batch prefermentation. The differences in fermentation behavior are illustrated in Figure 7. These findings are incongruous with previous findings on the two strains in the cofermentation of steam-pretreated wheat straw (230), indicating that the cofermentation performance of individual strains is also dependent on variations in cultivation conditions and the characteristics of the pretreated biomass.

The hybrid SHCF designs that were investigated were able to sustain xylose utilization throughout the fermentation at various inhibitor concentrations when yeast viability was preserved, even though xylose utilization rates declined after the exhaustion of glucose. However, the investigated designs revealed a trade-off between promoting xylose conversion and maintaining the viability of the pitched yeast. Fed-batch designs promoted xylose utilization and reduced xylitol production but seemingly hampered yeast viability. Lowered viability implies reduced fermentative capacity, and thus, strategies that employed fed-batch strategies in either the prefermentation or the cofermentation, or both, effected lower xylose utilization. Ethanol yield was maximized with a balance between substrate feeding to promote xylose utilization and measures to sustain yeast viability.

A weakness of the proposed method was the required separation of xylose-rich hydrolysate liquor and cellulose-rich solids after pretreatment, which necessitated high solids loadings in the enzymatic hydrolysis to successfully carry out the design. This provided a technological obstacle. The limitations imposed on the enzymatic hydrolysis by the high solids loadings, which could not be rectified in subsequent fermentation steps, constrained the ethanol yield. The outcome of the enzymatic hydrolysis thus had effects that were carried over to the cofermentation step and increased the variability and the spread of the cofermentation results (**Paper II**). Ensuring sufficient enzymatic hydrolysis is imperative in successfully carrying out this conversion strategy.

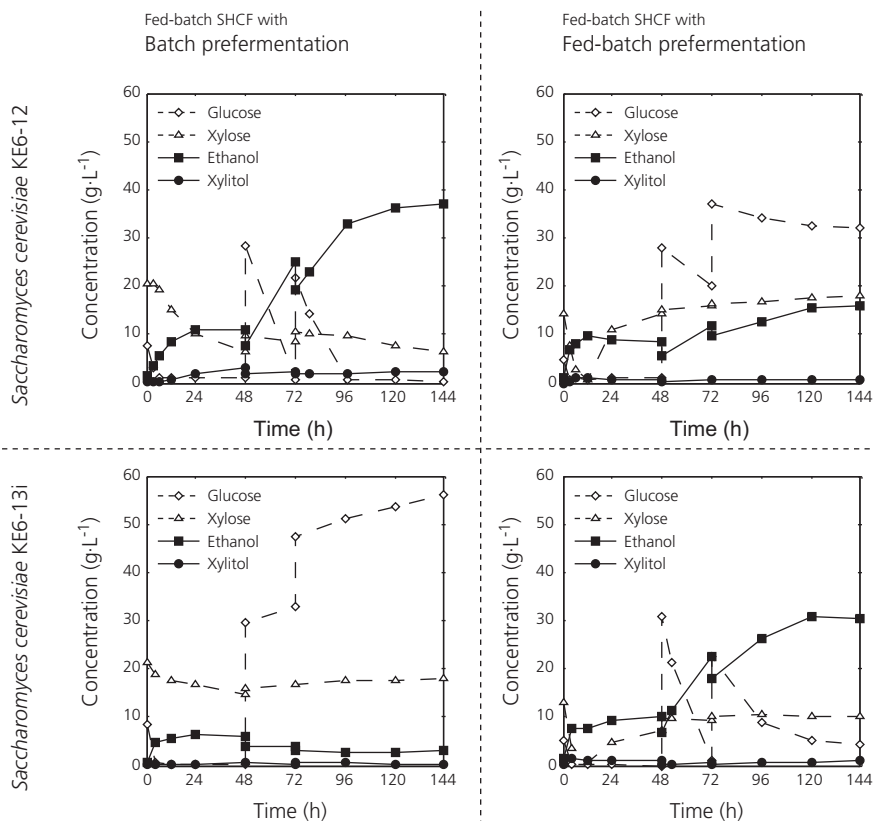


Figure 7. Effect of prefermentation mode on cofermentation performance of two *S. cerevisiae* strains. Time courses for hybrid SHCF designs with batch or fed-batch prefermentation of hydrolysate liquor and two additions of prehydrolyzed solids employing xylose-fermenting *S. cerevisiae* KE6-12 and KE6-13i. The fermentation configuration is elaborated on in **Paper II**.

Sequential targeting in hybrid SSCF strategies

Fed-batch SSCF, in combination with various process modifications, has been established as a feasible approach to obtain high ethanol yields in the cofermentation of biomass-derived glucose and xylose (133, 138-140, 222, 236). However, declining xylose consumption rates and incomplete xylose utilization are frequently observed in SSCF (139, 222, **Paper IV**). In **Paper III**, an established fed-batch SSCF strategy was extended to encompass the sequential targeting of xylose and glucose conversion to overcome these limitations. The strategy utilized batch prefermentation to convert a significant fraction of the available xylose in the pretreatment hydrolysate liquor into ethanol and a fed-batch design for the subsequent conversion of cellulose to ethanol.

The combination of batch prefermentation and fed-batch SSCF balanced the promotion of xylose utilization and sustained viability, as found in **Paper II**. With the two-step hybrid SSCF design, the xylose-fermenting capacity could be sustained throughout the prefermentation and SSCF. Higher ethanol yields and xylose utilization were obtained compared with the reference SSCF with substrate and enzyme feeding (**Paper III**) and previous studies on SSCF of steam-pretreated agricultural residues with substrate and enzyme feeding (139, 222, **Paper IV**) that employed *S. cerevisiae* TMB3400 (240) and its progenies (194). An ethanol yield exceeding 90% of the theoretical maximum, based on the available glucose and xylose, was achieved. The improved ethanol yield was attributed to sustained xylose-fermenting capacity, high xylose utilization (>90%), and low xylitol production (<0.05 g·g⁻¹, based on consumed xylose).

Although the hybrid SSCF strategy improved the results in comparison with the reference cases, it has inherent drawbacks. The suboptimal conditions for enzymatic hydrolysis that are needed to accommodate simultaneous saccharification and cofermentation are associated with a risk of rendering the hydrolysis rate-limiting for the ethanol production. This effect was observed in **Paper V**, where lower ethanol titers and yields and higher residual glucan content in the lignocellulosic residues after fermentation were observed with this strategy as compared with SSCF with prehydrolysis. The effect was also evidenced by differences in fermentation outcome between the strategies employed in **Papers II** and **III**. Prefermentation, followed by fed-batch SSCF, exhibited longer fermentation times before stagnation of ethanol production. This indicated that the hydrolysis rate restricted the ethanol productivity. In addition, prefermentation followed by fed-batch SSCF resulted in higher residual xylose concentrations in the fermentation broth than prefermentation followed by SHCF, effecting lower xylose utilization. This trend has also been observed in more conventional SSCF and SHCF configurations (227).

Process perspectives

From process and economical perspectives, there are a number of aspects to consider when choosing between cofermentation strategies. With high ethanol yields, final ethanol concentrations for cost-efficient downstream processing can be reached with relatively low WIS loads. In **Papers II** and **III**, ethanol concentrations above 40 g·L⁻¹ were obtained, which is generally regarded as the lower limit for cost-effective recovery of ethanol, with a WIS load of 10 wt%. However, the improvement in ethanol yields by SSCF in **Paper III**, as compared with the SHCF-derived strategy in **Paper II**, reinforced the notion that SSCF typically results in higher ethanol process yields than SHCF (203, 227).

The sequential targeting of xylose and glucose has beneficial effects on xylose utilization and ethanol yields (**Papers II** and **III**). However, this improvement comes

at a cost. Segmentation of the cofermentation process may prolong the fermentation time, which reduces the ethanol productivity of the conversion process and, thus, the ethanol production for a given fermentor capacity. From a process perspective, a trade-off between ethanol yield and productivity may exist with this methodology—a trade-off that needs to be addressed with optimization, based on economic considerations.

Furthermore, the fractionation of the slurry was linked to the possibility of targeting xylose and glucose conversion sequentially and keeping the viscosity low in the bioreactor, by either fed-batch SSCF or separate hydrolysis. However, the separation of xylose-rich hydrolysate liquor from cellulose-rich solids implies additional investments in additional processing steps and thus additional processing costs. In opting for fractionation, the improvement in ethanol yield from doing so must at least offset the incurred costs if the process economics are to be improved. SSCF designs with similar characteristics as that employed in **Paper III** can be implemented without fractionation of the pretreated material, as demonstrated by Jin et al. (140). However, this would imply the use of enzyme feeding schemes to selectively liberate hemicellulosic and cellulosic sugars in succession in a batch SSCF design, and higher solids concentrations can be expected throughout most of the cofermentation. The drawback with this approach is that it requires the prediction of the hydrolytic release of sugars, and also implies prolonged overall hydrolysis time and constrained ethanol productivity. A higher solids concentration also implies higher viscosity, which could thus impair the mixing regimen— especially at high solids loadings.

6. Process development for large-scale requirements

In recent years, production plants for second-generation bioethanol have been constructed in North America, Europe, China, and Brazil, and more are planned (241, 242). Production plants on the commercial and commercial-demonstration scale are currently operated or planned by, among others, DuPont, POET-DSM, Iogen, Mascoma, and Biochemtex/Beta Renewables in North America (241, 242); Raízen, and GranBio in South America (243); New Tianlong Industry Co (244), COFCO (245), Guozhen Group Co (243), and ShangDong LongLive (245) in China; and Biochemtex/Beta Renewables, Energochemica, Abengoa Bioenergy, Inbicon-Dong, Borregaard, and Clariant in Europe (241, 242). They are, or might become, pioneering plants that facilitate the deployment of production capacity with various feedstocks in different locations.

However, to realize the benefits of second-generation ethanol production and meet policy targets regarding resource utilization, energy security, and the reduction of greenhouse gas emissions, it is necessary to expand the commercialization and reach market penetration. One of the key factors for continued growth is the de-risking of the investment, so as to attract investment and avoid prohibitive risk premiums on the capital costs that are entailed by perceived risk (246). Pilot- and demonstration-scale plants contribute to building up the necessary knowledge and experience to de-risk projects (246). Furthermore, the commercialization of second-generation bioethanol is expected to accelerate the learning curve and reduce the cost of existing technology (31), similar to the learning curve effect that has substantially lowered the production costs of first-generation bioethanol in Brazil (247, 248). Commercialization and continuous operation provide the economic incentives and opportunities for improvements, debottlenecking, and innovation, which likely can not be duplicated on a lab scale. Deployment of production plants on a commercial scale will serve as proof of concept to de-risk further investment in second-generation bioethanol.

While incremental improvements are likely most efficiently made at scale by the industry, it is worthwhile to increase the knowledge of lignocellulosic conversion systems. Technologies that can reduce risk, reduce costs, identify potential advances,

and improve conversion efficiencies are still needed to improve profitability and make second-generation bioethanol competitive with its substitutes. From a process perspective, many of the challenges of process development for commercial-scale endeavors are related to the scale-up from the laboratory to implementation on a commercial scale, and scale-up is often the greatest single risk in process commercialization (246). In addition, several hurdles for the expanded deployment of production capacity still exist. Feedstock cost and availability are the driving factors that influence the selection of pioneer production plant locations, and these same factors will largely control the rate at which this industry grows and where (30). These issues are addressed in this chapter and in **Papers IV** and **V**.

6.1 Expanding the feedstock base

Techno-economical evaluations have shown that second-generation bioethanol plants typically need to be large to be profitable (249-251). Feedstock availability will have an impact on the scale of production and profitability, because the supply level of feedstocks influences the required scale of production to realize economies of scale (37). The pioneering plants will be located at the most advantageous locations available. In the short term, they are situated in areas where the bioethanol industry is already active and where existing crops and agricultural residues are available (252). In doing so, the deployment can take advantage of colocating with pre-existing first-generation ethanol facilities, making use of their farming and transportation infrastructure (30) and reaping the benefits of heat and stream integration (144, 253, 254). This significantly de-risks the undertaking of the project (249). This approach has been applied by POET-DSM for their second-generation ethanol plant Project Liberty in Emmetsburg, Iowa (255). The facility is colocated with a first-generation bioethanol plant that utilizes corn as feedstock; the second-generation plant uses corn stover and corn cobs sourced from nearby acreage (255).

However, as the deployment of additional plants and additional capacity increases, so will the competition for feedstock and deployment sites. New deployments will be increasingly constrained by the lack of additional agricultural feedstocks (252), and less attractive locations will have to be explored. Given the low bulk density of lignocellulosic biomass, the feedstock sourcing radius for a plant will be constrained by the cost of transportation (37), which represents a diseconomy of scale (37). Geographically concentrated supply systems need to address the issues of resource availability, diversity, and competing uses, and any such supply system should benefit from diversifying the feedstock base to ensure supply, hedge the risks of crop failure, and minimize seasonality constraints and storage requirements (30). Another aspect of the supply is the price of feedstock, which constitutes a significant fraction of the total production cost (30, 36). The use of a diversified feedstock base enables the

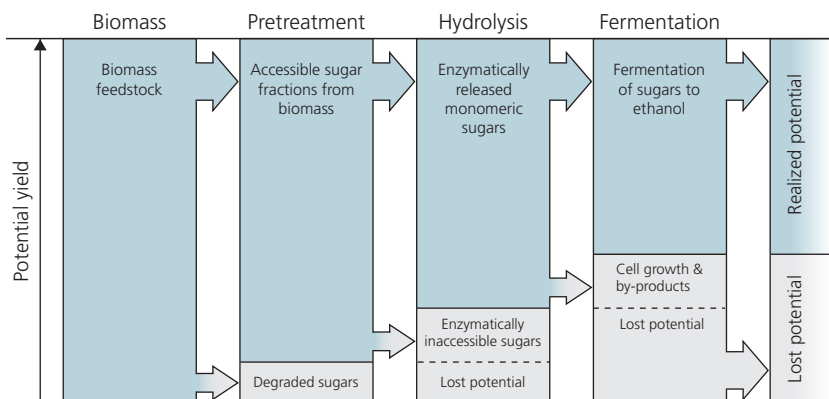


Figure 8. Schematic representation of yield losses in the integrated processing chain.

An overview of where losses of potential process ethanol yield in the chain of conversion steps are incurred.

input to be altered to minimize the cost of feedstock and the possibility of hedging against price changes and thus economical risk. All of the above call for diversification of the feedstock base. Diversifying feedstock sources increases the prospects of second-generation bioethanol contributing considerably to the fuel supply and increases the ability to reach policy goals regarding energy security and reduced greenhouse gas emissions.

From a process perspective, there are a number of restrictions in employing a broad feedstock base. A diversified feedstock base can be employed in a plant if the pretreatment technology is suitable for the individual feedstocks in the supply mix. Although some pretreatment methods, such as ionic liquid pretreatment (256), are claimed to be feedstock-agnostic, the pretreatment technologies that are being commercially pursued are not. The leading contenders for the pretreatment of agricultural residues are autocatalyzed and dilute acid-catalyzed steam pretreatment (77, 91), which have a proven broad range of applications (39). They are currently implemented by Biochemtex/Beta Renewables, POET-DSM, and Abengoa Bioenergy in their commercial-scale production facilities (91). The broad application range of steam pretreatments enables the use of a range of agricultural crops and residues as feedstock for the conversion process. Unfortunately, most feedstocks have different established optimal pretreatment conditions (257), which are founded on the attributes of the feedstock. The differences in optimal pretreatment conditions imply that the different feedstocks in a supply mix have to be processed in successive campaigns to overcome the recalcitrance of the lignocellulosic feedstock in the pretreatment and maximize the release of fermentable sugars in the process. However, there are also potential gains in blending different feedstocks and processing them concurrently.

The blending of time-dependent and location-specific feedstocks would enhance supply chain efficacy and stabilize the variance in feedstock composition. The feedstock blends can be formulated based on not only availability but also to target specific physiochemical characteristics. This allows a feedstock with lower potential process yields to enter the resource pool and meet acceptable compositional quality standards. The composition and consistency of a feedstock are important in attaining and maintaining desired process ethanol yields in the conversion process. The cellulose and hemicellulose content is critical to achieve the desired process ethanol production, and variations in ash content have implications for pretreatment efficacy, due to changes in buffering capacity (75). In addition, improved quality and consistency of the input contribute to the robustness of the process (75). It has also been suggested that there are synergistic effects in the steam pretreatment of feedstock blends of species that are physically and chemically heterogeneous (258). Higher sugar recoveries and yields after steam pretreatment and saccharification have been achieved with blends of hybrid poplar and wheat straw, as compared with the individual feedstocks (258). Nevertheless, the structural diversity of feedstock blends makes the concurrent processing of multiple feedstocks more challenging. In each step of the conversion process, there are potential losses that reduce the ethanol yield that can be derived from the employed feedstock (Figure 8). Structural differences between different feedstocks in a blend can give rise to unfavorable pretreatment efficacy for one of the components in the blend, and the effects propagate throughout the conversion chain. This could systematically decrease the potential ethanol yield in each step, as compared with a single-feedstock process, and lead to lower ethanol yields in the end. To realize the benefits of feedstock blending, it is of importance to develop conversion strategies with minimal negative impact on the performance of the pretreatment, saccharification, and fermentation and thus minimize the lost potential in ethanol yield (Figure 8).

In **Paper V**, concurrent processing of wheat straw and corn stover is investigated. The similar attributes of the feedstocks make them good candidates for feedstock blending and concurrent processing, encompassing dilute acid-catalyzed steam pretreatment, enzymatic hydrolysis, and cofermentation. The aim of the study was to obtain industrially relevant ethanol yields and titers that were comparable with those obtained with single feedstocks, preferably ethanol titers above 50 g·L⁻¹ and ethanol yields above 80% of the theoretical maximum. The single feedstocks and various blends thereof were pretreated under the same conditions—conditions that were derived from established optimal pretreatment conditions for the single feedstocks. Subsequently, the pretreated feedstocks were hydrolyzed and cofermented with 2 different SSCF strategies, emphasizing cofermentation (fed-batch SSCF with prefermentation) or enzymatic hydrolysis efficacy (batch SSCF with prehydrolysis).

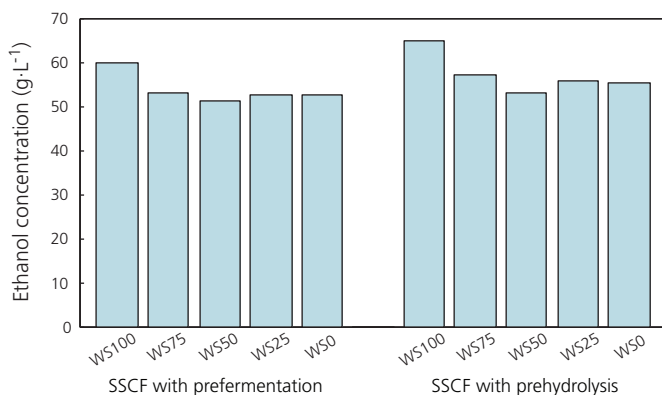


Figure 9. Ethanol titers after SSCF with pretreated wheat straw, corn stover, and blends thereof. Final ethanol titers after SSCF of wheat straw, corn stover, and various blends thereof (expressed above as percentage of wheat straw in the blend). SSCF was performed with either prefermentation, catering to the sugar preferences of the yeast, or prehydrolysis, emphasizing hydrolytic efficiency.

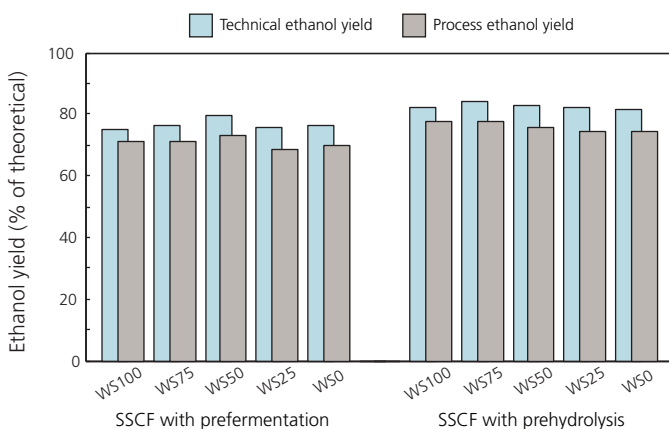


Figure 10. Ethanol yields after SSCF with pretreated wheat straw, cornstover, and blends thereof. Technical and process ethanol yields after SSCF of wheat straw, corn stover, and various blends thereof (expressed above as percentage of wheat straw in the blend). Technical ethanol yield is based on the sugar content in the pretreated material, and process ethanol yield is based on the sugar content in the feedstock. SSCF was performed with either prefermentation, catering to the sugar preferences of the yeast, or prehydrolysis, emphasizing hydrolytic efficiency.

Paper V shows that with the appropriately selected fermentative conversion configuration, few obstacles exist to the conversion of pretreated blends of corn stover and wheat straw in SSCF. Ethanol titers exceeded the desired 50 g·L⁻¹ (Figure 9) and similar technical ethanol yields that exceeded the desired 80% of the theoretical maximum were attained with individual feedstocks and all feedstock blends (Figure 10). However, the study suggests that restrictions to the process ethanol yield were

entailed to some extent by the uniform pretreatment conditions. Decreasing xylose recovery after pretreatment and decreasing glucan hydrolyzability were observed with increasing ratios of corn stover in the feedstock blend. This was reflected in the process ethanol yields (Figure 10). In spite of this, the process ethanol yields were remarkably robust across the whole range of feedstock blends. The process ethanol yields were in the range of 74% to 78% of the theoretical maximum. This suggests that wheat straw and corn stover could be used interchangeably, although the pretreatment conditions for corn stover and the blends might require fine-tuning to maximize the liberation of fermentable sugars.

Paper V further shows that the conversion process is highly intertwined and that the pretreatment was the determining step in the design of subsequent processing steps. The outcome of the pretreatment governs the choice of the integrated enzymatic hydrolysis and cofermentation configuration that elicits the highest process ethanol yield (Figure 10).

In addition, significantly lower technical ethanol yields were obtained after SSCF with prefermentation, which promotes the cofermentation efficacy, of steam-pretreated wheat straw in **Paper V** versus **Paper III**, even though the same cofermentation strategy, fermenting microorganism, and propagation strategy were employed. The principal difference is that the wheat straw in **Paper III** was pretreated under other conditions and with significantly greater severity. Although the recovery of glucose and xylose after pretreatment in **Paper III** is unknown, that the technical ethanol yield in **Paper III** was 25% higher suggests that the pretreatment conditions for maximizing the process ethanol yield might be different from those that maximize the yield of fermentable sugars after pretreatment and enzymatic hydrolysis, emphasizing the importance of clearly defining the pretreatment goals when optimizing the process. Furthermore, it stresses that balancing the intertwined trade-offs in the pretreatment, hydrolysis, and cofermentation is necessary to obtain industrially relevant ethanol titers and process ethanol yields and that the integrated process steps must be evaluated and optimized concurrently.

6.2 Cofermentation at scale

Scaling up fermentation processes from the lab to commercial scale has its own challenges. Factors, such as handling of feedstock, logistics, and process timing, that generally are not problems on the lab scale need to be addressed. Scaled-up processes imply larger vessels and, by necessity, the handling of greater amounts of materials. The logistics of such an operation imply longer time constants for operations, such as feeding pretreated feedstock to the bioreactors and longer mixing times. For many fermentation strategies developed on a lab scale, this means that feeding patterns need

to be modified to the reality of large-scale enterprises to mimic lab-scale designs. This is addressed in **Paper IV**. In addition, ethanol production and productivity on the demonstration and commercial scales are constrained by factors, such as the risk of contamination, reduced surface area-to-volume ratios for heat transfer, prolonged mixing times, and the power requirements for mixing (231).

Sterile or aseptic conditions can be maintained on the lab scale but are not a cost-effective option for commercial-scale production. Process design and handling of process streams that minimize the risk of microbial contamination are needed, since contamination leads to decreased process ethanol yields and the considerable loss of potential revenue. Methods of reducing the risk of microbial contamination might necessitate process modifications that reduce the conversion efficiency but are necessary to de-risk the operation, such as by lowering the pH during fermentation to suppress bacterial growth (259).

Many of the other constraining factors are related to the altered geometric and physical conditions in a scaled-up bioreactor. As the bioreactor volume is increased, the surface area per unit volume for heat transfer is reduced, which creates difficulties in controlling the temperature in the bioreactor. This poses a problem for microbial and biocatalytic processes, where stability and performance are highly temperature-dependent (45, 231). Mixing also becomes increasingly problematic with increasing scales, and the time constants generally increase (231, 260). This includes mixing times, which are crucial for the biochemical conversion process. Longer time constants for mixing and improper mixing regimens give rise to both temperature and concentration gradients in the bioreactor (260). This can form zones with enhanced stress conditions, such as hotspots with high or low pH or zonal overheating, which can have detrimental effects on the stability and productivity of microorganisms and biocatalysts (231, 260). Scaling up processes is often associated with reduced process performance in comparison with lab-scale technology (231). Less favorable mixing behaviors and conditions lead to decreased reproducibility and reduced yields and—in the end—diminished batch-to-batch consistency and product quality (260).

However, the conditions imposed by the transition to a larger scale need not be detrimental. For example, it has been shown that hydrolysis rates in the enzymatic hydrolysis of steam-pretreated spruce are faster on a demonstration scale than on a lab scale (261). Successful scale-up requires an understanding of the interactions between microorganisms and the biocatalyst and the chemical and physical conditions in the reactor to allow accurate predictions to be made as scales are changed.

The specific conditions imposed by a larger scale in the fermentation, which are usually not present on a smaller scale, make scale-up studies important for facilitating the transition between scales. It allows for the identification of specific conditions that occur in the scale-up and the parameters that have the largest impact on performance. Conversely, scale-down experiments are important to allow for an investigation of the

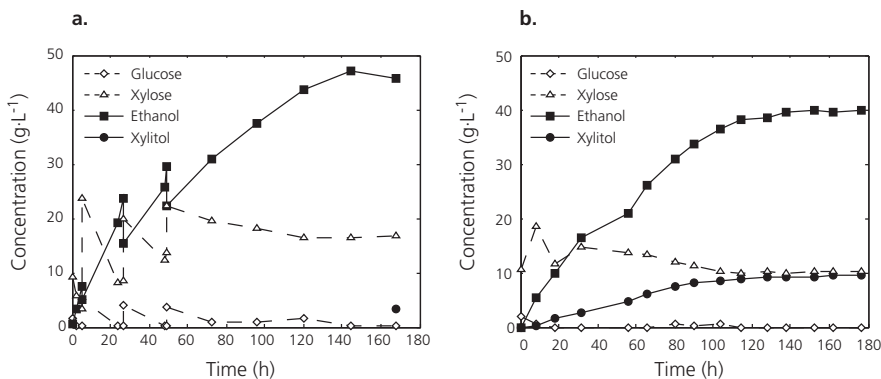


Figure 11. Fed-batch SSCF on two scales—process development and demonstration scale.

SSCF with prefermentation and fed-batch addition of substrate and enzymes for the conversion of steam-pretreated corncobs to ethanol in (a) process development-scale and (b) demonstration-scale bioreactors.

conditions. By mimicking the conditions on a smaller scale, cost-efficient investigations with higher throughput can be performed, and crucial data for scaling up can be obtained (260). Since carrying out experiments is expensive and time-consuming, especially when scaling up, process modeling and simulations can facilitate the cost-efficient evaluation of various process options. In order to model the process, data are needed, but convincing information over a significant range of conditions that could be encountered commercially is not obtained on a lab scale. Pilot- and demonstration-scale plants bridge the gap between the bench and commercial scales and contribute to acquiring the necessary knowledge and experience to de-risk commercial-scale projects (246).

In **Paper IV**, yeast propagation and SSCF strategies that were developed for cofermentation of steam-pretreated corncobs on the lab and bench scale (<1.5 L) were scaled up to the process development (30 L) and demonstration scale (10 m³). The employed SSCF strategy used prefermentation and substrate and enzyme feeding to meet the sugar consumption and conversion preferences of the xylose-fermenting strain of *S. cerevisiae*, which is elaborated on in **Paper III**. For demonstration-scale experiments, the SSCF strategy was adapted to the conditions for material handling and feeding at a demonstration scale facility, mimicking the development-scale feeding pattern. Differences in circumstances for handling feedstock and feeding the bioreactors are reflected in the time courses (Figure 11). The study showed that the SSCF strategy performed comparably on the process development and demonstration scales, thus verifying the scalability of the design. Comparable ethanol yields in the range of 60% to 70% of the theoretical maximum, based on the total available glucose and xylose, and similar cofermentation patterns were observed, as can be seen in Figure 11, and the final ethanol concentrations were adequate for cost-efficient

product recovery. These types of scale-up experiments are essential for providing data about the nonlinear effects of scaled parameters, which can be used to predict the behavior on a commercial scale and aid in developing solid principles for the design of biomass processing operations.

7. Conclusions and outlook

7.1 Conclusions

Industrial processes can be engineered to suit microorganisms, or the microorganism can be metabolically engineered to fit the process. A simple conversion process with tailored enzyme systems and fermenting microorganisms will likely reduce costs, but optimizing one strain for each combination of feedstock and pretreatment is not feasible. This is especially true if seasonal and regional variations in the composition and variable supply of individual feedstocks are taken into consideration. Process design provides the means to adapt the process to a microorganism, improve ethanol productivity and yields, and ensure consistent cofermentation results. This reduces the technological risk. In this thesis, various process design strategies to improve the conversion process and reduce technological risks were presented.

Short-term adaptation of xylose-fermenting *S. cerevisiae* with lignocellulosic hydrolysate during propagation provides a broad adaptive response, which improved xylose utilization, reduced by-product formation, and—in the end—increased the overall ethanol yield. The benefits came at the expense of a lower biomass yield in the propagation, due to the inhibition of growth in the presence of lignocellulosic hydrolysate during propagation. The optimal short-term adaptation, which offsets the improved performance and reduced biomass yield, has to be based on economic considerations.

The fermentation design can increase ethanol titers and process ethanol yields in the cofermentation of lignocellulosic hydrolysates. The choice of cofermentation strategy—SHCF, SSCF, or hybrid strategies—depends on the fermenting microorganism, feedstock, and pretreatment conditions. From a fermentative conversion standpoint, targeting xylose and glucose conversion in sequential cofermentation steps, made possible by slurry fractionation, maintained the xylose-fermenting capacity of xylose-fermenting *S. cerevisiae* in SHCF and SSCF and improved xylose utilization. However, multiple cofermentation steps may prolong the fermentation time and thus lower process ethanol productivity. In addition, substrate feeding strategies provide the means to improve xylose utilization, minimize by-product formation, and thus improve overall ethanol yields. However, continuous or repeat addition of substrate that contains inhibitors has a detrimental effect on yeast

viability. Ethanol yield is maximized by a balance between efficient xylose utilization and maintained yeast viability.

Fed-batch SSCF designs with substrate and enzyme feeding that were developed on a lab and bench scale required adaptation of the strategy to the conditions for material handling and feeding on the demonstration scale to mimic the process development-scale feeding pattern. The SSCF strategy performed comparably on the process development and demonstration scales. Comparable ethanol yields, based on total available glucose and xylose; similar cofermentation patterns; and similar final ethanol concentrations were obtained, thus verifying the scalability of the design. These kinds of scale-up experiments are essential for providing data that can be used to predict the behavior on a commercial scale.

Feedstocks with similar attributes can be blended to improve the quality of the feedstock input and expand the feedstock base for ethanol production, contributing to improved process consistency and feedstock supply. Wheat straw and corn stover can be blended at various ratios and processed concurrently by steam pretreatment and SSCF, eliciting comparable process ethanol yields across the whole range of feedstocks and feedstock blends. However, uniform pretreatment conditions for all blends can systematically disfavor one of the components in the blend, constraining the process ethanol yield for feedstock blends. Fine-tuning of the pretreatment conditions, based on the feedstock composition, might be required to maximize the liberation of fermentable sugars in each blend. Pretreatment was the determining step for the integrated process design, governing the choice of the subsequent SSCF design. The key to obtaining high ethanol titers and process yields is to balance the intertwined trade-offs in pretreatment, enzymatic hydrolysis, and cofermentation.

In conclusion, the ethanol yield in the cofermentation of biomass-derived glucose and xylose is maximized through multiple trade-offs along the integrated chain of steps in the conversion process. The choices that are ultimately made in each processing step will depend on economic considerations.

7.2 Outlook

Although process design begins with the characteristics of the available enzyme systems, fermenting microorganisms, and pretreatment methodologies, promising process designs provide a feedback loop for the development of enabling capabilities. These capabilities can improve the efficiency of cofermentation strategies. Several improvements in the attributes of enzyme systems and fermenting microorganisms are desirable:

- Although improved inhibitor tolerance and an extended range of sugars that can be converted by the fermenting microorganism are desirable traits, the greatest gains in cofermentation are likely to be attained through the development of higher conversion rates and true coconsumption capabilities. These two attributes will shorten fermentation times and eliminate conversion of the trailing xylose, which is a bottleneck.
- Development of enzyme systems and strains that can close the gap between optimal temperature and pH in enzymatic hydrolysis and cofermentation, respectively. This would eliminate some of the trade-offs that reduce the conversion efficiency of SSCF and allow for simpler cofermentation strategies.
- Development of more efficient enzyme preparations that allow for milder pretreatment conditions in the steam pretreatment step. Milder pretreatment would increase the recovery of fermentable sugars after pretreatment and thus the potential to obtain higher yields. It would also generate lower amounts of inhibitory compounds, which would improve fermentation performance.

From a process point of view, the development of robust propagation procedures that can adapt the fermenting microorganism to specific feedstocks, pretreatment methods, and pretreatment conditions and at the same time elicit high biomass yields, preferably propagated on lignocellulosic sugars, is of interest. Furthermore, it is of interest to investigate the concurrent processing of other location-specific feedstock blends to expand the feedstock base, enable feedstock upgrading, and identify potential synergies. Likely, the enabling technologies and demonstration of concepts will originate in the lab, while incremental improvements in the conversion process will be made in production facilities.

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RESEARCH

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Short-term adaptation during propagation improves the performance of xylose-fermenting *Saccharomyces cerevisiae* in simultaneous saccharification and co-fermentation

Fredrik Nielsen^{1*} , Elia Tomás-Pejó^{2,3}, Lisbeth Olsson² and Ola Wallberg¹

Abstract

Background: Inhibitors that are generated during thermochemical pretreatment and hydrolysis impair the performance of microorganisms during fermentation of lignocellulosic hydrolysates. In omitting costly detoxification steps, the fermentation process relies extensively on the performance of the fermenting microorganism. One attractive option of improving its performance and tolerance to microbial inhibitors is short-term adaptation during propagation. This study determined the influence of short-term adaptation on the performance of recombinant *Saccharomyces cerevisiae* in simultaneous saccharification and co-fermentation (SSCF). The aim was to understand how short-term adaptation with lignocellulosic hydrolysate affects the cell mass yield of propagated yeast and performance in subsequent fermentation steps. The physiology of propagated yeast was examined with regard to viability, vitality, stress responses, and upregulation of relevant genes to identify any links between the beneficial traits that are promoted during adaptation and overall ethanol yields in co-fermentation.

Results: The presence of inhibitors during propagation significantly improved fermentation but lowered cell mass yield during propagation. Xylose utilization of adapted cultures was enhanced by increasing amounts of hydrolysate in the propagation. Ethanol yields improved by over 30 % with inhibitor concentrations that corresponded to ≥ 2.5 % water-insoluble solids (WIS) load during the propagation compared with the unadapted culture. Adaptation improved cell viability by >10 % and increased vitality by >20 %. Genes that conferred resistance against inhibitors were upregulated with increasing amounts of inhibitors during the propagation, but the adaptive response was not associated with improved ethanol yields in SSCF. The positive effects in SSCF were observed even with adaptation at inhibitor concentrations that corresponded to 2.5 % WIS. Higher amounts of hydrolysate in the propagation feed further improved the fermentation but increased the variability in fermentation outcomes and resulted in up to 20 % loss of cell mass yield.

Conclusions: Short-term adaptation during propagation improves the tolerance of inhibitor-resistant yeast strains to inhibitors in lignocellulosic hydrolysates and improves their ethanol yield in fermentation and xylose-fermenting capacity. A low amount of hydrolysate (corresponding to 2.5 % WIS) is optimal, whereas higher amounts decrease cell mass yield during propagation.

Keywords: Yeast, Pre-adaptation, Propagation, Co-fermentation, Lignocellulose, Xylose

*Correspondence: fredrik.nielsen@chemeng.lth.se

¹ Department of Chemical Engineering, Lund University, P.O. Box 124, 221 00 Lund, Sweden

Full list of author information is available at the end of the article



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Background

One of the major hurdles in achieving economical fermentative conversion of lignocellulosic biomass to ethanol is the presence of inhibitory compounds that are generated during thermochemical pretreatment of biomass. Major inhibitors, such as weak organic acids, furfuraldehydes, and lignin derivatives, have adverse effects on the performance of microbial biocatalysts [1, 2]. Their inhibitory activity affects cellular growth and fermentation behavior, thus decreasing the longevity of the fermenting microorganism, ethanol productivity, and overall ethanol yield of the process [1].

Detoxifying the hydrolysate is one technique of overcoming the limitations that are imposed by such inhibitors [3]. However, many detoxification methods incur additional production costs and add complexity to the fermentation process [4, 5], decreasing the profitability of lignocellulosic ethanol production.

An alternative to detoxification is the use of fermenting microorganisms that can detoxify or tolerate inhibitors in situ without compromising ethanol productivity or yield. A combination of inhibitor-tolerant yeast strains and efficient feed strategies can lower the technological risk in the fermentative step of the lignocellulose-to-ethanol process. Since the economics of fermentation-based bioprocesses depends significantly on the performance of microbial biocatalysts, microbial performance is likely a key to sustainable and cost-competitive production of lignocellulosic ethanol.

Several approaches to developing *Saccharomyces cerevisiae* strains with improved tolerance to inhibitors have been described. Overexpression of homologous or heterologous genes that encode enzymes that confer resistance to specific inhibitors in yeast has improved their tolerance to lignocellulosic hydrolysates [6–8]. Improved tolerance to inhibitors has also been obtained in *S. cerevisiae* strains by evolutionary engineering [9, 10], a method that mimics natural selection by improving cellular properties through iterative genetic diversification and selection. In evolutionary engineering, microorganisms that are subjected to high inhibitor concentrations over extended periods acquire substantial tolerance to inhibitors due to random genetic changes [11].

Pre-emptive exposure to inhibitors can be used during cultivation to provide short-term adaptation and improved performance during fermentation. Whereas changes are incorporated into the genotype of a microorganism in long-term adaptation, short-term adaptation relies on the expressed phenotype and phenotypic heterogeneity. The phenotype that is induced during short-term adaptation primes a microorganism to function in presence of specific environmental factors [12, 13]. Physiologically, adaptation is effected in part by the induction

of genes that express a particular resistance phenotype in the presence of sublethal concentrations of inhibitors [7, 8, 14, 15]. The selective pressure exercised by inhibitors during short-term adaptation selects for phenotypes that are more resistant to inhibitors in the substrate.

One method of short-term adaptation of yeast is pre-adaptation—cultivating yeast under conditions that resemble the subsequent fermentation. Pre-adaptation can reduce inhibitory effects and increase the performance of yeast. Several examples of improvements in hexose fermentation have been noted with pre-adaptation of *S. cerevisiae*. Pre-adaptation of *S. cerevisiae* enhances its ability to detoxify or tolerate inhibitors in the media [16]. Yeast that are pre-adapted with hydrolysate liquor during propagation convert hexoses to ethanol faster, and detoxify furfural and 5-hydroxymethyl furfural (HMF) by metabolic conversion considerably faster than yeast that have been propagated in the absence of inhibitors [16]. Short-term adaptation of *S. cerevisiae* with added acetic acid in the pre-culture reduces fermentation times significantly in hexose fermentations with inhibitory levels of acetic acid [17].

In addition, adapting yeast during propagation elicits an adaptive response to inhibitory compounds in the hydrolysate. This is particularly important, because the exact composition of the hydrolysate, especially regarding lignin residues and derivatives, is seldom known and because it is poorly understood which individual compounds are most inhibitory.

Although the impact of short-term adaptation on hexose fermentation has been studied [16–18], the influence on co-fermentation of hexoses and pentoses has not been investigated extensively. Xylose fermentation capacity is affected to a greater extent by inhibitors than hexose fermentation capacity [19]. In using recombinant *S. cerevisiae* with the ability to co-ferment biomass-derived xylose and glucose, the effects of the propagation procedure on xylose and glucose consumption must be considered to realize the desired ethanol. Short-term adaptation during propagation has beneficial effects on the utilization of glucose and xylose in the co-fermentation of bagasse hydrolysates in terms of consumption and conversion [20], suggesting that this method is a feasible approach for increasing the resistance to fermentation inhibitors.

It is an attractive option to combine the use of inhibitor-tolerant strains with short-term adaptation to improve fermentation performance. However, the presence of inhibitors during cultivation on hydrolysate impedes growth [1], resulting in a lower cell mass yield compared to cultivation without inhibitors. Implicitly this means a higher cost of propagation of yeast for a specific fermenter capacity. To improve the economics of the process, the added cost must at least be offset by

improved performance of the pre-adapted yeast. Successful pre-adaptation has the potential to decrease yeast loads, shorten fermentation times and increase substrate loads.

This study examined the influence of pre-adaptation on yeast performance and overall ethanol yield from glucose and xylose in simultaneous saccharification and co-fermentation (SSCF) of steam-pretreated wheat straw. The objective was to determine the level of adaptation that is required to promote efficient co-fermentation of glucose and xylose in SSCF while maintaining cell mass yields during propagation. Short-term adaptation was performed by gradually adapting the yeast to inhibitor concentrations that resembled those in the fermentation. The aim was to minimize the hydrolysate requirements in the propagation to preserve high cell mass yields in the propagation step while still acquiring yeast that were adapted to the harsh fermentation environment. Select physiological properties of the cultivated yeast were monitored to identify changes that were induced by the propagation procedure and influenced ethanol productivity and yield during fermentation.

Results and discussion

In this study we correlated several traits of the propagated cells with their fermentation performance with respect to cell mass yield, cell proliferation, and physiological properties. In the next step, these hallmarks were examined with regard to SSCF to determine their effects on ethanol productivity and yield under relevant process conditions.

Propagation

Propagation was performed in fed-batch mode after an initial batch culture. During the propagation, hydrolysate amounts that corresponded to 0, 2.5, 5.0, or 10 % water-insoluble solids (WIS) load were added during the late feed phase. Propagation was evaluated in terms of final cell count, cell mass yield, viability, vitality, stress indicators, and expression of genes that conferred resistance to inhibitors.

Cell count and cell mass yield

The cell count at the end of propagation and the cell mass yield in the cultivation step were measured to determine the impact of pre-adaptation on cell proliferation. Cell mass yield declined with increasing amounts of hydrolysate liquor in the feed solutions (Fig. 1a). It decreased by 20 % with inhibitor concentrations in the feed that corresponded to 10 % WIS compared with the molasses reference, which was expected, because cell growth is suppressed by inhibitors that are generated during the thermochemical pretreatment [1]. The molasses solution

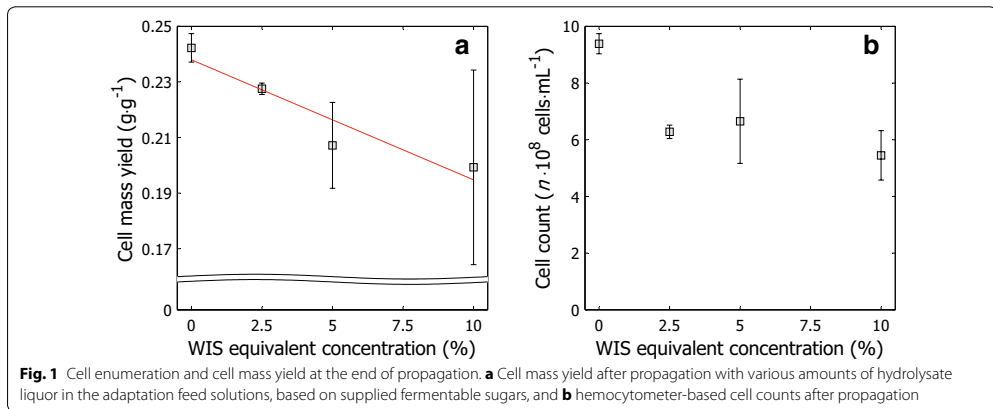
that was used in the batch cultivation phase contained weak organic acids (1.7 g L⁻¹ lactic acid and 0.4 g L⁻¹ acetic acid) that inhibit cell growth and potentially act synergistically [21].

The inhibitors from the molasses in the feed solutions (~5.2 g L⁻¹ lactic acid and ~1.2 g L⁻¹ acetic acid) contributed further to the background inhibitory activity in the propagation medium. The high concentration of inhibitors, especially acetic acid (10.2 g L⁻¹) and furfural (7.9 g L⁻¹), in the hydrolysate liquor that was introduced to the feed solutions suppressed growth further. Higher inhibitor concentrations in the feed solutions were expected to divert metabolic flux away from growth toward ATP formation to maintain intracellular pH and detoxify the hydrolysate, because weak organic acids lead to intracellular acidification and because cellular detoxification mechanisms are energy demanding.

The presence of xylose in the feed solutions that contained hydrolysate liquor possibly biased the data. Because the employed strain was able to grow aerobically on xylose but preferentially consume hexose sugars, the impact of xylose availability on cell mass yield becomes unclear. However, the xylose-supplemented molasses feed media elicited no significant differences in cell mass yield compared with the molasses reference (data not shown), thus indicating little to no effect of xylose on cell mass yield in propagation.

The differences in cell mass yields were due in part to the cultivation feed strategy. Because the difference in inhibitor concentrations between feed solutions was expected to affect specific growth rates, implementing a fixed feeding strategy for all propagation conditions would have created disparate cultivation conditions, and consequently, certain adapted cultures would have been cultivated under sub-optimal conditions. Overfeeding of substrate, due to a low critical specific growth rate under the prevailing cultivation conditions, causes cells to undergo respiratory–fermentative growth instead of targeted respiratory growth. Thus, cell mass yields will likely decrease as the carbon source is converted aerobically into ethanol—often referred to as the Crabtree effect [22]. Respiratory growth typically leads to a cell mass yield of approximately 0.5 g g⁻¹, compared with roughly 0.1 g g⁻¹ for aerobic fermentation. Respiratory growth can be ensured through optimization of the propagation feed rate and the use of an exponential feeding profile that keeps the specific sugar addition rate lower than the rate that offsets overflow metabolism.

The final cell counts, shown in Fig. 1b, and cell mass yields had disparate patterns. The cell count for the reference culture on molasses at the end of the cultivation was 9×10^8 cells mL⁻¹, and a downward shift to 5.4–6.3 $\times 10^8$ cells mL⁻¹ was obtained with inhibitors in the



feed solution (Fig. 1b). By microscopy, larger cells were generated in the presence of inhibitors. Although hemocytometer-based cell counts are prone to experimental error and large spreads, these results indicate lower cell proliferation in the presence of inhibitors.

Viability and vitality

Cell viability, the ability of cells to sustain metabolic activity and reproduce, was determined by methylene blue staining and cell counts using a hemocytometer. The percentage of viable cells increased with increasing amounts of hydrolysate liquor in the feed during the fed-batch phase of the cultivation (Fig. 2a). One explanation is that although fewer cells were produced, they were better equipped to survive. However, the frequency of budding cells display an opposing pattern with declining frequency with higher amounts of hydrolysate liquor in the feed (Fig. 2b), likely due to the suppression of cell growth and cell proliferation by inhibitors [1, 14, 19, 23].

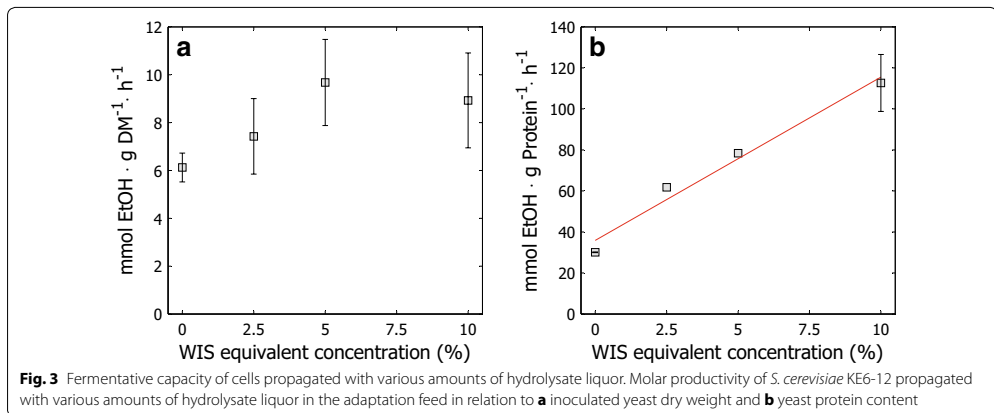
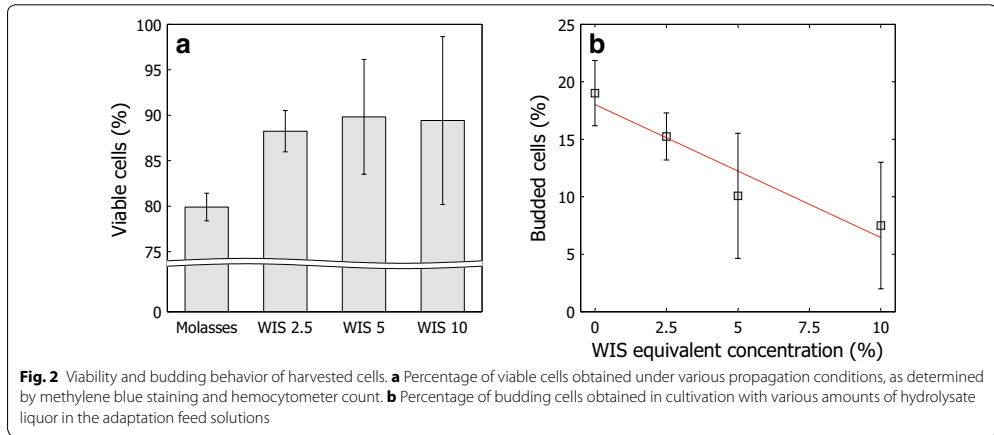
It has been suggested that furfural has transient effects and decreases cell replication without inhibiting cell activity [24]. Our results indicate that the metabolic activity improved with short-term adaptation, despite the curtailed ability to reproduce. Further, budding was seen in a small number of stained cells, indicating that some cells were susceptible to the dye but remained viable or that oxygen was present and the dye was reoxidized to its colored state. Both hemocytometer- and methylene blue staining-based counts tend to produce high levels of experimental error [25], hence a variance in the results was expected. Nevertheless, data on viability, although important, are insufficient—cells might be viable but weakly active and are unable to perform in fermentation.

Fermentative capacity tests were performed to assess the vitality of the cultivated yeast. Vitality reflects the physiological state of living cells and, in this instance, refers to the fermentation performance of the yeast. Increased fermentative capacity, in terms of ethanol productivity per gram of yeast dry matter, was obtained with increasing amounts of hydrolysate liquor in the feed (Fig. 3a). The greater fermentative capacity of adapted cells indicates that they were in a more metabolically active state. When the fermentative capacity was expressed as molar ethanol productivity per gram of intracellular protein, this trend became clearer (Fig. 3b). This result indicates that pertinent proteins were synthesized when the cells were subjected to selective pressure. The amount of synthesized intracellular proteins declined with increasing amounts of hydrolysate liquor in the feed solution (data not shown). These results suggest that adaptation enables yeast to produce cells with the proper levels of enzymes and proteins that are needed to maintain high metabolic activity and sufficient energy supplies for energy demanding detoxification and regulation of intracellular pH.

Adaptation-induced transcriptional changes

The expression levels of several genes were measured by quantitative PCR (qPCR) in cultures that were adapted with varying amounts of hydrolysate liquor. Genes that conferred resistance to furfuraldehydes and aliphatic acids and those that promoted growth under toxic conditions (*ZWF1*, *ADH6*, *ALD6*, and *ERG2*) were selected as proxies of adaptation in different cultures.

Previous studies have shown that *S. cerevisiae* converts furfural and HMF into their reduced or oxidized derivatives, which have lower toxicity against *S. cerevisiae* [26].

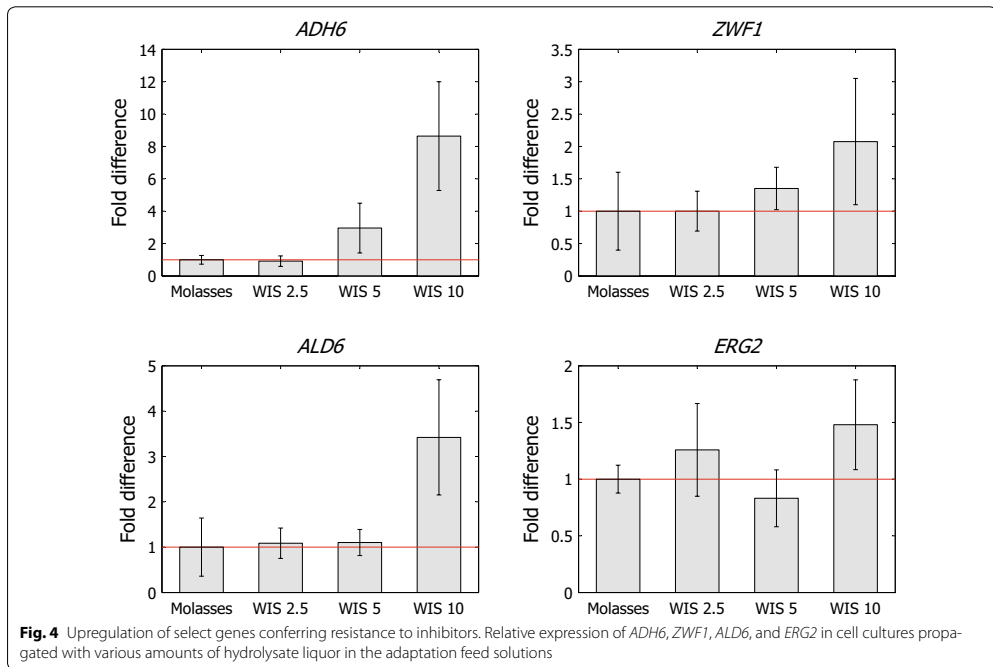


Cytoplasmic glucose-6-phosphate dehydrogenase, which is encoded by *ZWF1*, and cinnamyl alcohol dehydrogenase, encoded by *ADH6*, converts these furan derivatives into less toxic compounds [7, 8]. Yeast strains that over-express *ADH6* have also been shown to be able to grow in the presence of toxic aldehyde concentrations [27].

Gene expression of *ZWF1* and *ADH6* was similar between the reference culture and xylose-supplemented cultures (data not shown). Further, the expression of *ZWF1* and *ADH6* did not differ significantly between the reference cell culture and the culture that was pre-adapted with low hydrolysate liquor content (2.5 % WIS equivalent) (Fig. 4a, b). However, *ZWF1* and *ADH6* were upregulated with higher hydrolysate liquor content in the

feed (Fig. 4)—i.e., with inhibitor concentrations that corresponded to 5 and 10 % WIS mass fraction.

The cultures generated an adaptive response on a transcription level at furfuraldehyde concentrations that corresponded to 5 % WIS and were amplified by increased exposure to furfuraldehydes. *ZWF1* levels were marginally higher with adaptation at inhibitor concentrations that corresponded to 5 % WIS versus the reference state but increased twofold at 10 % WIS. *ADH6* increased threefold at inhibitor concentrations that corresponded to 5 % WIS compared with the reference, which was amplified to an eightfold increase with an inhibitor concentration of 10 % WIS equivalent. The upregulation of these genes reflects the adaptation of cells to environmental factors,



which is expected to improve growth and ethanol productivity in the presence of furfural and HMF [14].

The upregulation of *ZWF1* and *ADH6* can affect the distribution of products from the engineered XR/XDH pathway for xylose utilization. Fermentation of xylose to ethanol with recombinant *S. cerevisiae* is slow and exhibits a low ethanol yield, likely due to capacity limitations in the pentose phosphate shunt and an imbalance in redox co-factors created by the xylose catabolism [28]. The redox-neutral process requires NADPH (XR) and NAD⁺ (XDH) [28], which must be regenerated in separate processes. Xylitol formation and excretion can result from an imbalance in co-factors between the NAD(P)H-consuming XR and NADH-producing XDH reactions [29]. Increased activity of NAD(P)H-dependent alcohol dehydrogenase 6 (encoded by *ADH6*) and NAD(P)⁺-dependent glucose 6-phosphate dehydrogenase (encoded by *ZWF1*) in the presence of inhibitors changes the intracellular pool of NAD(P)H [30, 31]. Alterations in the NAD(P)H-pool and the co-factor balance between NAD(P)H and NAD⁺ can influence the product distribution from the engineered XR/XDH pathway and thus the extent of xylitol formation and excretion in recombinant *S. cerevisiae* [30–32].

Aldehyde dehydrogenases, such as the protein that is encoded by *ALD6*, constitute another class of enzymes that have beneficial effects on cell tolerance. The acetaldehyde dehydrogenase that is encoded by *ALD6* plays a critical role in the conversion of acetaldehyde to acetyl-CoA during growth on non-fermentable carbon sources [33] and in the breakdown of toxic aldehydes [14]. It has been shown that the *ALD6*-encoded NAD(P)⁺-dependent aldehyde dehydrogenase is upregulated in the presence of HMF and furfural [34]. In contrast to *ZWF1* and *ADH6*, *ALD6* was upregulated (by threefold) only at inhibitor concentrations that corresponded to 10 % WIS (Fig. 4), which might be an adaptive response to the stress imposed by critical levels of toxic compounds or metabolic readjustment to cope with environmental factors. Park et al. [14] proposed that the overexpression of *ALD6* mediates the recovery of yeast cell metabolism from HMF and furfural inhibition and thus increases ethanol production from lignocellulosic biomass that contained furan-derived inhibitors. Moreover, it has been shown that upregulation of *ALD6* enhances cell growth in media that contains furfural and HMF [14].

ERG2 mediates the biosynthesis of ergosterol and is one of several genes that are involved in the biosynthesis

of plasma membrane lipids that protect against acetic acid [35]. Upregulation of *ERG2* serves as a proxy for changes in the concentration of structural membrane components that confer resistance to acetic acid, for example. There was no significant change in *ERG2* levels at moderate concentrations of inhibitors in the feed (Fig. 4). However, at inhibitor concentrations that corresponded to 10 % WIS, *ERG2* was upregulated 1.5-fold compared with the reference (Fig. 4). The upregulation indicate alterations in the plasma membrane structure to withstand the hostile environmental conditions, which is likely to affect the tolerance of yeast to acetic acid, as reported for other chemical stresses [36].

Stress indicators: glycogen and trehalose

The trehalose and glycogen levels in *S. cerevisiae* are believed to be major determinants of stress resistance. These carbohydrates accumulate when growth conditions deteriorate as a means of adapting to various environmental conditions [37]. Trehalose, in particular, has been attributed a role in stress protection, which is a crucial mechanism in the adaptive response to a variety of physical and chemical stresses (e.g., nutritional limitations, heat, oxidative agents, and ethanol inhibition) in *S. cerevisiae* [38, 39]. The relative levels of glycogen and trehalose can be considered indicators of the stress to which cells have been subjected during cultivation [40] but also function as reserve compounds and protect cell integrity against several stressors [39].

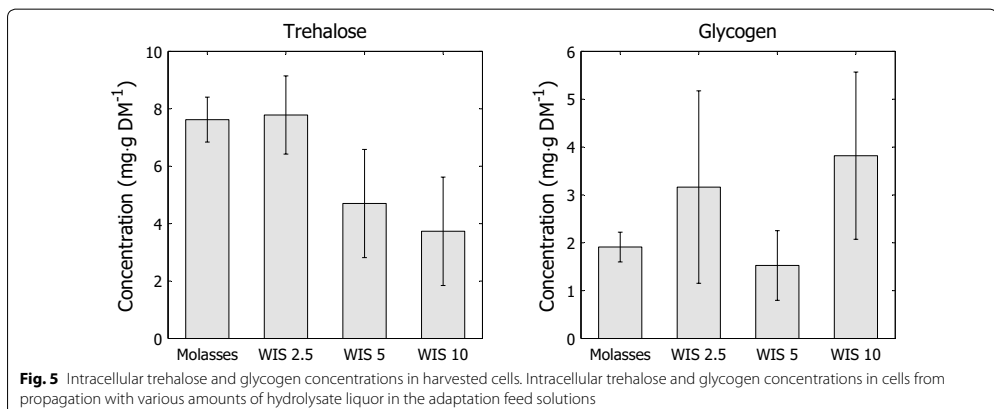
As shown in Fig. 5, there were no significant differences in the glycogen content of cells that were adapted with increasing amounts of hydrolysate in the feed. In contrast, intracellular trehalose levels decreased with increasing hydrolysate content during propagation

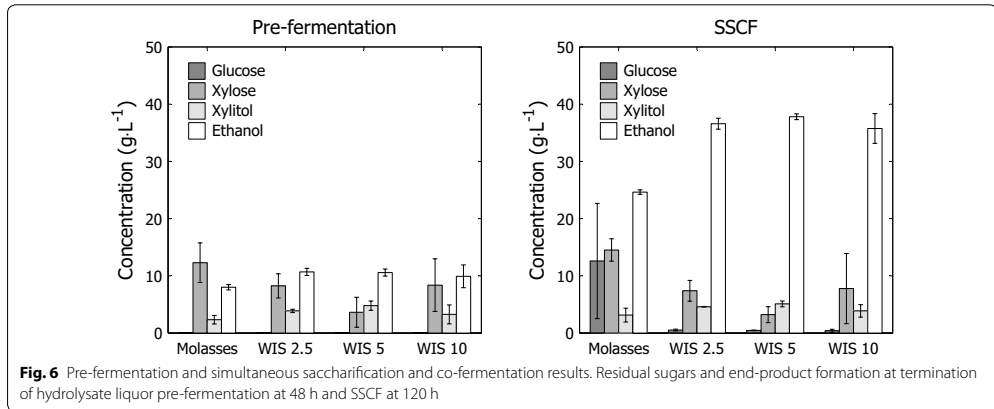
(Fig. 5). Because trehalose is considered to be a stress-induced molecule, the low concentrations of trehalose indicate less stress in adapted cultures due to the inhibitors. Considering the qPCR data, the reduced synthesis of stress-induced molecules might be attributed to an enhanced adaptive response. The decline in synthesized trehalose (Fig. 5) coincides with the upregulation of *ADH6*, *ZWF1*, and *ALD6* (Fig. 4).

It has been suggested that increased trehalose content in *S. cerevisiae* sustains cell viability during the initial stages of fermentation and thus results in higher carbohydrate utilization rates [41]. Elevated trehalose levels would thus improve the outcomes of the fermentative capacity tests and SSCF evaluation. In this study, this benefit was neither observed in the fermentative capacity tests (Fig. 3) nor in the SSCF experiments (Fig. 6). In these cases, performance improved and trehalose levels declined with increasing amounts of hydrolysate during the short-term adaptation.

Simultaneous saccharification and co-fermentation

Fermentation performance was evaluated using a hybrid SSCF design, comprising pre-fermentation of the hydrolysate liquor and SSCF with 2 additions of solid material, as described by Nielsen et al. [42]. This design allowed us to study fermentation behavior during hydrolysate fermentation and SSCF under the appropriate conditions for each process and has been applied successfully to obtain high ethanol yields (>90 % of theoretical maximum stoichiometric yield) in highly inhibitory, pretreated lignocellulosic material. Fermentation of steam-pretreated lignocellulosic materials by *S. cerevisiae* KE6-12 has been demonstrated in various fermentation modes [42–45]. In these studies, short-term adaptation





was performed during propagation with hydrolysate amounts that resembled the fermentation conditions. However, the effects on fermentation outcomes were not elucidated.

Pre-fermentation

All cultures depleted the available glucose during the pre-fermentation (Figs. 6a, 7). The disparity between different cultures appeared in the xylose utilization and end-product formation. Whereas the yeast cultures that were cultivated only on molasses and molasses that were supplemented with xylose utilized 40–50 % of the available xylose (Fig. 6; Table 1), the pre-adapted cultures showed greater xylose utilization and ethanol productivity

(Fig. 7). Xylose utilization improved with increasing amounts of hydrolysate liquor in the fed-batch propagation. However, in the pre-fermentations with yeast that was cultivated with an inhibitor concentration that corresponded to 10 % WIS, the variance in xylose utilization increased significantly, correlating with greater variance in cell mass yield, viability, and transcriptional changes with increasing inhibitor concentrations in the feed.

Pre-adapted cultures also produced over 30 % more ethanol than the reference culture, due to improved xylose utilization (Fig. 6; Table 1), which was, however, not mirrored by the ethanol yield. This result is attributed in part to xylitol excretion. The faster utilization of sugar and removal of furaldehydes from the liquid phase

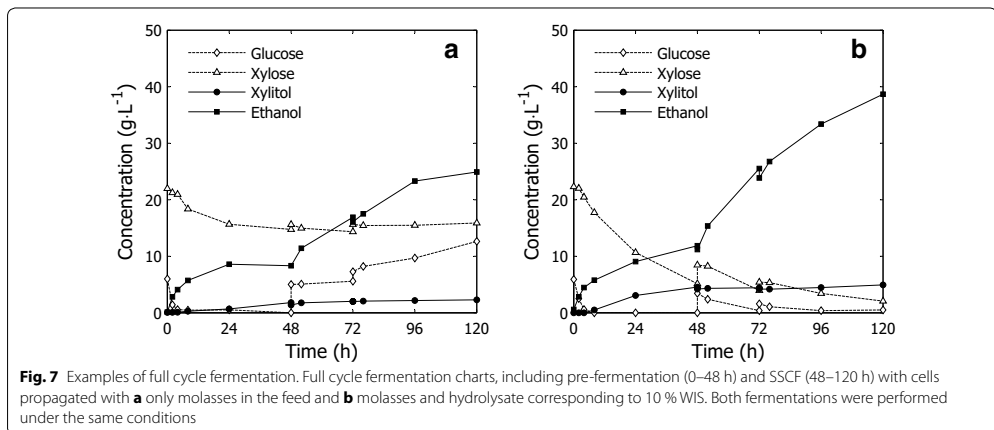


Table 1 Pre-fermentation and simultaneous saccharification and co-fermentation results

| | Pre-fermentation | | | | SSCF | | | |
|-----------|------------------------------|-----------------------------|------------------------------|------------------------------|------------------------------|-----------------------------|------------------------------|------------------------------|
| | Residual sugars | | End-products | | Residual sugars | | End-products | |
| | Glucose (g L ⁻¹) | Xylose (g L ⁻¹) | Ethanol (g L ⁻¹) | Xylitol (g L ⁻¹) | Glucose (g L ⁻¹) | Xylose (g L ⁻¹) | Ethanol (g L ⁻¹) | Xylitol (g L ⁻¹) |
| Molasses | BDL | 12.3 | 8.0 | 2.3 | 12.6 | 14.5 | 24.6 | 3.1 |
| WIS 2.5 % | BDL | 8.2 | 10.7 | 3.9 | 0.5 | 7.4 | 36.6 | 4.6 |
| WIS 5 % | BDL | 3.6 | 10.6 | 4.8 | 0.5 | 3.2 | 37.8 | 5.1 |
| WIS 10 % | BDL | 8.4 | 9.9 | 3.3 | 0.4 | 7.8 | 35.7 | 3.9 |

Residual sugars and end-product formation at termination of hydrolysate liquor pre-fermentation at 48 h and SSCF at 120 h

BDL below detection limit.

by adapted cultures (Fig. 8) demonstrates that the ability of the yeast strain to tolerate and transform inhibitors improved with short-term adaptation.

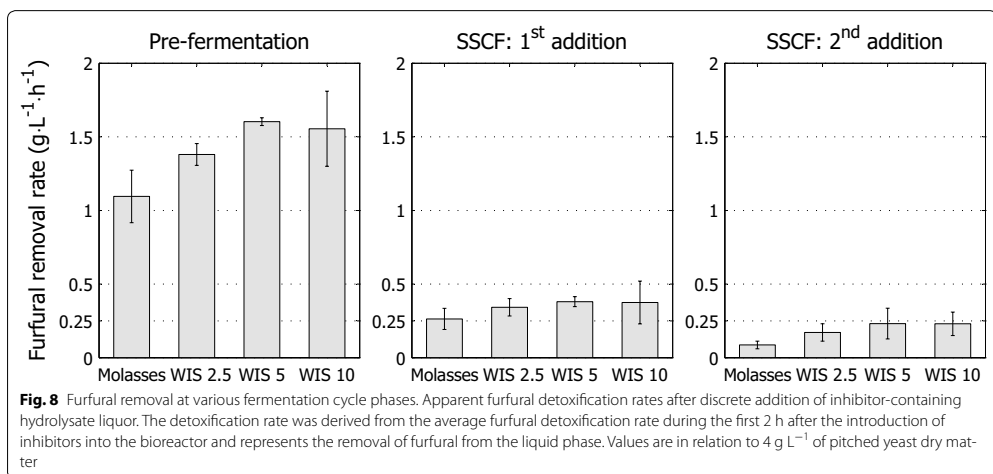
Simultaneous saccharification and co-fermentation

The effect of pre-adaptation became apparent after adding back the lignocellulosic solids during the SSCF. The lower substrate consumption rate in the unadapted cultures caused the accumulation of glucose and xylose in the fermenters, which resulted in low yields due to incomplete substrate utilization (Fig. 6; Table 1). No significant differences in performance were observed between the reference cultures with and without xylose supplement (data not shown), indicating that the presence of xylose in the feed media during propagation had little or no effect on the fermentation during SSCF.

The behavior of the unadapted cultures might be due to lack of adaptation, which would have increased their susceptibility to inhibitors. Based on the high concentration

of inhibitory compounds, the longevity of unadapted cultures could be diminished, impairing ethanol productivity. Considering the viability and vitality of the cultures after propagation, the decrease in performance during fermentation can be explained in part by the lower load of viable cells and the lower fermentative capacity in the unadapted cultures. The unadapted cultures were 80 % viable on average compared with 88–90 % for adapted cultures (Fig. 2); further, the molar ethanol productivity in the fermentation capacity test was 6.1 versus 7.4–9.7 mmol g DM⁻¹ h⁻¹ on average in adapted cells (Fig. 3).

The adapted cultures displayed rapid consumption of glucose and improved xylose utilization (Fig. 7), the latter of which can be linked to some extent to the upregulation of genes that confer resistance to furaldehydes and the ability of, e.g., furfural to act as an electron acceptor in the regeneration of co-factors that are necessary to maintain flux through the engineered XR/XDH pathway [31, 46]. The improvement in fermentation performance was



evidenced by the higher ethanol titers and lower marginal xylool excretion compared with the unadapted reference. Even with pre-adaptation at low inhibitor concentrations, the ethanol conversion improved significantly (Fig. 6 and Table 1)—the reference had an average ethanol concentration of 24.6 versus 35.7–37.8 g L⁻¹ for pre-adapted cultures (Fig. 6; Table 1).

The final ethanol concentrations and, thus, ethanol yield increased with pre-adaptation, and no significant difference in ethanol titer was observed between fermentations with cell cultures that were propagated with various amounts of hydrolysate in the fed-batch phase. Increasing amounts of hydrolysate liquor in the feed during pre-adaptation improved xylose utilization, although it was not reflected by higher ethanol titers. Arguably, pre-adaptation had a positive effect on the viability and vitality of the yeast during fermentation, allowing ethanol production to be sustained. This hypothesis is supported by the ability of various cultures to utilize xylose after each addition of solids. Higher rates of xylose utilization were maintained for longer periods with pre-adapted cultures, as were higher apparent furaldehyde detoxification rates (Fig. 8). These measures could be indicators of enhanced viability and vitality of the cells or cells that are better equipped for anaerobic metabolism.

Park et al. [14] reported that transcriptional upregulation of genes that confer resistance to inhibitors correlated with improved fermentative capacity. They found that the highest ethanol productivity was gained with upregulation of *ZWF1* and *ADH6*. On addition of furfural and HMF, *ZWF1* upregulation was associated with the highest specific growth rate and ethanol productivity. Notably, upregulation of *ZWF1*, *ALD6*, and *ADH6* in our experiments occurred during pre-adaptation with inhibitor concentrations that corresponded to 5 and 10 % WIS, but ethanol production was largely unchanged compared with cell cultures that were pre-adapted with 2.5 % WIS equivalent concentration. Because the improvement in fermentation even occurred for cultures that were pre-adapted with low hydrolysate liquor content, there was no correlation between adaptation-induced transcriptional changes and fermentation results. However, it should be noted that the transcription of few genes was investigated, and the resulting phenotypes were the product of a broader range of changes in gene expression.

Nevertheless, the fermentation results in SSCF correlate well with the increase in fermentative capacity and viability of the cultivated yeast at various levels of adaptation. Similar trends were seen in yeast viability at the end of the propagation and in the fermentation results in the SSCF, indicating that the improvement in ethanol yield was due in part to inoculation in the SSCF with higher amounts of viable yeast. Increased viability of the

cultivated yeast thus accounted for some of the improvement, whereas the remainder was attributed to improved fermentation performance, as indicated by the increased fermentative capacity. The limiting factor in obtaining high yields was most likely the ability to sustain viability in the culture throughout the fermentation cycle, through extended longevity of the cells or anaerobic growth. The assays did not determine the mechanisms that effected the improvements, but adaptation is clearly beneficial for fermentation in SSCF with steam-pretreated wheat straw.

Another concern is the increased variance in viability with higher hydrolysate liquor content in the feed during cultivation. Although this variability was not fully reflected in the SSCF ethanol titers, it was evidenced by the xylose utilization. Extensive conversion of xylose is a prerequisite for obtaining high ethanol yields in the conversion of lignocellulosic biomass to ethanol—more so when agricultural residues are utilized as substrate. Variability is also an issue from a research and industrial perspective. Reproducible cultivation with low variance ensures consistent performance of the fermenting microorganism and reduces the technological risk. Thus, it would be favorable to adapt the cells with low inhibitor concentrations to minimize hydrolysate consumption and variability in fermentation.

Conclusions

Adaptation during propagation improves the tolerance of inhibitor-resistant yeast strains and thus increases ethanol yields from glucose and xylose. The improved tolerance of pre-adapted cells resulted in faster and more complete xylose utilization during fermentation. The pre-adapted cells also upregulated genes that conferred inhibitor resistance and experienced greater viability and vitality. The positive effects on ethanol yield in SSCF were observed even for yeast that was adapted at low inhibitor concentrations. Adaptation at higher concentrations of inhibitors than necessary resulted in overall loss of fermentable sugars, due to lower cell mass yield, because more sugars were required to propagate enough yeast for a specific fermenter capacity. Increased variability in cultivation outcome and fermentation was also seen with higher amounts of inhibitors in the pre-adaptation process, which constitutes a technological risk.

Methods

Raw material and pretreatment

Wheat straw slurry with a water-insoluble solids (WIS) content of 13.7 % mass fraction was obtained from SEKAB E-Technology AB (Örnsköldsvik, Sweden). The wheat straw was impregnated with dilute H₂SO₄ to pH 2 and steam-pretreated at 186 °C for 8 min. The hydrolysate liquor was separated from the solid fraction with

a hydraulic press (HP5 M, Fischer Maschinenfabrik GmbH). All solids were retained in the filter cake, and a WIS mass fraction of 48 % was obtained in the solid fraction. The compositions of the solid fraction and hydrolysate liquor are listed in Table 2.

Microorganism

The utilized non-commercial *Saccharomyces cerevisiae* KE6-12 strain (Taurus Energy AB) harbors genes from *Scheffersomyces stipitis* that encode xylose reductase (XR) and xylitol dehydrogenase (XDH) and overexpresses endogenous xylulokinase (XK), enabling xylose conversion. The stock culture aliquots contained a mass fraction of 20 % glycerol and were stored at -80°C .

Cultivation procedure

Pre-cultures

The pre-cultures were cultivated in 250-mL shake flasks with 150 mL of sterile minimal medium that contained 20 g L⁻¹ glucose and xylose, 7.5 g L⁻¹ (NH₄)₂SO₄, 3.75 g L⁻¹ KH₂PO₄, and 0.75 g L⁻¹ MgSO₄·7H₂O. The medium was supplemented with 1 mL L⁻¹ vitamin solution and 10 mL L⁻¹ trace element solution, the composition of which has been reported by Taherzadeh et al. [47]. The pH of the medium was adjusted to 5.5 with 5 M

NaOH solution and inoculated with 300 μL of stock culture aliquots. The pre-culture was incubated at 30 °C on an orbital shaker (Lab-Therm, Kühner) at 180 rpm for 24 h.

Propagation

The propagations were performed in 2-L Labfors bioreactors (Infors AG) in a sequential aerobic process: batch cultivation on sugar beet molasses, followed by fed-batch cultivation on wheat straw hydrolysate and sugar beet molasses (Nordic Sugar). The molasses contained 0.411 g g⁻¹ of fermentable sugars (sucrose, fructose, and glucose), lactic acid (0.034 g g⁻¹), and acetic acid (0.011 g g⁻¹). The batch cultivations had a 0.5 L working volume with a 50 g L⁻¹ molasses solution that was supplemented with 23.5 g L⁻¹ (NH₄)₂SO₄, 3 g L⁻¹ KH₂PO₄, 2.25 g L⁻¹ MgSO₄·7H₂O, 33 μg L⁻¹ biotin, and 120 ppm Vitahop (BetaTec). The batch cultivation was carried out with a constant aeration rate of 1 vvm and an agitation rate of 700 rpm, and pH was maintained at 5.2. The batch phase was concluded when all sugars were consumed, as indicated by the evolution of carbon dioxide and oxygen in the reactor gas effluent.

Adaptation of the cultivated yeast to fermentation conditions was performed during the fed-batch phase by introducing hydrolysate liquor into the feed solution, as per Alkasrawi et al. [16]. Molasses was the primary carbon source in the feed solutions, and the reference feed solution contained 150 g L⁻¹ of molasses. Various amounts of hydrolysate liquor were added to yield inhibitor concentrations in the feed solutions that were equivalent to those in an SSCF with WIS loads of 2.5, 5, and 10 % mass fraction. A constant amount of fermentable sugars (sucrose, fructose, and glucose) was achieved throughout the range of feed solutions by altering the molasses concentration to offset the contribution of hydrolysate-derived glucose. Experiments with reference feed solution that was supplemented with 14.5 g L⁻¹ D-xylose were performed to determine whether the presence of xylose, without the influence of inhibitors, in the propagation step affected yeast performance.

The feed solution was pulse-added to the bioreactor for 20 h to a final working volume of 1.5 L. The feeding pattern was discretized around a constant dilution rate trajectory (0.056 h⁻¹). The agitation rate was maintained at 700 rpm, and the bioreactor was sparged at a constant aeration rate of 1 vvm, based on final volume. The pH was maintained at 5.2 by automatic addition of sterile 2.5 M NaOH solution.

Harvest

Samples were withdrawn for various analytical assays and for preparation of inocula for the SSCF experiments.

Table 2 Composition of hydrolysate liquor and water-insoluble solids

| | |
|---|------|
| Steam-pretreated material (% of dry matter) | |
| Glucan | 46.8 |
| Xylan | 4.7 |
| Galactan | 1.7 |
| Arabinan | BDL |
| Mannan | BDL |
| Lignin | 31.8 |
| Lignin ash | 9.5 |
| Hydrolysate liquor (g L ⁻¹) | |
| Glucose | 11.6 |
| Xylose | 36.1 |
| Galactose | 3.7 |
| Arabinose | 3.8 |
| Mannose | 1.5 |
| Formic acid | 1.5 |
| Acetic acid | 10.2 |
| Levulinic acid | 0.05 |
| 5-hydroxymethyl furfural (HMF) | 1.0 |
| Furfural | 7.9 |

Composition of structural carbohydrates and lignin in the water-insoluble fraction of the pretreated material and sugar composition and prevalence of inhibitory compounds in the hydrolysate liquor. The composition was determined per NREL [53, 54]

BDL below detection limit

The cultivated cells were harvested by centrifugation (3800×g, 10 min) and washed with sterile 9 g L⁻¹ NaCl solution. The cell pellets were resuspended in sterile 9 g L⁻¹ NaCl solution, yielding a cell dry matter concentration of 120 g L⁻¹.

Hybrid simultaneous saccharification and co-fermentation

The fermentation experiments were performed in sterilized 2-L Labfors bioreactors (Infors AG) with a final working volume of 1.5-L. SSCF was performed per Nielsen et al. [42]. A WIS load of 10 % mass fraction and an enzyme load of 10 FPU g⁻¹ WIS⁻¹, based on final weight, were applied. The bioreactors were inoculated with a yeast load of 4 g L⁻¹ of yeast dry weight, based on the final volume, and the pH was maintained at 5.2 automatically with sterile 2.5 M NaOH solution. The hydrolysate liquor was pre-fermented at 30 °C with an initial addition of 2 FPU g⁻¹ WIS⁻¹ Cellic CTech2 enzyme solution (Novozymes AS). Half of the solid fraction was added back after 48 h with 8 FPU g⁻¹ WIS⁻¹ Cellic CTech2 enzyme solution and elevation of the temperature to 35 °C. The remaining solids were added back to the fermenter after 72 h, and the SSCF was terminated after 120 h.

Analytical procedures

Methylene blue staining and cell enumeration

Samples of cultivation broth were dyed with methylene blue (Sigma-Aldrich Chemie GmbH). The cell suspension was diluted 100 times with 9 g L⁻¹ NaCl solution to maintain cell integrity and dyed with 0.3 g L⁻¹ methylene blue. The samples were incubated at room temperature for 5 min. Total cells, dyed cells, and budded cells were counted on a hemocytometer in a Bürker chamber.

Fermentative capacity

The fermentative capacity test was conducted per Jørgensen et al. [48]. Cells were harvested from 110 mL of broth by centrifugation (3800×g, 10 min); washed with 100 mL CBS medium, pH 6.5, without glucose or (NH₄)₂SO₄, [49]; and resuspended in 110 mL of the same media. The cell suspension was transferred to an anaerobic shake flask and incubated at 30 °C on an orbital shaker (Lab-Therm, Kühner). After 5 min, 5 mL of glucose solution (200 g L⁻¹) was added. Samples were withdrawn every third minute for 30 min and centrifuged (16,000×g, 3 min), and the supernatant was retained, filtered (0.20 µm, GVS Filter Technology Inc), and stored at -20 °C until analysis of the ethanol concentration. The cells in the remaining fermentation broth were harvested to analyze protein content. Ethanol productivity was regressed based on the linear correlation between ethanol concentration and time for the full sample range, and

related to the amount of inoculated yeast dry matter and its protein content.

Total protein measurements

Cells from the fermentative capacity assay were harvested to analyze protein content by centrifugation (960×g, 3 min), washed with sterile distilled water, and frozen immediately in liquid nitrogen. Cell samples were stored at -20 °C until analysis. In preparation for the assay, the cells were thawed, washed twice with distilled water, and suspended in TBS (200 mM Tris, 1.36 M NaCl, pH 7.6) together with acid-washed glass beads. The cells were disrupted in a FastPrep Instrument (MP Biomedicals) for 20 s and kept on ice for 2 min. The cycle was repeated 6 times. The suspension was centrifuged (20,000×g, 5 min, 4 °C), and the supernatant analyzed with regard to protein content by Bradford method [50] on a microplate reader (FLUOstar Omega, BMG Labtech). Bovine serum albumin (Sigma-Aldrich, Cat. No. A3803) was used as the standard.

Trehalose and glycogen measurements

Cells were harvested from 20 mL of cultivation broth by centrifugation (960×g, 3 min), washed twice with 5 mL sterile 9 g L⁻¹ NaCl solution, resuspended in 1 mL 20 mM sodium acetate buffer (pH 4.8), and frozen immediately in liquid nitrogen. The samples were then stored at -80 °C until analysis. Approximately 10 mg (dry weight) of cells was resuspended in defined volumes of 0.25 M Na₂CO₃ and incubated at 95 °C for 4 h under constant agitation in a thermomixer (Comfort, Eppendorf). Acetic acid (1 M) and sodium acetate (0.2 M) were added to the incubated samples to yield a solution with 62.5 mM Na₂CO₃, 0.15 M acetic acid, 0.12 M sodium acetate, and a pH of 5.2.

Aliquots of sample solution were treated with 0.119 U mL⁻¹ trehalase (Megazyme K-TREH 11/12) and 2.85 U mL⁻¹ of amyloglucosidase (Sigma-Aldrich, Cat. No. A7420). Hydrolysis of trehalose and glycogen was performed under constant agitation overnight at 37 and 57 °C, respectively, in a thermomixer. The supernatant was withdrawn after centrifugation (5000×g, 3 min), and the liberated glucose in the trehalose and glycogen assays was measured using the Glucose GOD/PAP kit (Biosis, Cat. No. 000919) with an external glucose standard.

Quantitative PCR

Cells from 10 mL of cultivation broth were harvested by centrifugation (960×g, 3 min), washed twice with sterile 9 g L⁻¹ NaCl solution, frozen immediately in liquid nitrogen, and stored at -80 °C until analysis. RNA was extracted using the RNeasy kit (Qiagen) with DNase treatment per the manufacturer's protocol. The samples

Table 3 Sequences of oligonucleotide primers in the quantitative PCR assay

| Gene | Forward primer | Reverse primer |
|--------------|------------------------------|------------------------------|
| <i>ADH6</i> | GTCCTGGTGGTATCGGCAGTATGGGTA | ATGTCGGTAAGGGAGGAAGCACAGACTA |
| <i>ALD6</i> | ACCCAAGAGAAAGAGGCCGTCTACTAAG | GCTCTAAGGTGGTGAAGTTCATGTAGCC |
| <i>ERG2</i> | GCCGAAGTTTACACTCTGGTATGACTC | TCCTTGGCAGTCAGGTAGACAGTTCTAT |
| <i>ZWF1</i> | GACATTACTGATATCTGCGGGTCTGCT | GGGAACCTGGAAGGGTCTCTGATAAAG |
| <i>TAF10</i> | TACCCGAATTTACAAGAAAAGATAAGA | ATTTCTGAGTAGCAAGTGCTAAAAGTC |

were subjected to reverse transcription and the cDNA was used for qPCR.

Expression of *TAF10*, *ADH6*, *ALD6*, *ZWF1*, and *ERG2* was quantified using Brilliant II SYBRGreen QPCR Master Mix, 0.5 μ M of forward and reverse primer, and 2 μ L cDNA. The qPCR experiments were performed on a Stratagene Mx3005P. The qPCR program comprised an initial denaturation for 10 min at 95 °C and amplification for 40 cycles of 1 min at 65 °C followed by 1 min at 72 °C for elongation of the amplicons. *TAF10*, used as an internal reference gene to derive ΔC_T values for the samples, was stably expressed in all samples, because its C_T value did not vary significantly. The primer sequences were designed from the sequences in the Saccharomyces Genome Database (<http://www.yeastgenome.org/>) and are listed in Table 3. Data on relative quantification of the genes were evaluated using the comparative $\Delta\Delta C_T$ -method. Fold-differences were expressed as $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_{T, \text{sample}} - \Delta C_{T, \text{calibrator}}$.

HPLC analysis

Extracellular metabolites, inhibitors, and sugars were measured by high-performance liquid chromatography (HPLC) on a Shimadzu HPLC system that was equipped with an RID-10A refractive index detector (Shimadzu). Samples for carbohydrate analysis with low pH (from hydrolysates) were pH-adjusted to 5 with $\text{CaCO}_3(\text{s})$ and centrifuged in 10-mL tubes ($960 \times g$, 5 min). Samples from the fermentation experiments, with adequate pH, were centrifuged in 2-mL Eppendorf tubes at $16,000 \times g$ for 3 min. All supernatants were filtered through 0.20- μ m syringe filters (GVS Filter Technology Inc.) and stored at -20 °C until analysis.

Extracellular metabolites, organic acids, and degradation products in hydrolysate liquors and fermentation broths were analyzed by isocratic ion-exchange chromatography on an Aminex HPX-87H column (Bio-Rad Laboratories) with a Cation-H Bio-Rad micro-guard column (Bio-Rad Laboratories) at 50 °C. The eluent was 5 mM H_2SO_4 at a flow rate of 0.5 mL min^{-1} . Sugars and xylitol in wheat straw hydrolysate liquor and fermentation broth were quantified by isocratic

ion-exchange chromatography on an Aminex HPX-87P column (Bio-Rad Laboratories) with a De-Ashing Bio-Rad micro-guard column (Bio-Rad Laboratories) at 85 °C. Millipore water was used as eluent at a flow rate of 0.5 mL min^{-1} .

Dry matter and water-insoluble solids content measurements

Water-insoluble solids (WIS) and dry matter content (DM) of solids were measured per standardized laboratory procedures (LAP) that were developed by the National Renewable Energy Laboratory (NREL) [51, 52].

The dry matter mass fraction of the cultivation broths was measured by filtering 10 mL of fermentation broth through a 0.45- μ m membrane filter (Whatman GmbH). The retentate was washed with 15 mL distilled water, and the filters were vacuum-dried for 2 min and dried overnight at 105 °C. Dry samples were cooled in a desiccator for 4 h and weighed on an analytical balance.

Composition of hydrolysate liquor and water-insoluble solids

Soluble carbohydrates, monomeric sugars that were released into solution and hydrolysis degradation products were quantified by acid hydrolysis and HPLC per NREL [53]. Further, structural carbohydrate, lignin, and ash contents of the water-insoluble fraction of the wheat straw slurries were measured by two-step hydrolysis method by NREL [54].

Abbreviations

DM: dry matter; FPU: filter paper unit; HMF: 5-hydroxymethylfurfural; HPLC: high-performance liquid chromatography; LAP: laboratory analytical procedure; qPCR: quantitative polymerase chain reaction; SSCF: simultaneous saccharification and co-fermentation; TBS: tris-buffered saline; WIS: water-insoluble solids.

Authors' contributions

FN participated in the conception and design of the study; performed the cell culture propagations, fermentations, and analytical assays; and wrote the manuscript. ETP participated in the conception and design of the study and performed the protein, trehalose, glycogen, and qPCR assays. LO participated in the conception and design of the study and critically reviewed the drafted manuscript. OW assisted in interpreting the data, participated in the editing and preparation of the final manuscript, and critically reviewed the manuscript. All authors have commented on the manuscript. All authors read and approved the final manuscript.

Author details

¹ Department of Chemical Engineering, Lund University, P.O. Box 124, 221 00 Lund, Sweden. ² Department of Biology and Biological Engineering, Industrial Biotechnology, Chalmers University of Technology, SE-412 96 Göteborg, Sweden. ³ Present Address: Unit of Biotechnological Processes for Energy Production, IMDEA Energy, 28935 Móstoles, Spain.

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Competing interests

The study was part of the project "Process development for combined pentose and hexose fermentation", which was co-financed by Taurus Energy AB. LO is a technical advisor for Taurus Energy AB. Remaining authors declares no financial competing interests, and all authors declare that they have no non-financial competing interests.

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Paper II

RESEARCH ARTICLE

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Prefermentation improves ethanol yield in separate hydrolysis and cofermentation of steam-pretreated wheat straw

Fredrik Nielsen, Guido Zacchi, Mats Galbe and Ola Wallberg* 

Abstract

Agricultural residues, such as wheat straw, are feasible substrates for ethanol fermentation provided that pentoses and hexoses can be converted efficiently. Separate hydrolysis and cofermentation (SHCF) constitute a framework for improvement of conversion efficiency, because it permits independent optimization of the enzymatic hydrolysis and cofermentation steps. A drawback is that the high glucose concentrations present in SHCF repress xylose utilization and constrain ethanol yields. To improve xylose utilization the xylose-rich hydrolyzate liquor was separated from glucose-rich solids and the phases were cofermented sequentially. Prefermentation of the xylose-rich hydrolyzate liquor followed by fed-batch cofermentation of glucose-rich prehydrolyzed solids enabled sequential targeting of xylose and glucose conversion. The aim was to improve the xylose conversion by lowering the glucose repression of the xylose uptake. Various prefermentation configurations and feed patterns for prehydrolyzed solids were examined. Prefermentation increased ethanol yields overall, and fed-batch prefermentation reduced xylitol production. The best results were obtained by balancing promotion of efficient xylose conversion with maintained yeast viability. Fed-batch prefermentation and a single addition of prehydrolyzed solids, elicited an ethanol yield of $0.423 \text{ g}\cdot\text{g}^{-1}$ and a xylitol yield of $0.036 \text{ g}\cdot\text{g}^{-1}$.

Keywords: *Saccharomyces Cerevisiae*, Xylose, Cofermentation, Prefermentation, Prehydrolysis, Lignocellulose, Ethanol

Background

Fermentative conversion of lignocellulosic biomass into ethanol provides a sustainable alternative that could partially replace traditional petroleum refining, but to successfully implement lignocellulosic technologies economic sustainability must be ensured. High final ethanol concentration and high ethanol yield has been identified as key factors for improved process economics [1]. To achieve these, efficient hydrolysis and fermentation as well as utilization of a variety of sugars present in the feedstock are necessary.

The main obstacles to efficient cofermentation of lignocellulose-derived sugars are the limitations of the microbial physiology that restricts efficient conversion of various substrates [2] and the ability to cope with a

variety of inhibitors [3]. The wild-type strain of *Saccharomyces cerevisiae* is tolerant to many inhibitors that are generated by thermochemical pretreatment, but it is largely unable to convert pentoses into ethanol without genetic modification [4]. Exogenous genes that encode for xylose reductase (XR) and xylitol dehydrogenase (XDH) [5, 6], as well as xylose isomerase [7], have been introduced into the *S. cerevisiae* genome to enable assimilation of xylose. However, fermentation of xylose to ethanol by engineered *S. cerevisiae* is slower and generally results in lower ethanol yields than glucose fermentation [8]. This is likely because of limitations in capacity in the pentose phosphate shunt [9] and an imbalance in redox cofactors in engineered XR/XDH-pathways [10]. The cofactor imbalance between the NAD(P)H-consuming XR and NADH-producing XDH catalyzed reactions restricts flux through the engineered pathway, and causes xylitol production [11, 12]. Improvements have been

*Correspondence: ola.wallberg@chemeng.lth.se
Department of Chemical Engineering, Lund University,
P.O. Box 124, 221 00 Lund, Sweden

made by altering the cofactor specificity of XR towards NADH [13, 14], and by overexpression of endogenous xylokinase [9]. However, the distribution of products and the rate of conversion remain dependent on the balance and turnover of cofactors. The xylose conversion capacity, as well as tolerance of inhibitors, have been further improved in engineered *S. cerevisiae* strains by evolutionary engineering and adaptation strategies [15], but the slow cellular uptake of pentoses remains a constraint for efficient xylose conversion. Xylose is taken up by non-specific hexose transport mechanisms [16], and because their affinity for glucose is many-fold higher than for xylose [10] excessive amounts of glucose competitively inhibit the transporters and prevent efficient uptake of xylose. However, low concentrations of glucose have been shown to enhance the xylose uptake rate [17], which imply coconsumption [18]. The enhanced xylose uptake rate has been attributed to improved cofactor recycling [19], and the induction of genes expression for transporter systems [19] and glycolytic enzymes [20].

Fermentation design can provide a tool to improve xylose utilization and conversion efficiency by accommodating the substrate consumption patterns of the fermenting microorganism. Thus, various strategies have been proposed to optimize the conversion of biomass-derived glucose and xylose to ethanol, where enzymatic hydrolysis and fermentative conversion can be performed either sequentially (separate hydrolysis and cofermentation, or SHCF) or simultaneously in a single vessel (simultaneous saccharification and cofermentation, or SSCF). Opting for either strategy is generally a trade-off between optimal temperatures and inhibitory glucose concentrations during hydrolysis on the one hand (SHCF) and sub-optimal temperatures and ethanol-inhibited cellulolysis on the other (SSCF). Whereas some studies have shown that SSCF-based designs generally result in higher yields [18, 21], the separate hydrolysis in SHCF-based designs enable optimization of the process conditions in the individual steps. Performing separate hydrolysis eliminates rate limiting effects of the hydrolysis on conversion rates, and problems associated with high viscosity during fermentation are alleviated by prior liquefaction of the solids. These properties become increasingly important as the solids load is increased in the process. Both strategies have advantages, and the choice is strain and feedstock dependent. Modifications to the fundamental strategies have been implemented to improve fermentation performance and substrate utilization [22–24]. Fed-batch design has been implemented to promote coconsumption in SHCF [22, 23]. Fed-batch designs, where a glucose-rich feed supported the xylose utilization, improved the overall ethanol yields, and lowered xylitol production in co-fermentation of steam-pretreated wheat straw with

strains of xylose-fermenting *S. cerevisiae* [23]. Further, prefermentation has been implemented as a modification to SSCF to improve xylose utilization and ethanol yields [24, 25]. Depletion of glucose in the liquid fraction of whole spruce slurry, prior to enzyme addition in SSCF, reduced the competitive inhibition of the xylose uptake and increased ethanol yields [24]. The authors presupposed that the process significance would be even greater with xylose-rich feedstocks. When pretreated agricultural residues are used as substrate, which have higher xylose content than spruce, the use of prefermentation can be extended to encompass substantial xylose conversion. The high xylose concentration, in combination with low glucose concentration, in the hydrolyzate liquor provide glucose-to xylose ratios during prefermentation that kinetically favor xylose uptake [17]. By separating the hydrolyzate liquor from the lignocellulosic solids, the advantages of an SHCF strategy can be combined with the beneficial conditions for xylose conversion in prefermentation. The combined strategy features sequential targeting of xylose and glucose conversion with optimal temperatures and customization of the enzymatic hydrolysis and fermentation steps individually. It has previously been demonstrated with a 2-step batch-SSCF of AFEX-pretreated switchgrass that sequential targeting of xylose and glucose conversion improve xylose utilization and ethanol yields [25].

In this study, various SHCF-based cofermentation strategies for the conversion of glucose and xylose to ethanol were examined. Two wheat straw slurries with various inhibitor concentrations, prepared by dilute acid catalyzed steam-explosion, were used. The pentose-rich hydrolyzate liquor in the slurries was separated from the hexose-rich solids to enable sequential targeting of xylose and glucose conversion. Cofermentation was performed by a xylose-fermenting and inhibitor tolerant strain of *S. cerevisiae* in a 2-step process, where prefermentation of the hydrolyzate liquor was followed by feeding of enzymatic hydrolysate, which consisted of prehydrolyzed unwashed solids. The hypothesis was that xylose could be converted with greater efficiency and render higher ethanol yields under the more favorable conditions for xylose conversion in the sequential fermentation steps than by conventional SHCF. Various feed patterns during prefermentation and for the addition of enzymatic hydrolyzate were examined to improve the xylose conversion and maximize the ethanol yield.

Methods

Microorganisms

Fermentation was performed with the non-commercial recombinant *Saccharomyces cerevisiae* KE6-12 strain (Taurus Energy AB), which harbors genes from

Scheffersomyces stipitis (formerly *Pichia stipitis*) that encode for xylose reductase (XR) and xylitol dehydrogenase (XDH) and overexpressing endogenous xylulokinase (XK), thus enabling xylose conversion. The strain was developed by evolutionary engineering [26] on the industrial strain *S. cerevisiae* TMB3400 [27] to improve inhibitor tolerance and xylose conversion capacity. Stock culture aliquots contained a mass fraction of 20 % glycerol in water and were stored at $-80\text{ }^{\circ}\text{C}$.

Raw material and preprocessing

Two batches of wheat straw that were pretreated with dilute acid-catalyzed steam explosion under various conditions were procured from SEKAB E-Technology AB (Örnsköldsvik, Sweden). The slurries were denoted *severe* or *mild* slurry, respectively, based on the relative inhibitor concentrations in the hydrolyzate liquor. The *severe* slurry was prepared by impregnation with dilute H_2SO_4 , pH 2.4, and steam-pretreatment at $190\text{ }^{\circ}\text{C}$ for 15 min, and had a water-insoluble solids (WIS) content of 13.9 wt % and a total dry-matter (DM) content of 20.1 wt %. The *mild* slurry was prepared by impregnation of wheat straw with dilute H_2SO_4 , pH 1.7, and steam-pretreatment at $187\text{ }^{\circ}\text{C}$ for 8 min, and had a WIS content in the range of 11.1–12.7 wt % and a total DM content in the range of 17.5–18.2 wt %. The pH of the slurries was adjusted to 5 with 12.5 M NaOH, and the hydrolyzate liquor was separated from the solids by filtration using a hydraulic press (HP5 M, Fischer Maschinenfabrik). The unwashed solid fraction of the *severe* slurry had a WIS content of 34 wt % after filtration, versus between 38 and 44 wt % WIS in the unwashed solid fraction of the *mild* slurry.

Cultivation of yeast

The precultures were cultivated in 250 ml shake flasks with 150 ml of sterile minimal medium, containing $20\text{ g}\cdot\text{L}^{-1}$ glucose, $20\text{ g}\cdot\text{L}^{-1}$ xylose, $7.5\text{ g}\cdot\text{L}^{-1}$ $(\text{NH}_4)_2\text{SO}_4$, $3.75\text{ g}\cdot\text{L}^{-1}$ KH_2PO_4 , and $0.75\text{ g}\cdot\text{L}^{-1}$ $\text{MgSO}_4 \cdot 7\text{ H}_2\text{O}$. The media was supplemented with $1\text{ mL}\cdot\text{L}^{-1}$ vitamin solution and $10\text{ mL}\cdot\text{L}^{-1}$ trace element solution, per Taherzadeh et al. [28]. The pH of the medium was adjusted to 5.5 with 5 M NaOH in all precultures, all of which were inoculated with 300 μl of the stock cell aliquots. The preculture was incubated at $30\text{ }^{\circ}\text{C}$ on an orbital shaker (Lab-Therm, Kühner) at 180 rpm for 24 h.

The cultivations were performed in a sterilized 2 L Labfors bioreactor (Infors AG) equipped with two six-blade Rushton turbines. The reactor diameter to impeller diameter ratio was 3, and the reactor height to diameter ratio was 1.7. The yeast was propagated with aerobic batch cultivation on molasses followed by aerobic fed-batch cultivation on wheat straw hydrolyzate liquor and molasses. The batch cultivation was performed with $50\text{ g}\cdot\text{L}^{-1}$

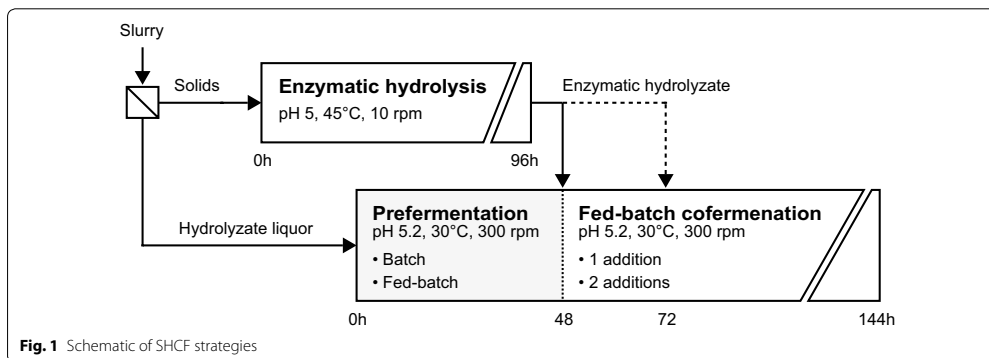
molasses solution that was supplemented with $23.5\text{ g}\cdot\text{L}^{-1}$ $(\text{NH}_4)_2\text{SO}_4$, $3\text{ g}\cdot\text{L}^{-1}$ KH_2PO_4 , $2.25\text{ g}\cdot\text{L}^{-1}$ $\text{MgSO}_4 \cdot 7\text{ H}_2\text{O}$, $33\text{ }\mu\text{g}\cdot\text{L}^{-1}$ biotin, and 120 ppm Vitahop (BetaTec). The molasses (Nordic Sugar A/S) contained 40 wt % fermentable sugars (sucrose, fructose, and glucose), lactic acid ($0.034\text{ g}\cdot\text{g}^{-1}$), and acetic acid ($0.008\text{ g}\cdot\text{g}^{-1}$). The cultivation was initiated by inoculation with the preculture. The batch cultivation was performed with 0.5 L working volume, with a constant aeration rate of 1 vvm, pH maintained at 5.2 and an agitation rate of 700 rpm. The batch phase was concluded when all sugars were consumed, as indicated by the evolution of carbon dioxide and oxygen in the bioreactor gas effluent.

The fed-batch phase was initiated after fermentable sugars were depleted in the batch phase. The feed solution comprised diluted wheat straw hydrolyzate liquor that was supplemented with $150\text{ g}\cdot\text{L}^{-1}$ of molasses. The hydrolyzate liquor in the feed solution brought about inhibitor concentrations in the final working volume that corresponded to the concentrations in a broth with a 7.5 wt % WIS load. The purpose of the hydrolyzate liquor in the fed-batch phase was to improve yeast tolerance by short-term adaptation of the cultivated yeast to the environmental conditions in the fermentation experiments, per Nielsen et al. [29]. The feed solution was pulse-fed to the bioreactor at a constant rate for 20 h to a final working volume of 1.5 L. The reactor was aerated by sparging at a constant rate of 1 vvm, based on the final volume, and the pH was maintained at 5.2 automatically with sterile 2.5 M NaOH solution.

The propagated yeast was harvested by centrifugation ($3800\times g$, 10 min) and washed with $9\text{ g}\cdot\text{L}^{-1}$ sterile NaCl solution. The cell pellets were resuspended in sterile $9\text{ g}\cdot\text{L}^{-1}$ NaCl solution to yield inocula with a cell dry matter concentration of $120\text{ g}\cdot\text{L}^{-1}$.

Enzymatic hydrolysis of the solid fractions

The unwashed solid fractions from the filtered slurries of pretreated wheat straw were hydrolyzed enzymatically prior to the cofermentation (Fig. 1) in a Terrafors rotating drum reactor (Infors AG) that was agitated by free-fall mixing, yielding a glucose-rich enzymatic hydrolyzate. The unwashed solid fractions of the *severe* and *mild* slurries, containing both solids and hydrolyzate liquor, were diluted with distilled water. The dilution of the solid fraction of the *severe* slurry yielded a WIS load of 20 wt % in the enzymatic hydrolysis, and the dilution of the solid fractions of the *mild* slurry yielded WIS loads of 26 and 32 wt % in the enzymatic hydrolyses, dependent on the WIS content in the original slurry. The dilution was constrained by the target 10 wt % WIS in the SHCF experiments. Hydrolysis was performed at $45\text{ }^{\circ}\text{C}$ for 96 h with an enzyme load of $9\text{ FPU}\cdot\text{g}^{-1}$ WIS and constant



reactor revolution at 10 rpm. Cellic CTec2 enzyme preparation (Novozymes A/S) with a filter paper activity of $98 \text{ FPU}\cdot\text{g}^{-1}$, as determined per Adney and Baker [30], was dispersed in the dilution-water to promote even distribution of enzymes. The pH was maintained at 5 by manual addition of 5 M sterile NaOH solution.

Separate hydrolysis and cofermentation

All cofermentations were performed in sterilized 2 L Labfors bioreactors (Infors AG) equipped with an anchor impeller and a pitched six-blade turbine. The reactor diameter to impeller diameter ratio was 1.5 for the anchor impeller and 1.7 for the pitched six-blade turbine, and the reactor height to diameter ratio was 1.7. All cofermentations were supplemented with $0.5 \text{ g}\cdot\text{L}^{-1}$ $(\text{NH}_4)_2\text{HPO}_4$, $0.125 \text{ mL}\cdot\text{L}^{-1}$ Vitahop (BetaTec), and $0.4 \text{ mL}\cdot\text{L}^{-1}$ Antifoam RD Emulsion (Dow Corning), all based on the final volume. The pH in the fermentation broths were maintained at 5.2 by automatic addition of sterile 2.5 M NaOH solution.

SHCF with prefermentation

The prefermentation of hydrolyzate liquor followed by fed-batch cofermentation of enzymatic hydrolyzate was performed with a final working weight of 1.5 kg; a total WIS load of 10 wt %, based on the final weight; a yeast load of $5 \text{ g}\cdot\text{L}^{-1}$ of dry matter (DM), based on the final volume; and an overall enzyme load of $10 \text{ FPU}\cdot\text{g}^{-1}$ WIS.

The fermentations were performed sequentially by prefermentation of the hydrolyzate liquor, followed by cofermentation of the enzymatic hydrolyzate (Fig. 1). The prefermentation of the hydrolyzate liquor was performed using either of two feed strategies: batch or fed-batch. In batch prefermentation, the entire amount of separated hydrolyzate liquor was supplied at outset and cofermented for 48 h. For fed-batch prefermentation, a

two-step process was adopted. Approximately 25 wt % of the separated hydrolyzate liquor was cofermented in batch for 12 h, and the remaining 75 wt % was fed linearly to the fermentor for the subsequent 36 h. The amount of hydrolyzate liquor at outset was set to surpass minimum required liquid level in the fermentor ($\geq 200 \text{ mL}$) to ensure reliable pH and temperature control. Regardless of mode, the full yeast load, $5 \text{ g}\cdot\text{L}^{-1}$, was pitched at outset and $1 \text{ FPU}\cdot\text{g}^{-1}$ WIS of Cellic CTec2, based on total ingoing WIS, was added after 4 h to hydrolyze solubilized oligosaccharides. The hydrolyzate liquor was cofermented under anaerobic conditions at 30°C for 48 h, and the bioreactor was agitated at 300 rpm.

Two feed strategies were used for the following fed-batch cofermentation of the enzymatic hydrolyzate: (i) all enzymatic hydrolysate was added after 48 h, and (ii) half of the enzymatic hydrolyzate was added after 48 h, and the remaining half added after 72 h. Enzymes, equivalent to a total enzymatic activity of $9 \text{ FPU}\cdot\text{g}^{-1}$ WIS, were carried over with the addition of enzymatic hydrolyzate, resulting in total cellulolytic activity of $10 \text{ FPU}\cdot\text{g}^{-1}$ WIS, based on the total ingoing WIS content. The temperature was maintained at 30°C , and agitation was maintained at 300 rpm. The experiments were terminated after a total fermentation time of 144 h.

Model fermentations

Model cofermentations were performed with a final working weight of $\sim 1.4 \text{ kg}$ using *mild* and *severe* hydrolyzate liquor from the pretreated wheat straw slurries. The hydrolyzate liquor was diluted with distilled water to inhibitor and sugar concentrations that corresponded to a WIS load of 10 wt %. Further, the hydrolyzate liquor was supplemented with glucose, corresponding to 81 % yield in enzymatic hydrolysis of the solid fraction. The supplemented hydrolyzate liquor mimicked the composition

of enzymatically hydrolyzed slurry. An enzyme load of 2 FPU·g⁻¹ WIS was applied to hydrolyze solubilized oligosaccharides, and the fermentor was inoculated with a yeast load of 5 g·L⁻¹ DM. All components were added at the outset. The model cofermentation mimicked a batch SHCF, but avoided the bias from influence of solid material and hydrolysis limitations in the cofermentation. The hydrolyzate liquor was cofermented under anaerobic conditions at 30 °C for 144 h, and the bioreactor was agitated at 300 rpm.

Analytical procedures

Extracellular metabolites, inhibitors, and sugars were measured by high-performance liquid chromatography (HPLC) on a Shimadzu HPLC system that was equipped with an RID-10A refractive index detector (Shimadzu). Samples for carbohydrate analysis with low pH (from hydrolyzate liquors) were adjusted to pH 5 with CaCO₃ and centrifuged in 10 mL tubes (960×g, 5 min). All samples were centrifuged (16,000×g, 3 min), and the supernatants filtered through 0.20 μm syringe filters (GVS Filter Technology). Filtered samples were stored at -20 °C until analysis.

Extracellular metabolites, organic acids, and degradation products in the hydrolyzate liquors and fermentation broth were analyzed by isocratic ion-exchange chromatography using an Aminex HPX-87H column (Bio-Rad Laboratories) at 50 °C. The eluent was 5 mM H₂SO₄, applied at a flow rate of 0.5 mL·min⁻¹. Sugars and xylitol in the hydrolyzate liquors and fermentation broth were quantified by isocratic ion-exchange chromatography on an Aminex HPX-87P column (Bio-Rad Laboratories) at 85 °C. Deionized water was used as the eluent at a flow rate of 0.5 mL·min⁻¹.

Dry matter content (DM) of solids and water-insoluble solids (WIS) were measured per Sluiter et al. [31], and Sluiter et al. [32], respectively. Soluble carbohydrates, monomeric sugars that were released into solution during pretreatment, and pretreatment degradation products were quantified by acid hydrolysis and HPLC per Sluiter et al. [33]. Further, structural carbohydrates, lignin, and ash contents of the water-insoluble fraction of the wheat straw slurries were measured per Sluiter et al. [34].

Calculation of yields

Ethanol yields were calculated at two levels: an overall yield and a metabolic yield. The overall ethanol yield was based on total supplied glucose and xylose; i.e. the sum of glucose and xylose present in the slurries, including monomers, oligomers, and polymers. The metabolic ethanol yield was based on consumed glucose and xylose. The mass of glucose and xylose available in the form of

polymers in the WIS were corrected with factors 1.111 and 1.136, respectively, to account for the addition of water to the monomeric units during hydrolysis. The percentage of maximum theoretical ethanol yield was based on a theoretical stoichiometric yield of 0.51 g·g⁻¹ on glucose and xylose.

Results and discussion

Material preprocessing and composition

Wheat straw was pretreated using dilute acid-catalyzed steam pretreatment at two conditions to yield two slurries denoted *mild* and *severe* slurry, based on the relative inhibitor concentrations in the hydrolyzate liquor. The lower inhibitor concentrations in the *mild* slurry was attributed to lower degradation, due to the shorter hold-up time, and also to some extent to higher dilution in the pretreatment. The *severe* slurry had a water-insoluble solids (WIS) content of 13.9 wt %, versus 11.1 to 12.7 wt % for the *mild* slurry. The variation between barrels of the *mild* slurry was likely due to sedimentation in the storage vessel at the demonstration-scale pretreatment site. The *mild* slurry with WIS content of 12.7 wt % was used in the evaluated fermentations of *mild* slurry. The *mild* slurry with lower WIS content (11.1 wt %) was used to illustrate the limitations with the proposed cofermentation strategy. The applied pretreatments solubilized mainly hemicellulosic sugars. Consequently, hydrolyzate liquors that were rich in xylose and hemicellulosic oligomers, and cellulose-rich solids were obtained. The composition of the WIS fractions and the sugar and inhibitor concentrations of the hydrolyzate liquors are listed in Table 1.

The separation of hydrolyzate liquors from the lignocellulosic solids by filtration, in combination with the compositional differences between the phases (Table 1), enabled the sequential targeting of xylose and glucose conversion. The hydrolyzate liquor contained most of the xylose and inhibitors and the unwashed lignocellulosic solids harbored mainly cellulose and lignin. This allowed the use of feeding schemes that mitigate the impact of inhibitors and effect more favorable glucose-to-xylose ratios for xylose utilization could be implemented.

Enzymatic hydrolysis of the solid fractions

The enzymatic hydrolysis of the retained solids after filtration was performed at high WIS loads, because of the separation of hydrolyzate liquor from the lignocellulosic solids. The dilution of the solid fractions was further constrained by the intended WIS load of 10 wt % in the SHCF experiments and the aim to maximize separation between the bulk fractions of glucose and xylose.

The unwashed solid fraction of the *severe* slurry was diluted from a WIS content of 34 wt % in the retained solids to the 20 wt % used in the enzymatic hydrolysis.

Table 1 Composition of structural carbohydrates and lignin in the water-insoluble fractions of the pretreated materials and sugar compositions and prevalence of inhibitory compounds in hydrolyzate liquors

| | Severe wheat straw slurry | Mild wheat straw slurry |
|---|---------------------------|-------------------------|
| Steam-pretreated material (% of dry weight) | | |
| Glucan | 51.4 | 50.7 |
| Xylan | 1.7 | 1.0 |
| Galactan | 0.0 | BDL |
| Arabinan | 0.1 | 0.05 |
| Mannan | 0.1 | 0.07 |
| Acid-soluble lignin | 0.7 | 0.6 |
| Acid-insoluble lignin | 33.8 | 29.8 |
| Lignin ash | 6.9 | 10.2 |
| Total determined | 94.6 | 92.4 |
| Hydrolyzate liquor (g·L ⁻¹) | | |
| Glucose | 14.5 | 8.7 |
| Xylose | 32.6 | 35.4 |
| Galactose | BDL | 0.9 |
| Arabinose | 3.5 | 1.8 |
| Mannose | 1.8 | 0.7 |
| Formic acid | 1.6 | 0.8 |
| Acetic acid | 8.5 | 5.6 |
| Levulinic acid | BDL | 0.5 |
| HMF | 1.3 | 0.3 |
| Furfural | 7.7 | 3.7 |

BDL below detection limit

The unwashed retained solids from the *mild* slurry was diluted from a WIS content of 44 to the 26 wt % WIS applied in the enzymatic hydrolysis. Sufficient water for dilution was available to provide even distribution of enzyme preparation and sufficient mixing. Liquefaction became apparent after a few hours, and an extensive degradation of solids occurred during the enzymatic hydrolysis. The enzymatic hydrolysis was performed to produce a glucose-rich enzymatic hydrolysate, and not evaluated further.

Performing the enzymatic hydrolyses at high WIS loads imply the risk of lower sugar yields in hydrolysis. It has been shown for several lignocellulosic substrates that greater substrate loads decrease the corresponding hydrolysis yield [35]. The effect has been attributed to product inhibition [36, 37], inhibition by sugar-derived inhibitors and lignin [38, 39], and mass transfer limitations and other effects that are related to the increased WIS loads [40]. However, inhibition primarily affects the hydrolysis rate—not the maximum conversion or yield—given sufficient time. Minor inefficiencies in the hydrolysis could be rectified with the employed cofermentation

strategy, because the enzymes were carried over from the enzymatic hydrolysis to the subsequent cofermentation step, and provided an additional period of hydrolysis. Despite the possibility to partially rectify hydrolysis inefficiencies obtaining high sugar yields in the hydrolysis was important to successfully carry out the devised SHCF-design.

Strategies for separate hydrolysis and cofermentation

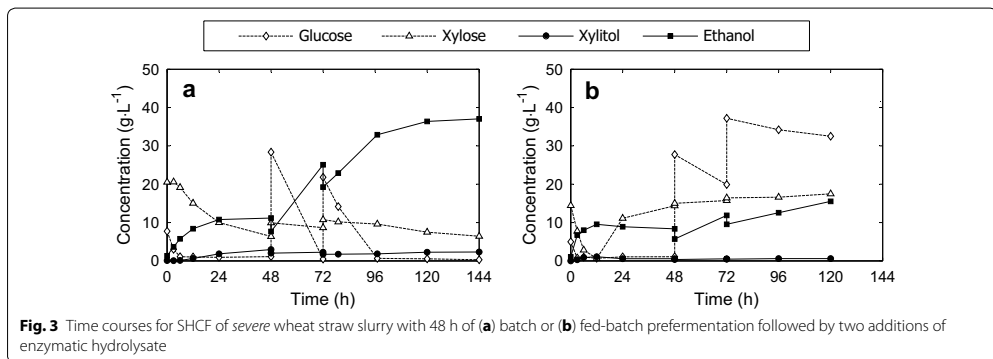
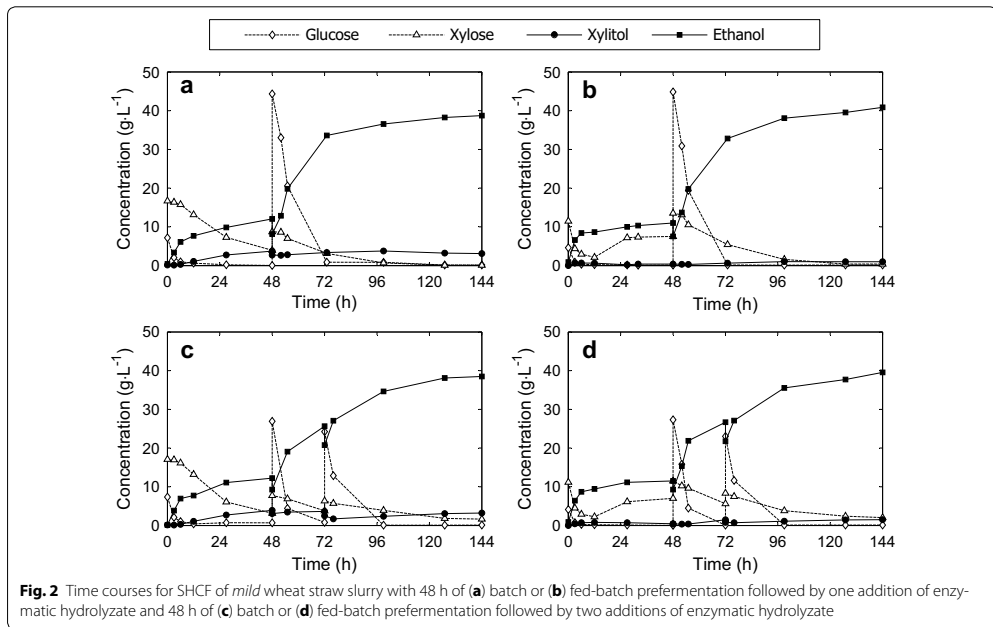
In the first cofermentation step, the prefermentation, xylose conversion was targeted. The prefermentation of the hydrolyzate liquors separated from *mild* and *severe* slurry were conducted in batch and fed-batch. In the second cofermentation step, the fed-batch cofermentation, glucose conversion was targeted, and remaining xylose coconsumed. The enzymatic hydrolyzate obtained from the enzymatic hydrolysis of the solid fractions, which contained high amounts of glucose, was fed to the prefermented hydrolyzate liquor. One or two additions of enzymatic hydrolyzate were investigated. The aim was to elucidate the impact of number of additions of enzymatic hydrolyzate, independently and in combination with different prefermentation configurations, on xylose utilization and ethanol yield.

Xylose utilization in batch prefermentation

Batch prefermentation of the separated *mild* hydrolyzate liquor resulted in depletion of glucose and consumption of 88 % of the xylose. The deviation from the mean in the experiments was less than 1 %. The consumed xylose equaled 69 % of the total available xylose in the used slurry. Batch prefermentation of separated *mild* hydrolyzate liquor elicited higher xylose utilization than with *severe* hydrolyzate liquor. The glucose in the *severe* hydrolyzate liquor was depleted and 77 % of the available xylose in the hydrolyzate was consumed, which equaled 48 % of the total available xylose in the used slurry. The substantial consumption of xylose during batch prefermentation reduced the extent of xylose coconsumption needed after the addition of enzymatic hydrolyzate.

The addition of enzymes after the depletion of measured glucose in the fermentation broth was intended to supply low amounts of monomeric glucose from solubilized oligomers to promote coconsumption and facilitate xylose uptake. However, declining xylose uptake rates were seen in the batch prefermentations of *mild* and *severe* hydrolyzate liquors after depletion of measured glucose (Figs. 2a, c, 3a), indicating that not enough glucose was available in the solubilized oligomers to sustain coconsumption and facilitate xylose utilization.

Batch prefermentation of *mild* and *severe* hydrolyzate liquor elicited metabolic ethanol yields of 0.34 and 0.33 g·g⁻¹, respectively. The higher ethanol



concentrations and metabolic ethanol yields obtained with *mild* hydrolysate liquor were attributed to the higher xylose utilization (Table 2) and the lower inhibitor concentrations (Table 1), respectively.

Xylose utilization in fed-batch prefermentation

To address the inability to sustain coconsumption of xylose and glucose by liberating glucose from the solubilized oligomers in the hydrolysate liquors, the fed-batch

prefermentation strategy was implemented. The fed-batch layout was intended to continuously supply low amounts of glucose and promote coconsumption. The glucose-to-xylose ratio of the hydrolysate liquors was such (Table 1) that a low measured glucose concentration in the bioreactor could be maintained with a feed of hydrolysate liquor (Figs. 2b, d, 3b), which kinetically favored xylose consumption [17]. However, lower xylose utilization was observed with fed-batch prefermentation,

Table 2 Summary of prefermentation results

| | Severe wheat straw slurry | | Mild wheat straw slurry | |
|---|---------------------------|-----------|-------------------------|-----------|
| | Batch | Fed-batch | Batch | Fed-batch |
| Residual sugars and end-products [g·L ⁻¹] | | | | |
| Glucose | 0.1 | 0.1 | 0.0 | 0.1 |
| Xylose | 6.4 | 14.4 | 3.7 | 7.3 |
| Ethanol | 11.2 | 8.4 | 12.1 | 11.2 |
| Xylitol | 2.9 | 0.6 | 3.8 | 0.4 |
| Metabolic ethanol yield [g·g ⁻¹] ^a | 0.329 | 0.322 | 0.342 | 0.353 |
| % of theoretical | 64.4 | 63.1 | 67.1 | 69.2 |
| Xylose utilization [%] | 77.3 | 48.6 | 88.2 | 76.9 |
| Xylitol yield [g·g ⁻¹] ^b | 0.136 | 0.041 | 0.137 | 0.017 |

^a Based on consumed glucose and xylose and related to a maximum theoretical yield of 0.51 g·g⁻¹

^b Based on consumed xylose

as compared with batch, with both hydrolyzate liquors (Table 2). On average 77 and 49 % of xylose available in the *mild* and *severe* hydrolyzate liquor, respectively, was consumed; as compared to 88 and 77 %, respectively, in batch. The deviation from the mean in fed-batch prefermentation experiments was less than 3 %. The underlying reason for the lower xylose consumption was that the feed rate of xylose-rich hydrolyzate liquor exceeded the xylose uptake rate. In combination with preferential consumption of glucose, this resulted in the accumulation of xylose during the fed-batch phase of the prefermentation (Figs. 2b, d, 3b). Although lower xylose utilizations were achieved with fed-batch prefermentation, substantial fractions of total available xylose in the slurries were consumed. The consumed xylose in *mild* and *severe* hydrolyzate liquor after fed-batch prefermentation corresponded to 60 and 31 %, respectively, of total available xylose.

The lower xylose consumptions in fed-batch prefermentation were mirrored in the obtained ethanol concentrations (Table 2), but did not have a significant effect on the metabolic ethanol yields (Table 2). Metabolic ethanol yields of 0.353 and 0.322 g·g⁻¹ were obtained with fed-batch prefermentation of *mild* and *severe* hydrolyzate liquor, respectively, as compared to 0.342 and 0.329 g·g⁻¹ with batch prefermentation. The deviations from the mean metabolic ethanol yields were less than 2 % in all instances. Fed-batch prefermentations were also accompanied with lower xylitol production than batch prefermentation. Batch prefermentation of *mild* and *severe* hydrolyzate liquor resulted in xylitol yields of 0.136 and 0.134 g·g⁻¹, respectively, whereas only 0.017 g·g⁻¹ respectively 0.041 g·g⁻¹ were produced in fed-batch

prefermentation. The deviations from the mean in batch prefermentation experiments were less than 2 %, and less than 7 % in fed-batch prefermentation experiments. The difference in xylitol production between the prefermentation configurations was attributed to the higher xylose consumption rate in batch prefermentation. High xylose consumption rates create a metabolic bottleneck because of an imbalance of cofactors in engineered XR/XDH-pathways [11, 12], whereas it has been shown that lower consumption rates suppress xylitol production [41]. The lower xylose consumption in fed-batch prefermentation in combination with continuous availability of low concentrations of furaldehydes, which act as external electron sinks for the regeneration of cofactors [42], likely alleviated the cofactor imbalance.

Effect of prefermentation on overall xylose utilization

The selected feed strategy during prefermentation had several intertwined consequences on fermentation performance during the subsequent fed-batch cofermentation of enzymatic hydrolyzate. The different prefermentation configurations had different impacts on fermentative capacity of the yeast during the fed-batch cofermentation of enzymatic hydrolyzate, which was primarily attributed to changes in the viability.

Comparison of overall cofermentation outcome between batch and fed-batch prefermentation of *severe* hydrolyzate liquor followed by two additions of enzymatic hydrolyzate (Fig. 3) exemplify the different consequences in a high inhibitor concentration context. Batch prefermentation followed by fed-batch cofermentation of enzymatic hydrolyzate elicited a xylose utilization of 71 % and an overall ethanol yield of 0.381 g·g⁻¹. In contrast, the corresponding strategy with fed-batch prefermentation resulted in low glucose conversion and, seemingly, no xylose consumption after fed-batch prefermentation of *severe* hydrolyzate liquor (Fig. 3b). Fed-batch prefermentation supplied lower initial concentrations of inhibitors than batch prefermentation, but the continuous feed of hydrolyzate liquor seemingly exhausted the yeast. The continuous feed of inhibitors during fed-batch prefermentation exceeded the detoxification rate, which resulted in the accumulation of non-metabolized furaldehydes (data not shown). The furaldehydes were not fully converted during the sequent fed-batch cofermentation of enzymatic hydrolyzate. In contrast, the yeast was resilient to the higher initial inhibitor concentrations in batch prefermentation. The yeast was capable of promptly detoxifying the hydrolyzate liquor during batch prefermentation and after each addition of enzymatic hydrolyzate (data not shown). The decreased viability and fermentative capacity of the yeast was likely due to ceased growth and inhibitory effects [3]. The yeast cells

were assumed to be particularly sensitive to exposure to inhibitors during the prefermentation of xylose-rich hydrolyzate liquor, because the conversion of xylose does not support growth efficiently [43].

When hydrolyzate liquor and enzymatic hydrolyzate from *mild* slurry was utilized, the xylose fermenting capacity was sustained throughout 144 h of cofermentation. However, lower xylose utilization was obtained after fed-batch cofermentation of enzymatic hydrolyzate when fed-batch prefermentation was applied, as compared to the corresponding strategy with batch prefermentation (Table 3). Fed-batch prefermentation with one or two additions of enzymatic hydrolyzate elicited xylose utilizations of 98 and 91 %, respectively; whereas the corresponding cofermentations with batch prefermentation elicited xylose utilizations of 97 and 93 %, respectively. The deviations from the means for experiments employing fed-batch prefermentation was less than 3 %. The primary reason was higher residual xylose concentrations at the end of fed-batch cofermentations of enzymatic hydrolyzate when fed-batch prefermentation was applied (Table 3). This suggests that fed-batch prefermentation negatively influences the xylose fermenting capacity.

Effect of prefermentation on overall xylitol production

Strategies employing fed-batch prefermentations elicited lower xylitol yields than those with batch prefermentation (Table 3). The xylitol production was decreased with fed-batch prefermentation, regardless of whether *mild* or *severe* slurry was used, and effected lower overall xylitol yields at the end of the fed-batch cofermentation of

enzymatic hydrolyzate (Table 3). Because of the sequential targeting of xylose and glucose conversion in the fermentation steps, the xylitol productions during prefermentation were major determinants of total xylitol production. After prefermentation little xylitol was produced, because glucose was the predominant substrate and remaining xylose was mainly coconsumed.

For all practical purposes xylitol production represents a loss of carbon that could be converted to ethanol. The lower xylitol production in strategies employing fed-batch prefermentation contributed to the xylose conversion efficiency and, thus, the higher overall ethanol yields obtained. Despite lower xylose utilization the overall ethanol yields were higher than for strategies employing batch prefermentation (Table 3). The higher overall ethanol yields were correlated with the decreased xylitol production obtained with fed-batch prefermentation.

Effect of number of additions of enzymatic hydrolyzate

The multiple additions of enzymatic hydrolyzate during the fed-batch cofermentation provided the means to lower glucose concentrations in the fermentation broth, as compared to a single addition. Fed-batch SHCF of steam-pretreated wheat straw has previously been shown to enhance xylose utilization [22, 23]. However, regardless of chosen prefermentation configuration a single addition of enzymatic hydrolyzate elicited higher xylose utilization and ethanol yields than corresponding fed-batch cofermentations with two additions (Table 3). The yeast exhibited decreased xylose fermenting capacity with repetitive addition of *mild* enzymatic hydrolyzate

Table 3 Summary of results after prefermentation of hydrolyzate liquor followed by fed-batch cofermentation of enzymatic hydrolyzate

| Fig. | Description | Residual sugars and end-products | | | | Xylose utilization | Yields | | |
|--------|----------------------------|----------------------------------|-------------------|-------------------|-------------------|--------------------|----------------------|----------------------|-------------------|
| | | Glucose | Xylose | Xylitol | Ethanol | | Ethanol ^a | Xylitol ^b | |
| | | g·L ⁻¹ | g·L ⁻¹ | g·L ⁻¹ | g·L ⁻¹ | % | g·g ⁻¹ | % | g·g ⁻¹ |
| | <i>S, Model</i> | 0.1 | 5.3 | 2.0 | 28.9 | 75.9 | 0.346 | 67.9 | 0.120 |
| | <i>M, Model</i> | 0.1 | 1.2 | 5.7 | 32.2 | 96.1 | 0.360 | 70.6 | 0.191 |
| 2a | <i>S, B, 2</i> | 0.3 | 6.4 | 2.3 | 37.0 | 70.6 | 0.381 | 74.8 | 0.135 |
| 2b | <i>S, FB, 2</i> | 32.5 | 17.5 | 0.6 | 16.0 | ND | 0.164 | 32.2 | ND |
| 3a | <i>M, B, 1</i> | 0.1 | 0.1 | 3.1 | 38.7 | 98.0 | 0.401 | 78.5 | 0.112 |
| 3b | <i>M, FB, 1</i> | 0.1 | 0.4 | 1.0 | 40.9 | 96.9 | 0.423 | 82.9 | 0.036 |
| 3c, 4b | <i>M, B, 2</i> | 0.1 | 1.6 | 3.2 | 38.5 | 92.4 | 0.398 | 78.1 | 0.123 |
| 4a | <i>M, B, 2^c</i> | 0.5 | 4.7 | 1.4 | 27.3 | ND | 0.287 | 56.2 | ND |
| 3d | <i>M, FB, 2</i> | 0.1 | 2.0 | 1.5 | 39.6 | 90.9 | 0.409 | 80.2 | 0.058 |

S severe slurry; *M* mild slurry; *B* batch prefermentation; *FB* fed-batch prefermentation; 1 1 addition of enzymatic hydrolyzate; 2 2 additions of enzymatic hydrolyzate, ND not determined

^a Ethanol yield based on total supplied glucose and xylose and related to the maximum theoretical yield (0.51 g·g⁻¹)

^b Xylitol yield based on total consumed xylose

^c 12, M, B, 2 with insufficient prehydrolysis

that contained inhibitors. The trend was evidenced by higher final residual xylose concentrations (Table 3).

Because xylose is converted at lower rates than glucose [8], the fermentation time becomes of essence. The distributed multiple additions of enzymatic hydrolyzate effectively reduce the average time available for conversion of xylose that resides in the enzymatic hydrolyzate. Another aspect relates to the addition of inhibitors. The measured inhibitors were predominantly present in the separated hydrolyzate liquors, and were to a large extent converted to less toxic entities during the prefermentation. However, contributions of inhibitors were made with every addition of enzymatic hydrolyzate, because 20–30 wt % of the total amount of hydrolyzate liquor in the slurry after pretreatment remain with the solids after the filtration. In the experiment series with *mild* slurry the yeast was able to convert furfural irrespective of prefermentation mode and number of additions of enzymatic hydrolyzate. HMF, on the other hand, was only fully converted with batch prefermentation and one addition of enzymatic hydrolyzate (data not shown). Fed-batch prefermentation decreased the conversion of HMF, and in combination with two additions of enzymatic hydrolyzate no HMF was converted after prefermentation (data not shown). The trend in furfuraldehyde conversion was correlated to that of the xylose fermenting capacity. The aggregated effect of fed-batch prefermentation and multiple additions of enzymatic hydrolyzate elicited increasingly higher residual xylose concentrations (Table 3), and thus lower xylose utilization. This finding was indicative of that decreases in viability of the yeast occurred with both continuous and repetitive addition of substrate that contained inhibitors.

Maximization of ethanol yield

The highest overall ethanol yield was obtained with low concentrations of inhibitor in the slurry, because it permitted feeding schemes that maximized xylose conversion efficacy while the viability of the fermenting microorganism was sustained. The best results were obtained with fed-batch prefermentation and a single addition of enzymatic hydrolyzate, with an overall ethanol yield of $0.423 \text{ g}\cdot\text{g}^{-1}$, xylose utilization of 98 %, and the lowest xylitol production at $0.036 \text{ g}\cdot\text{g}^{-1}$. The deviations from the means were below 4 % in all instances. The strategy combined lower xylitol productions and higher ethanol yields elicited by fed-batch prefermentation with the lower effect of inhibitors associated with one addition of enzymatic hydrolyzate. A trade-off existed between promoting efficient xylose conversion with substrate feeding and maintaining yeast viability. Ethanol yield was maximized with a balance between them.

The reduction in residual xylose concentrations and decreased xylitol production (Table 3), compared to the model fermentations, indicate that the sequential targeting of xylose and glucose conversion is a feasible way to improve xylose conversion with SHCF-based conversion strategies. The proposed strategy leaves degrees of freedom in the design to implement feeding schemes that accommodate the traits of various fermenting microorganisms, as to mitigate the inhibitory effects, sustain yeast viability and maximize xylose conversion.

Method limitations

The necessitated high WIS enzymatic hydrolysis constituted a weakness of the proposed method, because the hydrolysis outcome had profound effect on the cofermentation. The shortcoming was evidenced by the difference between cofermentations of *mild* slurry with an original WIS content of 11.1 and 12.7 wt % (Table 3; Fig. 4). Batch prefermentation followed by two additions of enzymatic hydrolyzate was applied as cofermentation strategy.

The dilution of the retained solids after filtration of the *mild* slurry with the lower WIS content (11.1 wt %) was severely constrained by the required WIS load of 10 wt % in the SHCF. Hence, the retained solids were only diluted to a WIS load of 32 wt % in the enzymatic hydrolysis step. The enzymatic hydrolysis resulted in inferior hydrolysis because of various reasons associated with high WIS applications [35–37]. With inferior hydrolysis the lignocellulosic solids that remained in the enzymatic hydrolyzate were partially hydrolyzed and fermented simultaneously during the fed-batch cofermentation and the benefits of the preparatory hydrolysis of the unwashed solids waned. The simultaneous hydrolysis and cofermentation was undesirable in the SHCF, because the solids were partially hydrolyzed under suboptimal conditions at 30 °C during the fed-batch cofermentation. The drawback was not necessarily a lower yield given sufficient time, but that hydrolysis rate became a limiting factor for the fermentation rate. This constrained the final ethanol yield for the limited cofermentation period. An increase in temperature during the fed-batch cofermentation of the enzymatic hydrolyzate would have been needed to accommodate hydrolysis of remaining solids and avoid severe limitations to the fermentation rate. The illustrated limitation puts emphasis on the necessity of high sugar yields in preparatory enzymatic hydrolysis to implement the sequential targeting of xylose and glucose conversion with this strategy.

Conclusions

Prefermentation of hydrolyzate liquor followed by fed-batch separate hydrolysis and cofermentation improved ethanol yields, yet batch and fed-batch prefermentation

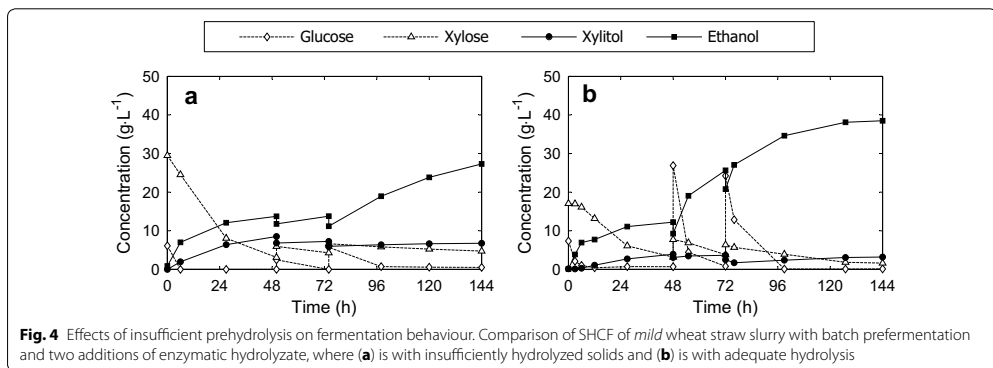


Fig. 4 Effects of insufficient prehydrolysis on fermentation behaviour. Comparison of SHCF of mild wheat slurry with batch prefermentation and two additions of enzymatic hydrolyzate, where (a) is with insufficiently hydrolyzed solids and (b) is with adequate hydrolysis

had different impacts on the cofermentation. Under influence of lower inhibitor concentrations fed-batch prefermentation resulted in lower xylitol production during all steps of the fermentation and prompted higher final ethanol yields compared to corresponding cofermentations with batch prefermentation. Under influence of high inhibitor concentrations, sustained fermentation capacity was paramount to obtain improved ethanol yield. *S. cerevisiae* KE6-12 was resilient to high inhibitor concentrations, but succumbed to continuous exposure to inhibitors. Regardless of type of slurry, continuous feed during prefermentation of the hydrolyzate liquor and multiple additions of enzymatic hydrolyzate—and their combination—appeared to hamper the fermentative capacity and exhaust the cells. The viability of the yeast, not glucose repression of xylose metabolism, appeared to be the limiting factor for higher ethanol yields in the cofermentations. The best results were obtained with mild slurry, applying fed-batch prefermentation and a single addition of enzymatic hydrolyzate. An ethanol yield of $0.423 \text{ g}\cdot\text{g}^{-1}$, based on supplied glucose and xylose, and the lowest xylitol production, $0.036 \text{ g}\cdot\text{g}^{-1}$, was obtained. A trade-off existed between promoting xylose conversion with substrate feeding and maintaining yeast viability, and ethanol yield was maximized with a balance between them.

Abbreviations

DM: dry matter; FPU: filter paper unit; HMF: 5-hydroxymethylfurfural; HPLC: high-performance liquid chromatography; SHCF: separate hydrolysis and cofermentation; SSCF: simultaneous saccharification and cofermentation; vvm: gas volume flow per unit of liquid volume per minute; WIS: water-insoluble solids; XDH: xylitol dehydrogenase; XK: xylulokinase; XR: xylose reductase.

Authors' contributions

FN participated in the conception and design of the study, performed the experiments and wrote the manuscript. GZ participated in the conception and design of the study and critically reviewed the manuscript. OW and MG assisted in interpreting the data, participated in the editing and preparation of

the final manuscript, and critically reviewed the manuscript. All authors read and approved the final manuscript.

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Competing interests

The study was part of the project "Process development for combined pentose and hexose fermentation", which was co-financed by Taurus Energy AB. GZ is a shareholder and member of the board at Taurus Energy AB. Remaining authors declares no financial competing interests and all authors declare that they have no non-financial competing interests.

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Paper III

Sequential targeting of xylose and glucose conversion in fed-batch simultaneous saccharification and cofermentation of steam-pretreated wheat straw for improved xylose conversion to ethanol

Fredrik Nielsen, Guido Zacchi, Mats Galbe, and Ola Wallberg

Department of Chemical Engineering, Lund University
P.O. Box 124, SE-221 00, Lund, Sweden

Abstract

Efficient conversion of both glucose and xylose in lignocellulosic biomass is necessary to make second-generation bioethanol from agricultural residues competitive with first-generation bioethanol and gasoline. Simultaneous saccharification and cofermentation (SSCF) is a promising strategy for obtaining high ethanol yields, to which modifications have been proposed to improve the ethanol yield. However, with this method, the xylose-fermenting capacity and viability tend to decline over time and restrict the xylose utilization.

In this study, we examine ethanol production from steam-pretreated wheat straw using an established SSCF strategy with substrate and enzyme feeding that was applied to steam-pretreated corn cobs. Based on our findings, we propose an alternative SSCF strategy to sustain the xylose-fermenting capacity and improve the ethanol yield. By separating xylose-rich hydrolyzate liquor from glucose-rich solids and then cofermenting the phases sequentially, xylose and glucose conversion can be targeted in succession. Because the xylose-fermenting capacity declines over time, while glucose is still converted, it can be advantageous to target xylose conversion upfront. The proposed strategy comprises prefermentation of the xylose-rich hydrolyzate liquor from the pretreatment followed by fed-batch SSCF. The proposed strategy sustained the xylose-fermenting capacity. Xylose utilization above 90% and overall ethanol yield above 90% of theoretical, based on supplied glucose and xylose, were achieved.

Keywords: SSCF, cofermentation, prefermentation, *Saccharomyces cerevisiae*, lignocellulose, xylose, ethanol.

1. Introduction

The production of fuel ethanol from lignocellulosic raw materials is associated with technological and economic hurdles that must be addressed to make lignocellulosic fuel ethanol competitive with first-generation fuel ethanol and gasoline. Raw material costs constitute a substantial part of the total production cost [1], necessitating efficient use of the raw material to improve the process economics. High ethanol yield and final ethanol concentration are key factors for improved profitability [2], and the major technological challenges to achieving them are linked to the biology and chemistry of the processing steps in using the raw materials efficiently. Accessing and converting the high xylose content in hardwood and agricultural feedstocks to ethanol are central for improving the process ethanol yield and economics.

The overall performance of the fermentation step depends largely on the tolerance of the fermenting microorganism to inhibitors and its ability to efficiently convert a variety of substrates to ethanol. Wild-type *Saccharomyces cerevisiae* has high ethanol productivity and high tolerance to ethanol and inhibitory compounds in lignocellulosic hydrolyzates under anaerobic conditions. However, it is largely unable to utilize xylose as substrate [3]. Introduction of exogenous genes coding for xylose reductase (XR) and xylitol dehydrogenase (XDH) [4, 5] or xylose isomerase [6], which catalyzes the transformation of xylose to xylulose, confers the ability to ferment pentose. Ethanol productivity and yield during fermentation have been further improved by overexpression of endogenous xylulokinase (XK) [7] and engineering of the cofactor specificity of XR [8, 9]. Furthermore, the tolerance of *S. cerevisiae* to inhibitors and its ability to convert xylose have been enhanced by evolutionary engineering [10, 11] and short-term adaptation during propagation [12].

However, xylose is generally fermented by *S. cerevisiae* to ethanol at lower rates [13] and lower yields than glucose [13, 14]. This has been attributed to capacity limitations in the pentose phosphate shunt [7] and mismatched cofactor dependency during xylose catabolism in engineered XR/XDH-strains [15]. The cofactor imbalance between NAD(P)H-consuming XR and NADH-producing XDH reactions effects the production of xylitol [16, 17].

Limited uptake of pentoses by *S. cerevisiae* entails another constraint on the conversion efficiency. Xylose is assimilated by nonspecific hexose transporters [18], and because their affinity for glucose is many-fold higher than for xylose [15], excessive amounts of glucose competitively inhibit the transporters and prevent efficient uptake of xylose. However, a continuous supply of glucose at concentrations below the maximum glucose uptake rate promotes xylose consumption [19], which implies that coconsumption occurs [20]. The increased xylose uptake rates have been attributed to improved cofactor generation [21] and the induction of genes for

transporter systems [21] and glycolytic enzymes [22]. Maintaining a low, but non-zero, supply of glucose would thus increase xylose utilization.

Low glucose concentrations promote coconsumption, rendering simultaneous saccharification and cofermentation (SSCF) an attractive process configuration for the cofermentation of biomass-derived glucose and xylose. The continuous release of glucose by enzymatic hydrolysis helps maintain low glucose concentrations, which kinetically favor xylose utilization in recombinant *S. cerevisiae* strains [19]. Typically, ethanol productivity [23] and yield are higher in SSCF than with separate hydrolysis and cofermentation [20, 24]. Ethanol yields in SSCF have been improved with modifications to the cofermentation strategy, such as fed-batch strategies [25], enzyme feeding [26], and prefermentation [27] and combinations thereof [28-30]. These modifications were introduced to keep glucose concentrations in the bioreactor low. Prefermentation depletes glucose in the liquid fraction, and substrate and enzyme feeding maintain low glucose concentrations during SSCF, which favors xylose uptake and conversion. However, declining xylose-fermenting capacity [28, 29] and exhaustion of the fermenting microorganism [31] have been observed on repeat addition of substrates that contain inhibitors, restricting xylose utilization and, ultimately, limiting the ethanol yield.

Combining prefermentation, feeding of cellulolytic enzymes, and fed-batch SSCF have been established as a feasible approach for obtaining high ethanol yields for cofermentation of biomass-derived glucose and xylose [28, 29]. In this study, we revisited the fed-batch SSCF strategy that was proposed by Koppram et al. [29], which was used for cofermentation of glucose and xylose from steam-pretreated corncobs. We applied this method to steam-pretreated wheat straw and made modifications to it to overcome the observed limitations of fed-batch SSCF [28, 29]. Pentose-rich hydrolyzate liquor was separated from hexose-rich solids after pretreatment, and the phases were fermented sequentially by coupling prefermentation of the hydrolyzate liquor with cofermentation of the unwashed solids in fed-batch SSCF. Consequently, we could target xylose conversion and glucose conversion sequentially, and create opportunities for sustaining the xylose-fermenting capacity and improving xylose conversion.

2. Materials and Methods

2.1 Microorganism

Fermentation was performed with the noncommercial xylose-fermenting *Saccharomyces cerevisiae* strain KE6-12 (Taurus Energy AB), which harbors genes from *Scheffersomyces stipitis* (formerly *Pichia stipitis*) that encode for xylose reductase (XR) and xylitol dehydrogenase (XDH) and overexpresses endogenous xylulokinase (XK). The strain was developed by evolutionary engineering [32] of the industrial strain *S. cerevisiae* TMB3400 [33] to improve its tolerance to inhibitors and xylose conversion capacity. Stock culture aliquots contained a mass fraction of 20% glycerol in water and were stored at -80°C.

2.2 Raw material and preprocessing

Two batches of wheat straw that were pretreated by dilute acid-catalyzed steam pretreatment were procured from SEKAB E-Technology AB (Örnsköldsvik, Sweden). The slurries were denoted *mild* and *severe*, respectively, based on the relative inhibitor concentrations in the hydrolyzate liquor. The *mild* slurry was prepared by impregnation of the wheat straw with dilute H₂SO₄, pH 1.7, and steam pretreatment at 187°C for 8 min. The pretreated material had a water-insoluble solids (WIS) content of 11.1 wt%. The *severe* slurry was impregnated with dilute H₂SO₄, pH 2.4, and steam pretreated at 190°C for 15 min. The pretreated material had a WIS content of 13.9 wt%. The compositions of the slurries are summarized in Table 1. The pH of the slurries was adjusted to 5 with 12.5 M NaOH.

The hydrolyzate liquor in slurries that were intended for fed-batch SSCF with prefermentation of the hydrolyzate liquor was separated from the solids by filtration using a hydraulic press (HP5M, Fischer Maschinenfabrik). The solid fraction of the *severe* slurry had a WIS content of 34 wt% after filtration versus 38 wt% WIS for that of the *mild* slurry.

2.3 Cultivation of yeast

The precultures were cultivated in 250-ml shake flasks with 150 ml of sterile minimal medium, containing 20 g·L⁻¹ glucose, 20 g·L⁻¹ xylose, 7.5 g·L⁻¹ (NH₄)₂SO₄, 3.75 g·L⁻¹ KH₂PO₄, and 0.75 g·L⁻¹ MgSO₄, that was supplemented with 1 mL·L⁻¹ vitamin solution and 10 mL·L⁻¹ trace element solution, per Taherzadeh et al. [34]. The pH of the medium was adjusted to 5.5 with 5 M NaOH. The precultures were inoculated

Table 1. Composition of steam-pretreated wheat straw materials.

Composition of structural carbohydrates in the WIS fraction and content of select sugars and inhibitors in the hydrolysate liquor.

| | <i>Mild</i> | <i>Severe</i> |
|--|-------------|---------------|
| WIS content in slurry (wt%) | 11.1 | 13.9 |
| Composition of WIS (wt%) | | |
| Glucan | 50.7 | 51.4 |
| Xylan | 1.0 | 1.7 |
| Lignin | 30.4 | 34.5 |
| Lignin ash | 10.2 | 6.9 |
| Content in hydrolysate liquor (g·L ⁻¹) | | |
| Glucose ^a | 8.7 | 14.5 |
| Xylose ^a | 35.4 | 32.6 |
| Acetic acid | 5.6 | 8.5 |
| HMF | 0.3 | 1.3 |
| Furfural | 3.7 | 7.7 |

^a Both monomeric and oligomeric forms are included.

with 300 μ l of stock cell aliquot and incubated at 30°C on an orbital shaker (Lab-Therm, Kühner) at 180 rpm for 24 h.

The cultivations were performed in a sterilized 2-L Labfors bioreactor (Infors AG) that was equipped with two 6-blade Rushton turbines. The reactor diameter:impeller diameter ratio was 3, and the reactor height:diameter ratio was 1.7. The yeast was propagated by aerobic batch cultivation on molasses, followed by aerobic fed-batch cultivation on wheat straw hydrolysate liquor and molasses. The batch cultivation was performed with 50 g·L⁻¹ molasses solution that was supplemented with 23.5 g·L⁻¹ (NH₄)₂SO₄, 3 g·L⁻¹ KH₂PO₄, 2.25 g·L⁻¹ MgSO₄·7 H₂O, 33 μ g·L⁻¹ biotin, and 120 ppm Vitahop (BetaTec). The molasses (Nordic Sugar A/S) contained 40 wt% of fermentable sugars (sucrose, fructose, and glucose), lactic acid (0.034 g·g⁻¹), and acetic acid (0.008 g·g⁻¹). The cultivation was initiated by inoculation with the preculture. The batch culture had a working volume of 0.5 L, a constant aeration rate of 1 vvm, and an agitation rate of 700 rpm. The pH was maintained automatically at 5.2 with sterile 2.5 M NaOH solution. The batch phase was concluded when all sugars were consumed, as indicated by the evolution of carbon dioxide and oxygen in the bioreactor gas effluent.

The fed-batch phase was initiated after fermentable sugars were depleted in the batch phase. The feed solution comprised diluted wheat-straw hydrolysate liquor that was supplemented with 150 g·L⁻¹ molasses. The hydrolysate liquor in the feed solution effected inhibitor concentrations in the final working volume that corresponded to the concentrations in a broth with a WIS load of 7.5 wt%. The purpose of including the hydrolysate liquor in the fed-batch phase was to improve the tolerance of the yeast

by short-term adaptation of the cultivated yeast to the environmental conditions in the fermentation experiments, per Nielsen et al. [12]. The feed solution was pulse-fed to the bioreactor at a constant rate for 20 h to a final working volume of 1.5 L. The reactor was aerated by sparging at a constant rate of 1 vvm, based on the final volume, and the pH was maintained at 5.2 automatically with sterile 2.5 M NaOH solution.

The propagated yeast was harvested by centrifugation (3800×g, 10 min) and washed with 9 g·L⁻¹ sterile NaCl solution. The cell pellets were resuspended in sterile 9 g·L⁻¹ NaCl solution to yield an inoculum with a cell dry matter concentration of 120 g·L⁻¹.

2.4 Simultaneous saccharification and cofermentation

2.4.1 Fed-batch SSCF of whole slurry

Fed-batch SSCF of whole slurry (Figure 1a) was performed with severe wheat straw slurry in a sterilized 30-L NLF22 bioreactor (Bioengineering AG) that was equipped with 2 pitched 6-blade turbines. The reactor diameter:impeller diameter ratio was 1.7, and the reactor height:diameter ratio was 2.75. Cofermentation was performed with a final working weight of 15 kg and a total WIS load of 10 wt%. The strategy was a modified version of the 30-L scale fed-batch SSCF of steam-pretreated corncobs that was reported by Koppram et al. [29]. The fed-batch SSCF was carried out at 35°C with 7 wt% of the total amount of slurry at the outset, inoculated with 5 g·L⁻¹ of yeast dry matter, and supplemented with 0.5 g·L⁻¹ (NH₄)₂HPO₄, 0.125 mL·L⁻¹ Vitahop (BetaTec), and 0.4 mL·L⁻¹ Antifoam RD Emulsion (Dow Corning), all based on the final volume.

Prefermentation was performed for 2 h to deplete glucose in the liquid fraction of the initial amount of slurry. The bioreactor was then fed with 3 additions of slurry—each 31 wt% of the total amount of slurry—after 5 h, 27 h, and 49 h. Cellic CTec2 enzyme preparation (Novozymes A/S), corresponding to a cellulolytic activity of 2.5 FPU·g⁻¹ WIS, was added after 2 h to hydrolyze lignocellulosic solids and spiked with an additional 2.5 FPU·g⁻¹ at 24 h, 48 h, and 72 h, yielding a total enzyme load of 10 FPU·g⁻¹ WIS, based on total ingoing WIS. Enzymes and substrate were fed to maintain low glucose levels in the bioreactor, as shown by Koppram et al. [29]. The agitation rate was set to 300 rpm throughout the fermentation, and pH was maintained at 5.2 by automatic addition of sterile 2.5 M NaOH solution.

2.4.2 Prefermentation followed by fed-batch SSCF

Prefermentation of hydrolyzate liquor followed by fed-batch SSCF (Figure 1b) was performed with mild and severe wheat straw slurry in sterilized 2-L Labfors bioreactors (Infors AG) that were equipped with an anchor impeller and a pitched 6-blade turbine. The reactor diameter:impeller diameter ratio was 1.5 for the anchor

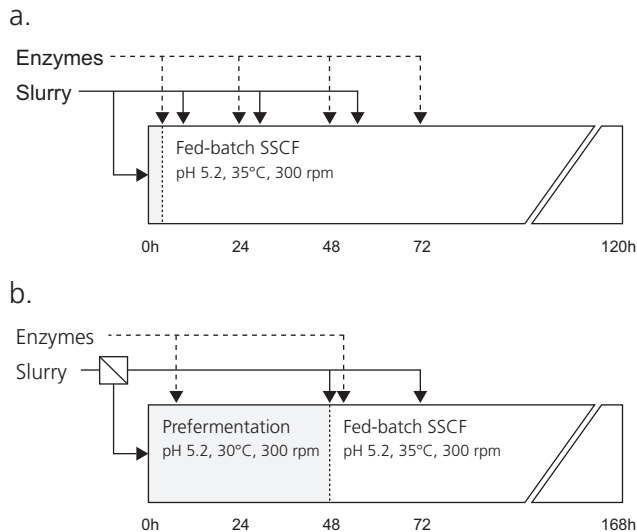


Figure 1. Schematics of SSCF strategies.

Schematic representation of (a) fed-batch SSCF with whole slurry and (b) fed-batch SSCF with prefermentation.

impeller and 1.7 for the pitched 6-blade turbine, and the reactor height:diameter ratio was 1.7. Cofermentation was performed with a final working weight of 1.5 kg and a total WIS load of 10 wt%.

The fermentation was conducted sequentially by batch prefermentation of the separated hydrolyzate liquor followed by fed-batch SSCF of the unwashed solids (Figure 1b). The separated hydrolyzate liquor was supplemented at the outset with $0.5 \text{ g}\cdot\text{L}^{-1}$ $(\text{NH}_4)_2\text{HPO}_4$, $0.125 \text{ mL}\cdot\text{L}^{-1}$ Vitahop (BetaTec), and $0.4 \text{ mL}\cdot\text{L}^{-1}$ Antifoam RD Emulsion (Dow Corning), all based on the final volume. The hydrolyzate liquor was inoculated with $5 \text{ g}\cdot\text{L}^{-1}$ of yeast dry matter, based on the final volume, and prefermented at 30°C for 48 h. An enzyme load of $2 \text{ FPU}\cdot\text{g}^{-1}$ WIS of Cellic CTec2, based on the total ingoing WIS, was added after 4 h to hydrolyze oligosaccharides in the hydrolyzate liquor.

After prefermentation—i.e. after 48 h—half of the unwashed solids was added to the prefermented hydrolyzate liquor. In conjunction was Cellic CTec2 added, yielding a total cellulolytic activity of $10 \text{ FPU}\cdot\text{g WIS}^{-1}$ based on the total ingoing WIS. The temperature was increased to 35°C to increase enzymatic activity. The remaining unwashed solids were added after 72 h. Agitation was maintained at 300 rpm throughout the fermentation, and pH was maintained at 5.2 by automatic addition of sterile 2.5 M NaOH solution. The fermentation was terminated after 168 h.

2.5 Analytical procedures

Extracellular metabolites, inhibitors, and sugars were quantified by high-performance liquid chromatography (HPLC) on a Shimadzu HPLC system that was equipped with an RID-10A refractive index detector (Shimadzu). Samples for carbohydrate analysis were pH-adjusted to 5, when needed, with CaCO_3 and centrifuged in 10-mL tubes ($960\times g$, 5 min). All samples were centrifuged ($16000\times g$, 3 min), and the supernatants were filtered through 0.20- μm syringe filters (GVS Filter Technology). The filtered samples were stored at -20°C until analysis. Extracellular metabolites, organic acids, and degradation products in the samples were analyzed by isocratic ion-exchange chromatography on an Aminex HPX-87H column (Bio-Rad Laboratories) at 50°C . The eluent was 5 mM H_2SO_4 , applied at a flow rate of $0.5\text{ mL}\cdot\text{min}^{-1}$. Sugars and xylitol in the samples were quantified by isocratic ion-exchange chromatography on an Aminex HPX-87P column (Bio-Rad Laboratories) at 85°C . Deionized water was used as the eluent at a flow rate of $0.5\text{ mL}\cdot\text{min}^{-1}$.

The dry matter content of solids and water-insoluble solids were measured per Sluiter et al. [35] and Sluiter et al. [36], respectively. Soluble carbohydrates and pretreatment degradation products in the hydrolyzate liquor were quantified per Sluiter et al. [37]. Further, structural carbohydrates, lignin, and ash content in the water-insoluble fraction of the wheat straw slurries were measured per Sluiter et al. [38].

2.6 Calculation of yields

Ethanol yields were calculated at 2 levels: an overall ethanol yield and a metabolic ethanol yield. The overall yield was based on total supplied glucose and xylose—i.e. the sum of glucose and xylose in the slurries after pretreatment, including monomers, oligomers, and polymers. The metabolic yield was based on consumed glucose and xylose. The mass of glucose and xylose that were available in the form of polymers in the WIS was corrected by the factors 1.111 and 1.136, respectively, to account for the hydration during the hydrolytic cleaving. The percentage of maximum theoretical ethanol yield was based on a theoretical stoichiometric yield of $0.51\text{ g}\cdot\text{g}^{-1}$ for glucose and xylose.

3. Results and discussion

In this study, a fed-batch SSCF strategy that was developed for cofermentation of steam-pretreated corncobs was applied to the cofermentation of steam-pretreated wheat straw. Based on the findings for the fed-batch SSCF method, an alternative strategy was proposed—sequential cofermentation with prefermentation of the hydrolyzate liquor followed by fed-batch SSCF—to improve the overall ethanol yield by increasing the consumption and conversion of xylose. Our cofermentation results were compared with those for fed-batch SSCF with steam-pretreated corncobs on a 30-L scale with *S. cerevisiae* KE6-12 [29] and steam-pretreated wheat straw on a lab-scale with the progenitor strain *S. cerevisiae* TMB 3400 [28]. The conditions and results of our fermentations and fermentations in referenced studies are summarized in Table 2 and Table 3, respectively. Further, the performance of prefermentation followed by fed-batch SSCF was compared with that of prefermentation followed by SHCF [31], which was performed using the same pretreated wheat straw.

3.1 Fed-batch SSCF of whole slurry

The fed-batch SSCF strategy for whole slurry (Figure 1a) that was derived from Koppram et al. [29] was unsuitable for cofermentation of severe wheat straw slurry—the fermentation performance declined over time and negligible xylose conversion and accumulation of glucose occurred in the later stages of the SSCF (Figure 2). Although xylose utilization was higher than reported by Koppram et al. [29]—65% versus 55%—the overall ethanol yield was 56% of the theoretical maximum (Table 3), lower than the 69% in Koppram et al. [29].

The lower ethanol yield was likely connected to differences between pretreated raw materials (Table 2). Repeat addition of slurry, and thus inhibitors, seemingly exhausted the fermenting microorganism, as observed for the strain in Nielsen et al. [31], and xylose-fermenting capacity declined with each addition of *severe* slurry during the fed-batch phase (Figure 2). The declining viability and xylose consumption prevented high overall ethanol yields from being obtained with this SSCF strategy.

Declining xylose-fermenting capacity throughout the cofermentation was also noted by Koppram et al. [29] and during cofermentation of steam-pretreated wheat straw with the progenitor strain *S. cerevisiae* TMB3400 [28], likely due to decreasing viability of the pitched yeast. The inhibitors, in combination with the synergism between the ethanol concentration and fermentation temperature that was needed to facilitate the enzymatic hydrolysis during SSCF [39], likely adversely affected the viability. In our fed-batch SSCF of whole slurry, the decrease in viability caused

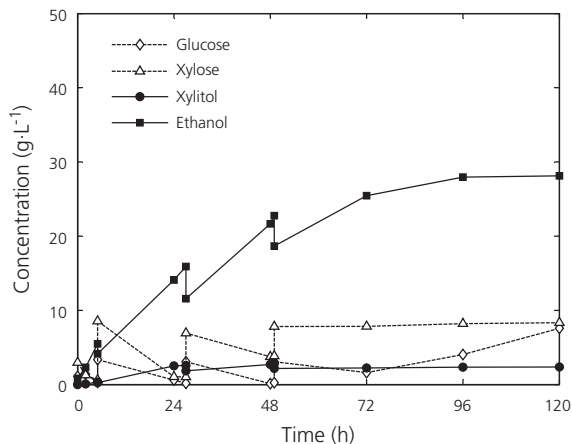


Figure 2. Fed-batch SSCF of whole *severe* wheat straw slurry.

Time course of fed-batch SSCF of whole *severe* wheat straw slurry with 2 h of prefermentation and enzyme feeding. Enzyme additions were made after 2 h, 24 h, 48 h, and 72 h, and slurry was added after 5 h, 27 h, and 49 h of cofermentation.

glucose to accumulate in the bioreactor after 72 h of cofermentation and on (Figure 2). However, Olofsson et al. [28] showed that lower viability was not the sole reason for the decline in xylose-fermenting capacity by pitching fresh yeast in the late stages of the SSCF, failing to observe any improvement in xylose consumption. Xylose consumption was negligible after 48 h in our fed-batch SSCF (Figure 2), although HMF and furfural were promptly converted after each addition of unwashed solids (data not shown), and glucose conversion maintained for at least 96 h, indicating that decrease in xylose-fermenting capacity occurred in part for other reasons, as noted by Olofsson et al. [28].

3.2 Fed-batch SSCF with prefermentation

To overcome the limited xylose conversion in the late fermentation phase and improve the overall ethanol yield, we devised a sequential fermentation strategy, comprising batch prefermentation of the xylose-rich hydrolyzate liquor followed by fed-batch SSCF (Figure 1b). The crude separation between xylose-rich hydrolyzate liquor and glucose-rich lignocellulosic solids enabled us to develop a fermentation strategy in which conversion of xylose and glucose could be targeted sequentially.

3.2.1 Fed-batch SSCF with prefermentation of *severe* hydrolyzate liquor

The results of the cofermentation of *severe* wheat straw slurry with the proposed strategy are shown in Figure 3. The overall ethanol yield was improved by 49%

compared with fed-batch SSCF of whole slurry, which was attributed to the sustained xylose-fermenting capacity and, thus, greater xylose utilization (Table 3).

The batch prefermentation of xylose-rich *severe* hydrolyzate liquor depleted glucose and consumed 73% of the xylose in the hydrolyzate liquor, equaling 45% of the total available xylose in the hydrolyzate liquor and WIS. The metabolic ethanol yield after prefermentation was 63.9% of the theoretical maximum, based on consumed sugars, and 12.5% of the consumed xylose was converted to xylitol. Further, the *severe* hydrolyzate liquor was detoxified *in situ* during prefermentation, thus relieving the inhibitory burden on the pitched yeast during fed-batch SSCF. In addition, the lower temperature during prefermentation—30°C versus 35°C during SSCF—likely helped to sustain the viability of *S. cerevisiae* during fermentation, as shown by Torija et al. [40].

The subsequent fed-batch SSCF resulted in an ethanol concentration of 41.9 g·L⁻¹ after 144 h, which corresponded to an overall ethanol yield of 84% of the theoretical maximum (Table 3). Solubilized glucose was depleted, and the residual xylose concentration was 1.5 g·L⁻¹. Xylose utilization reached 92% of the total available xylose, and 12.6% of consumed xylose was converted to xylitol (Table 3). No further gains in ethanol titers or yield were obtained with prolonged fermentation after 144 h (Figure 3), although xylose consumption was sustained. The xylose consumption after 144 h and on was primarily coupled to xylitol excretion. A change in end-product distribution occurred after 96 h, at which point xylitol production increased. Approximately 40% of all xylitol was produced after the 96-h point in the fed-batch SSCF. This change coincided with the depletion of measured glucose (Figure 3) and furfural in the fermentation broth (data not shown). The increase in xylitol production was likely due to predominantly xylose being consumed and a cofactor imbalance in the XR/XDH pathway [17].

By separating the xylose-rich hydrolyzate liquor from the solids and, as a first step, preferment it under advantageous conditions, 45% of all available xylose was consumed. Subsequently, the glucan was hydrolyzed enzymatically to glucose and converted during the fed-batch SSCF, and most of the remaining xylose was coconsumed. Because the fermenting microorganism loses its xylose-fermenting capacity over time [28] but still converts glucose to ethanol, it was advantageous for the overall xylose utilization to target xylose conversion first and subsequently glucose conversion. The concept of sequential targeting of xylose and glucose conversion has been demonstrated by Jin et al. [30]. They showed that xylose consumption and ethanol yield was improved with 2-step batch-SSCF of AFEX-pretreated switchgrass with independent xylanase and cellulase feeding.

Xylose utilization was further promoted by the low glucose concentrations in the fermentation broth throughout the fed-batch SSCF (Figure 3). In our sequential cofermentation strategy, we used a simpler feeding scheme than for fed-batch SSCF

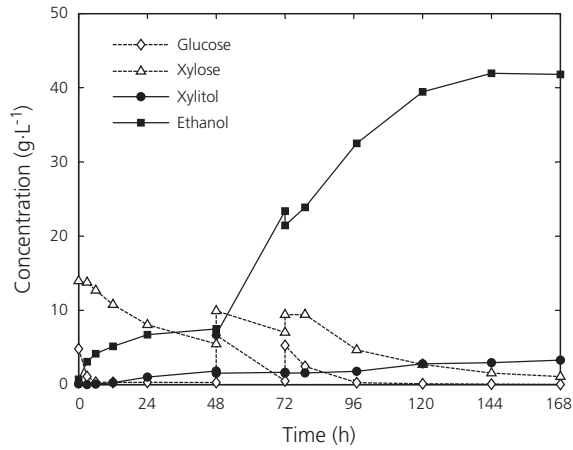


Figure 3. SSCF with batch prefermentation of *severe* hydrolysate liquor.

Time course of SSCF with 48-h batch prefermentation of *severe* hydrolysate liquor and fed-batch addition of unwashed *severe* solids at 48 h and 72 h.

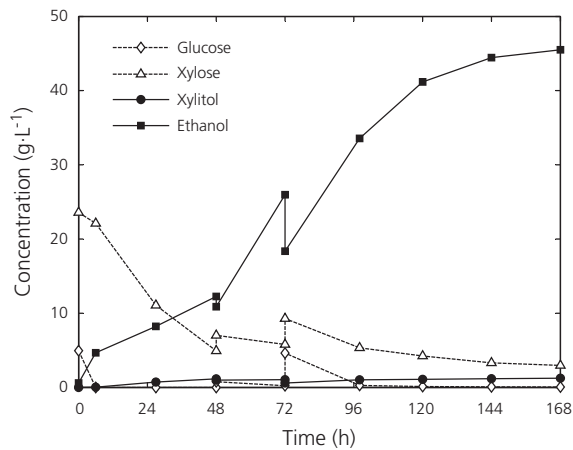


Figure 4. SSCF with batch prefermentation of *mild* hydrolysate liquor.

Time course of SSCF with 48-h batch prefermentation of *mild* hydrolysate liquor and fed-batch addition of unwashed *mild* solids at 48 h and 72 h.

of whole slurry. Whereas fed-batch SSCF of whole slurry used several additions of substrate and enzymes to maintain low glucose concentrations, the entire enzyme load was added prior to the fed-batch SSCF phase in our strategy—to prevent the hydrolysis rate from limiting ethanol production and yield—and only 2 additions of unwashed solids were performed. The feed of unwashed solids during fed-batch SSCF was used to restrict the hydrolytic release of glucose in the bioreactor. The number of additions of unwashed solids that we made during fed-batch SSCF was a tradeoff

between capping the glucose concentration in the fermentation broth and preventing exhaustion of the microorganism. This tradeoff exists, because continuous feeding and repeat addition of substrate that contains inhibitors adversely affect the viability of *S. cerevisiae* KE6-12 [31]. Similarly, the choice of batch prefermentation of the hydrolyzate liquor was a tradeoff between conserving viability and minimizing xylitol production, because fed-batch prefermentation results in lower overall xylitol yields than batch prefermentation but is detrimental to the viability of the fermenting microorganism [31].

3.2.2 Fed-batch SSCF with prefermentation of *mild* hydrolyzate liquor

Prefermentation and fed-batch SSCF of hydrolyzate liquor and unwashed solids from *mild* wheat straw slurry (Figure 4) were performed to determine the performance at lower inhibitor concentrations. The overall ethanol yield after prefermentation and fed-batch SSCF with *mild* slurry improved significantly compared with cofermentation of *severe* slurry with the same strategy.

The batch prefermentation of *mild* hydrolyzate liquor resulted in the depletion of glucose and the consumption of 83 wt% of the available xylose in the hydrolyzate liquor, of which 4.8 wt% was converted to xylitol. The xylose that was consumed during prefermentation was equal to 62 wt% of total available xylose. The increase in xylose utilization and lower xylitol production reflected improvements compared to prefermentation of *severe* hydrolyzate liquor. A metabolic ethanol yield of 78.6 % of the theoretical maximum was obtained after prefermentation.

The subsequent fed-batch SSCF resulted in an ethanol concentration of 45.5 g·L⁻¹ after 168 h of cofermentation, which corresponded to an overall ethanol yield of 92.4% of the theoretical maximum yield (Table 3). Solubilized glucose was depleted, and the residual xylose concentration was 2.9 g·L⁻¹. Xylose utilization reached 91 wt% of the total ingoing xylose, of which 4.4 wt% of consumed xylose was converted to xylitol (Table 3). Furaldehydes were promptly converted to their less toxic reduced form after each addition of substrate (data not shown), and the ethanolic cofermentation of glucose and xylose was sustained throughout the 168 h of cofermentation. The lower xylitol production compared with *severe* slurry was in part caused by lower xylose consumption in the late SSCF fermentation phase. The ethanol production after 144 h and on with *mild* slurry was based largely on the hydrolytic release of monosaccharides from lignocellulosic solids (Figure 4), in contrast to *severe* slurry, for which primarily solubilized xylose was consumed and converted to xylitol (Figure 3).

The lower WIS content of the *mild* slurry—11.1 wt% compared with 13.9 wt% WIS in the *severe* slurry—required a lower dilution to obtain a WIS load of 10 wt% in the cofermentation. As a result, higher sugar concentrations were obtained during prefermentation with *mild* hydrolysate. Despite the greater dilution of the *severe*

hydrolysate liquor, inhibitor concentrations remained higher than with *mild* hydrolysate liquor. However, the differences in ethanol yield cannot be explained solely by the differences in inhibitor concentration. Alternatively, the higher xylose-to-glucose ratio of the *mild* hydrolysate liquor seemed to have a positive impact on ethanol production. The effect of lower inhibitor concentration in the original *mild* slurry was more pronounced during the fed-batch SSCF step, in which considerably lower inhibitor concentrations were observed. Inhibitors were added to the fermentation broth with each addition of unwashed solids, because a fraction of the hydrolysate liquor remained with the solids after filtration.

The overall ethanol yield improved significantly with *mild* slurry, which was attributed to weaker inhibitory effects and lower xylitol production (Table 3). The difference in cofermentation outcomes between *mild* and *severe* wheat straw slurry confirmed the importance of pretreatment methods that disrupt the inherent recalcitrance of lignocellulosic material and simultaneously generate low concentrations of inhibitory degradation products. Keeping inhibitor concentrations low, while maintaining hydrolyzability, is paramount for improving ethanol yields.

3.3 Comparison with other studies

Regardless of fermenting microorganism, pretreatment, and raw material, the fermentation behavior in fed-batch SSCF of whole slurry was similar—xylose consumption declined over time. The inhibitor concentrations in *mild* and *severe* slurry were comparable with those in fed-batch SSCF in Olofsson et al. [28] and Koppram et al. [29], respectively. Taking these results into consideration, the sequential targeting of xylose and glucose conversion with prefermentation of hydrolyzate liquor followed by fed-batch SSCF improved the process compared with fed-batch SSCF of whole slurry. With our strategy, the xylose-fermenting capacity was sustained throughout the cofermentation, regardless of inhibitor concentrations in the slurries. Xylose utilizations that exceeded 90% of the total available xylose were obtained with both slurries.

Olofsson et al. [28] applied a considerably shorter fermentation time, 100 h, but xylose consumption after 40 h and on was limited. Koppram et al. [29] used a similar fermentation time as in our study but observed much lower xylose consumption than with prefermentation followed by fed-batch SSCF (Table 3). The improved xylose utilization with prefermentation of the separated hydrolyzate liquor followed by fed-batch SSCF with unwashed solids, thus, contributed significantly to the improved the overall ethanol yield.

Table 2. Summary of experimental conditions.

Summary of experimental conditions in this study as well as in Koppram et al. [29] and Olofsson et al. [28]. The following abbreviations are used: STEX: Acid-catalyzed steam explosion, PF: Prefermentation, CC: Corn cobs, WS: Wheat straw

| | Experimental conditions | | | | | | Inhibitor concentrations | | |
|--------------------------------|-------------------------|--------------------------|-----------------|--------------|------------------------------------|--|-------------------------------------|-----------------------------|----------------------------------|
| | Strain | Substrate | Duration [h] | WIS [wt%] | Yeast load [g·L ⁻¹] | Enzyme load [FPU·g ⁻¹ WIS] | Acetic acid [g·L ⁻¹] | HMF [g·L ⁻¹] | Furfural [g·L ⁻¹] |
| Fed-batch SSCF [28] | TMB3400 | STEX-WS | 100 | 11 | 4 | 20 | 2.5-3.4 | 0.2-0.5 | 3.1-4.9 |
| Fed-batch SSCF [29] | KE6-12 | STEX-CC | 168 | 10 | 5 | 15 | 8.3 | 1.9 | 4.0 |
| Fed-batch SSCF (2.4.1) | KE6-12 | STEX-WS <i>Severe</i> | 120 | 10 | 5 | 10 | 8.5 | 1.3 | 7.7 |
| Fed-batch SSCF with PF (2.4.2) | KE6-12 | STEX-WS <i>Severe</i> | 144 | 10 | 5 | 10 | 8.5 | 1.3 | 7.7 |
| Fed-batch SSCF with PF (2.4.2) | KE6-12 | STEX-WS <i>Mild</i> | 168 | 10 | 5 | 10 | 5.6 | 0.3 | 3.7 |

Table 3. SSCF end-results.

Residual sugar, end-product formation, and ethanol and xylitol yields after SSCF. The following abbreviations are used: STEX: Acid-catalyzed steam explosion, PF: Prefermentation, CC: Corn cobs, WS: Wheat straw, n.r.: Not reported.

| | Substrate | Residual sugars and end-products | | | | Yields | | | Xylose utilization [%] |
|--------------------------------|--------------------------|----------------------------------|--------------------------------|---------------------------------|---------------------------------|--|-----------------------------|------|---------------------------|
| | | Glucose [g·L ⁻¹] | Xylose [g·L ⁻¹] | Xylitol [g·L ⁻¹] | Ethanol [g·L ⁻¹] | Ethanol ¹ [g·g ⁻¹] | Xylitol ² [%] | | |
| Fed-batch SSCF [28] | STEX-WS | n.r. | n.r. | ≤2 | 38 | 0.35 | 69 | 9 | 49 |
| Fed-batch SSCF [29] | STEX-CC | 0.5 | 16 | 3.4 | 47 | 0.35 | 69 | 11 | 55 |
| Fed-batch SSCF (2.4.1) | STEX-WS <i>Severe</i> | 7.6 | 8.3 | 2.4 | 28.1 | 0.287 | 56.3 | 14.5 | 66 |
| Fed-batch SSCF with PF (2.4.2) | STEX-WS <i>Severe</i> | 0.0 | 1.5 | 2.9 | 41.9 | 0.428 | 84.0 | 12.6 | 92 |
| Fed-batch SSCF with PF (2.4.2) | STEX-WS <i>Mild</i> | 0.1 | 2.9 | 1.2 | 45.5 | 0.474 | 92.4 | 4.4 | 91 |

¹ Ethanol yield based on total supplied glucose and xylose and related to the maximum theoretical yield (0.51 g·g⁻¹).

² Xylitol yield based on consumed xylose.

3.4 SSCF vs. SHCF

To evaluate SSCF that is coupled to prefermentation versus SHCF, the outcomes were compared to those of Nielsen et al. [31], which examined the same pretreated raw material and fermenting microorganism. With prefermentation of the hydrolyzate liquor followed by fed-batch SSCF or SHCF the xylose-fermenting capacity was sustained throughout the cofermentation, thus effecting high xylose utilization. However, the ethanol concentrations and overall ethanol yields that were obtained with fed-batch SSCF of *mild* and *severe* slurry exceeded those obtained with

SHCF that was coupled to prefermentation [31]. The SHCF strategy elicited overall ethanol yields of 83% and 75% of the theoretical maximum, respectively, for cofermentation of *mild* and *severe* slurry, whereas the SSCF strategy resulted in overall ethanol yields of 92% and 84%, respectively (Table 3). The improvement in ethanol yields reinforced the notion that SSF typically results in higher yields than SHF [20, 24].

Further, the suboptimal conditions for hydrolysis that are needed to accommodate simultaneous saccharification and fermentation imply the risk of rendering the hydrolysis rate-limiting for ethanol production. Prefermentation followed by fed-batch SSCF had longer fermentation times before stagnation of ethanol formation, indicating that hydrolysis was rate-limiting for ethanol production. Prefermentation followed by fed-batch SSCF also resulted in higher residual xylose concentrations than prefermentation followed by SHCF [31], which resulted in lower xylose utilization. This trend was also observed for SSCF and SHCF without prefermentation by Olsson et al. [20].

3.5 Process considerations

With regard to process and economic perspectives, there are several aspects to consider. With high ethanol yields, final ethanol concentrations for cost-efficient downstream processing can be reached with relatively low WIS loads. In this study, we obtained ethanol concentrations above $40 \text{ g}\cdot\text{L}^{-1}$, which is generally regarded as the lower limit for cost-effective recovery of ethanol [41, 42], with a WIS load of 10 wt%. Further, the fractionation of slurry was linked to the possibility of targeting xylose and glucose conversion in succession and concurrently maintaining low viscosity in the bioreactor by fed-batch SSCF, in which the viscosity decreases continuously by enzymatic degradation. Keeping the viscosity low ensures proper mixing, which becomes increasingly important as the WIS loads are increased above the applied 10 wt% WIS. Furthermore, prefermentation prolongs the fermentation time and consequently negatively affects ethanol productivity for a given fermentor capacity. Thus, there is a tradeoff between ethanol yield and productivity with this method.

4 Conclusions

Our SSCF strategy sustained xylose fermentation throughout fermentation, and prefermentation of hydrolyzate liquor followed by fed-batch SSCF improved xylose utilization and ethanol yield compared with fed-batch SSCF with enzyme feeding. With our strategy, an overall ethanol yield of 84% of the theoretical maximum was reached at higher inhibitor concentrations in the slurry, versus 92% at lower inhibitor concentrations. Xylose utilization exceeded 90% after SSCF for both slurries. The suggested SSCF strategy resulted in overall ethanol yields that superseded those in corresponding SHCF strategies, thus reinforcing the notion that SSCF in general attains higher yields.

List of abbreviations

DM: dry matter; FPU: filter paper unit; HMF: 5-hydroxymethylfurfural; HPLC: high-performance liquid chromatography; SHCF: separate hydrolysis and cofermentation; SSCF: simultaneous saccharification and cofermentation; vvm: gas volume flow per unit of liquid volume per minute; WIS: water-insoluble solids; XDH: xylitol dehydrogenase; XK: xylulokinase; XR: xylose reductase.

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Paper IV



RESEARCH

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Simultaneous saccharification and co-fermentation for bioethanol production using corncobs at lab, PDU and demo scales

Rakesh Koppram¹, Fredrik Nielsen², Eva Albers^{1,3}, Annika Lambert⁴, Sune Wännström⁴, Lars Welin³, Guido Zacchi² and Lisbeth Olsson^{1*}

Abstract

Background: While simultaneous saccharification and co-fermentation (SSCF) is considered to be a promising process for bioconversion of lignocellulosic materials to ethanol, there are still relatively little demo-plant data and operating experiences reported in the literature. In the current work, we designed a SSCF process and scaled up from lab to demo scale reaching 4% (w/v) ethanol using xylose rich corncobs.

Results: Seven different recombinant xylose utilizing *Saccharomyces cerevisiae* strains were evaluated for their fermentation performance in hydrolysates of steam pretreated corncobs. Two strains, RHD-15 and KE6-12 with highest ethanol yield and lowest xylitol yield, respectively were further screened in SSCF using the whole slurry from pretreatment. Similar ethanol yields were reached with both strains, however, KE6-12 was chosen as the preferred strain since it produced 26% lower xylitol from consumed xylose compared to RHD-15. Model SSCF experiments with glucose or hydrolysate feed in combination with prefermentation resulted in 79% of xylose consumption and more than 75% of the theoretical ethanol yield on available glucose and xylose in lab and PDU scales. The results suggest that for an efficient xylose conversion to ethanol controlled release of glucose from enzymatic hydrolysis and low levels of glucose concentration must be maintained throughout the SSCF. Fed-batch SSCF in PDU with addition of enzymes at three different time points facilitated controlled release of glucose and hence co-consumption of glucose and xylose was observed yielding 76% of the theoretical ethanol yield on available glucose and xylose at 7.9% water insoluble solids (WIS). With a fed-batch SSCF in combination with prefermentation and a feed of substrate and enzymes 47 and 40 g l⁻¹ of ethanol corresponding to 68% and 58% of the theoretical ethanol yield on available glucose and xylose were produced at 10.5% WIS in PDU and demo scale, respectively. The strain KE6-12 was able to completely consume xylose within 76 h during the fermentation of hydrolysate in a 10 m³ demo scale bioreactor.

Conclusions: The potential of SSCF is improved in combination with prefermentation and a feed of substrate and enzymes. It was possible to successfully reproduce the fed-batch SSCF at demo scale producing 4% (w/v) ethanol which is the minimum economical requirement for efficient lignocellulosic bioethanol production process.

Keywords: *S. cerevisiae*, SSCF, Prefermentation, Xylose

* Correspondence: lisbeth.olsson@chalmers.se

¹Department of Chemical and Biological Engineering, Industrial Biotechnology, Chalmers University of Technology, Göteborg SE-412 96, Sweden

Full list of author information is available at the end of the article



Background

The global CO₂ emissions in 2010 from fossil energy use grew at the fastest rate since 1969. The year 2010 also witnessed that the global oil production did not match the rapid growth in consumption [1]. These recent data further intensify worldwide concerns about greenhouse gas emissions and energy security for a sustained economic development. For a reduced dependence on oil from fossil reserves, use of biofuels such as bioethanol from abundantly available lignocellulosic biomass is of great interest nowadays because they will count towards meeting the mandate of 10% binding target for biofuels from renewable sources in the transport for all European member states by 2020 [2]. Along with this interest comes increased interest in commercializing ethanol production technology from inexpensive lignocellulosic feedstocks which includes wood biomass, agricultural and forestry residues, biodegradable fraction of industrial and municipal wastes. Irrespective of type, the basic structural composition of lignocellulosic biomass consists of cellulose, hemicellulose and lignin. The cellulose and hemicellulose that form the polysaccharide fraction are embedded in a recalcitrant and inaccessible arrangement [3] and therefore requires a pretreatment step to disrupt the structure and make it accessible for subsequent steps. Since lignocellulosic materials are very complex, not one pretreatment method can apply for all the materials. Several methods that are classified in to physical, physico-chemical, chemical and biological pretreatment have been investigated and an elaborate review on each of these methods has been presented by Taherzadeh and Karimi [4]. One of the most commonly used pretreatment methods is steam explosion, with the addition of H₂SO₄ or SO₂, which removes most of the hemicellulose, followed by enzymatic hydrolysis to convert cellulose to glucose [5,6].

The release of hexose and pentose sugars during pretreatment and enzymatic hydrolysis is often accompanied by liberation of compounds such as furans, weak organic acids and phenolics compounds [7] that inhibits growth, ethanol yield and productivity of fermenting microorganism, *Saccharomyces cerevisiae* [8-10]. Traditionally and industrially relevant microorganism for ethanol fermentation is *S. cerevisiae*, but its inability to consume pentose sugars like xylose and arabinose has led to intensive research on metabolic and evolutionary engineering to develop strains that can tolerate high concentration of inhibitors and ferment xylose and arabinose [11-15]. However, it has been shown that recombinant *S. cerevisiae* strain utilizing pentose sugar may lose its xylose consuming ability in a long term evolutionary engineering for inhibitor tolerance [15]. Consequently, to ensure that all properties are retained during evolutionary engineering requires careful design of the selection pressure.

The enzymatic hydrolysis can be performed simultaneously with the co-fermentation of glucose and xylose in a

process referred to as simultaneous saccharification and co-fermentation (SSCF). Besides reduced capital cost [16], SSCF process offers several advantages which include continuous removal of end-products of enzymatic hydrolysis that inhibit cellulases or β -glucosidases [17] and higher ethanol productivity and yield than separate hydrolysis and fermentation [18,19]. It is required to operate a SSCF process at high content of water-insoluble-solids (WIS) to achieve high concentrations of ethanol. However, it has been shown that at high WIS content ethanol yield was decreased due to increased mass transfer resistance and inhibitors concentration [20]. Operating SSCF in a fed-batch mode at high WIS content not only assists ease of mixing and produces high ethanol concentrations [21] but also offers a possibility to maintain glucose at low levels allowing efficient co-fermentation of glucose and xylose [22]. Lowering of glucose concentration can be achieved by initially fermenting free hexoses before adding enzymes to a SSCF process in a concept referred as prefermentation enhanced xylose uptake irrespective of batch or fed-batch SSCF [23]. These flexibilities offered by a SSCF process makes it a promising process option for bioethanol production from lignocellulosic materials.

The heterogeneity of raw materials together with a variety of pretreatment methods, lack of detailed understanding of dynamic changes of substrate during enzymatic hydrolysis and unavailability of microorganisms that can ferment a wide range of carbohydrates and can tolerate high concentrations of inhibitors produced from pretreated biomass makes SSCF a highly researched area yet to reach the commercial status. There come additional technical challenges when operating at larger scales which include longer times to add material into the reactor, longer mixing times and therefore concentration gradients are inevitable. On-site propagation of yeast in large volumes is needed which also increases the probability of contamination since lignocellulosic ethanol plants will not employ aseptic operating conditions. Moving cellulosic ethanol technology from the laboratory to a commercial scale biorefinery is an expensive proposition and requires process data at sufficient scale to obtain engineering and process guarantees. Some prominent players that are working on this proposition include Chemtex, Inbicon, DuPont cellulosic ethanol, POET-DSM advanced biofuels, Iogen, Abengoa Bioenergy, Mascoma and SEKAB. A category of feedstock that is of considerable interest is corn derived residues due to that it is inexpensive and available in abundance. Corncob is an agricultural residue and a byproduct of corn production. Currently, 12.1 billion tons and 120 million tons of corn are being produced in the US and China, respectively. About 70 million metric tons of corncobs are available annually accounting only from the US and China markets [21,24]. Removal of corncobs from the agricultural grounds does

not contribute to decreased soil organic matter since corncobs are low in nutrients.

In this work, a xylose fermenting *S. cerevisiae* strain was used in SSCF of pretreated corncobs with the objective of determining suitable conditions for co-consumption of glucose and xylose. Fed-batch mode of SSCF in combination with prefermentation was investigated at high WIS content. To validate the designed SSF process and verify the reproducibility at different scales, the process was scaled up from lab conditions to process development unit (PDU) (30 liters) and further to demo scale (10 m³).

Results and discussion

The SSCF concept is one of the interesting process options and the potential of such process for biological conversion of lignocellulosic raw materials to bioethanol in large scales has to the best of our knowledge not been reported previously. A promising xylose consuming strain of *S. cerevisiae* was selected from screening seven different recombinant *S. cerevisiae* strains. The glucose influence on xylose consumption of the selected strain was investigated by model SSCF with glucose or hydrolysate feed. The potential of fed-batch SSCF process in combination with prefermentation was finally demonstrated in 10 m³ demo scale bioreactors.

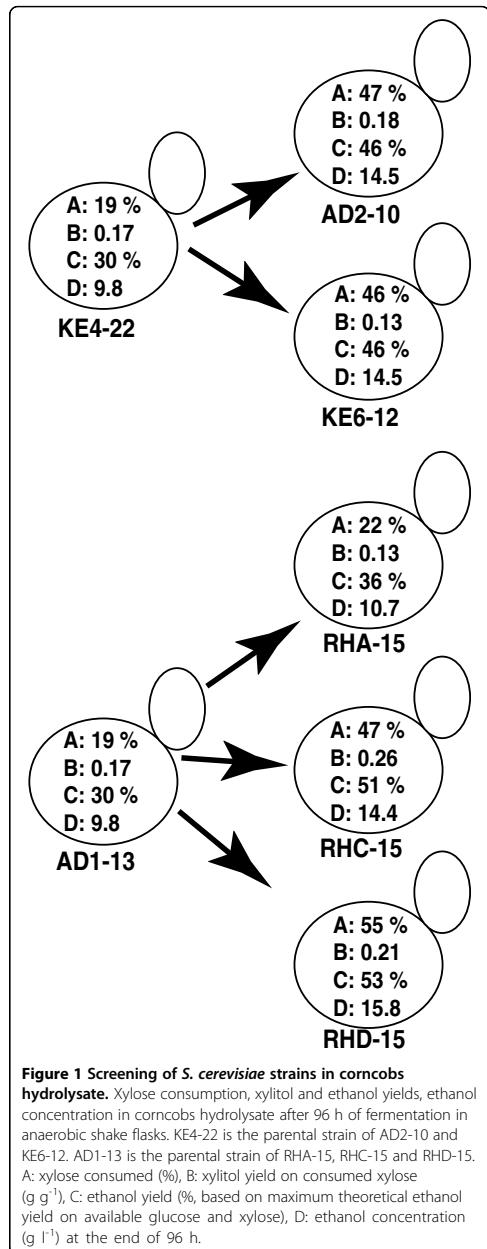
Screening and selection of *S. cerevisiae* strain

Anaerobic fermentation of corncob hydrolysate

The seven different *S. cerevisiae* strains (Table 1) were evaluated on their fermentation performance in corncobs hydrolysate in shake flasks equipped with glycerol loops. Since, xylose constitutes a significant proportion of mono-saccharides in corncobs hydrolysate xylose consumption and xylitol yield together with ethanol yield were determined (Figure 1) and used as parameters for strain selection. The strains, AD2-10, KE6-12 and RHC-15, RHD-15 displayed similar ethanol concentration, ethanol yield and performed better than their respective parental strains with regard to xylose consumption. The strain RHD-15 displayed the highest ethanol yield and xylose consumed. The strain KE6-12 stands alone among other strains in xylitol yield producing the lowest amount of xylitol from consumed xylose. Even though the screening revealed significant differences in fermentation of hydrolysate, it is important to evaluate the microbial performance in the

Table 1 *S. cerevisiae* strains used in this study

| Parental strain | Evolved strain |
|-----------------|----------------|
| KE4-22 | AD2-10 |
| | KE6-12 |
| AD1-13 | RHA-15 |
| | RHC-15 |
| | RHD-15 |



whole slurry in a SSCF process. The strains RHD-15 and KE6-12, due to their high ethanol and low xylitol yields, were therefore, selected as the preferred strains for subsequent investigations in the SSCF process.

SSCF of corncobs whole slurry

To assess the fermentation performance, the strains RHD-15 and KE6-12 were evaluated in a base case batch SSCF of corncobs whole slurry at 7.5% WIS for ethanol production. During the SSCF process, the glucose concentration was quickly reduced to less than 1 g l^{-1} within 10 h and thereafter, it was maintained at this level throughout the process (Figure 2a & 2b). Immediately after inoculation, both the strains started to consume xylose for a period of 72 h after which the xylose concentration in the reactor started to level off. After 96 h, the strain KE6-12 had consumed 37% of the available xylose and 30% of the consumed xylose was converted to xylitol (2.8 g l^{-1}) whereas, the strain RHD-15 had consumed 42% of the available xylose and 66% of the consumed xylose was converted to xylitol (6.4 g l^{-1}). An ethanol concentration of 21.9 and 21.5 g l^{-1} were achieved corresponding to a yield of 0.28 g g^{-1} and 0.27 g g^{-1} based on total available sugars for the strains KE6-12 and RHD-15, respectively. In comparison to RHD-15, strain

KE6-12 consumed marginally lower amount of xylose but, produced 56% less xylitol. Since, bioconversion of xylose to ethanol is one of the predominant requirements for an economical lignocellulosic bioethanol production, further fermentation and SSCF experiments were carried out with the strain KE6-12, unless otherwise stated. In screening experiments using corncobs hydrolysate, the strain RHD-15 performed relatively better than KE6-12, however, in SSCF using corncobs whole slurry both the strains resulted in similar ethanol yields and RHD-15 was clearly outperformed by KE6-12 in lower xylitol yields. The differences in results from the two screening experimental systems could be attributed to the differences in experimental conditions. The effect of pH on xylose consumption by a recombinant xylose utilizing *S. cerevisiae* has been previously shown that increasing the pH from 5.0 to 5.5 resulted in 46% increase in xylose consumption rate [25]. Screening using corncobs hydrolysate in shake flasks were performed at an initial pH 6.0 and 30°C which clearly resulted in higher xylose consumption compared to screening in SSCF where the pH was controlled at 5.0 and sub-optimal temperature of 35°C . It should be noted that often strain engineering and development results in a numerous strains and a high throughput screening of these strains in SSCF process in shake flasks could be impractical due to difficulties in mixing at high WIS content. The difference in two screening systems illustrate the importance of choice of experimental setup and conditions for screening to be as close as possible to that used in the actual experiments.

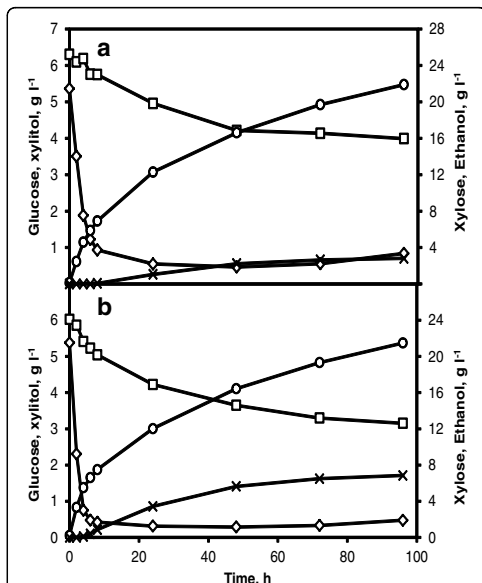


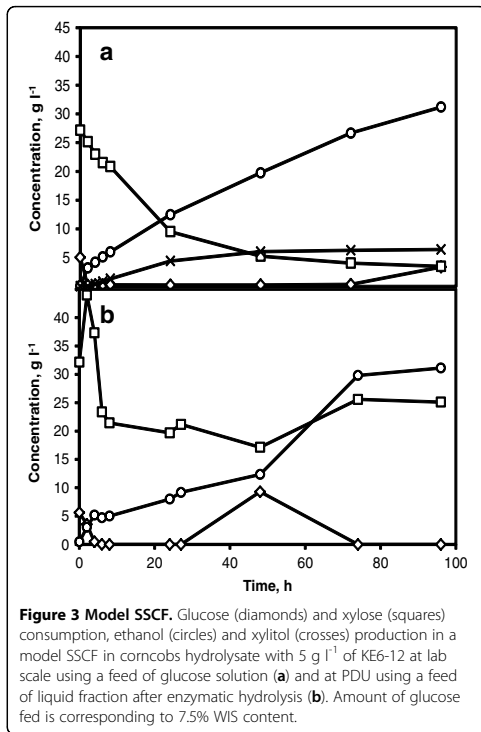
Figure 2 Screening of *S. cerevisiae* strains in corncobs whole slurry. Glucose (diamonds) and xylose (squares) consumption, ethanol (circles) and xylitol (crosses) production in SSCF at 7.5% WIS content, 5 g l^{-1} of yeast loading and 5 FPU gWIS^{-1} of enzyme loading using KE6-12 (a) and RHD-15 (b).

Model SSCF as a tool to design the SSCF process

In order to understand the effect of glucose on xylose consumption and to optimally design the SSCF process with effective xylose consumption a model SSF [26] with prefermentation [23] was performed. A model SSCF is a SSCF process without the addition of enzymes but fed with pure glucose solution or hydrolysate to the reactor mimicking the release of glucose during enzymatic hydrolysis of cellulose. Prefermentation is a concept where initially available free glucose was fermented before starting the feed.

Lab scale

Model SSCF in lab scale was performed in corncobs hydrolysate with a feed of 100 g l^{-1} glucose solution at a constant rate. A glucose feed corresponding to the amount of glucose from 7.5% WIS was started after 2 h of inoculation and terminated at 96 h. During the prefermentation period of 2 h, the glucose concentration was reduced to nearly 0 g l^{-1} and maintained at this level until 72 h (Figure 3a). Immediately after prefermentation, xylose was rapidly consumed until 48 h and thereafter, the concentration started to level off. After 96 h, 79% of xylose was consumed and 37% of consumed xylose was converted to xylitol (6.4 g l^{-1}).



An ethanol concentration of 31.2 g l⁻¹ was achieved corresponding to a yield of 0.38 g g⁻¹ based on total available sugars (75% of the theoretical yield). Higher ethanol concentration in model SSCF compared to batch SSCF may possibly be due to higher xylose consumption and also points to a direction that cellulose fibers were not completely hydrolyzed in batch SSCF to yield similar ethanol concentrations as that obtained in model SSCF.

PDU scale

A model SSCF in PDU scale similar to lab scale model SSCF was performed with a feed of hydrolysate from enzymatic hydrolysis. In order to completely hydrolyze cellulose fibers, enzymatic hydrolysis of solid fraction of corncoobs slurry was carried out at 50°C with enzyme loading of 6 FPU gWIS⁻¹. The liquid fraction remaining after filtering the enzymatically hydrolyzed solid fraction was used as a feed. Prefermentation in corncoobs hydrolysate was initiated by adding yeast and an enzyme solution corresponding to 3 FPU gWIS⁻¹. The glucose was rapidly consumed during the initial 10 h of prefermentation, reduced to near 0 g l⁻¹ and maintained at this level until 24 h (Figure 3b). A sharp increase in xylose concentration was

observed immediately after the addition of enzyme solution indicating the hydrolysis of xylan. Thereafter, the xylose was consumed quickly for 10 h, however, when the glucose was completely consumed the xylose consumption dramatically slowed down. This indicates that the consumption of glucose with maintained low concentration of glucose is beneficial for efficient xylose consumption. Previous study on fed-batch SSCF using xylose rich wheat straw has highlighted that maintaining low levels of glucose consequently increased consumed xylose twice as compared to a batch SSCF [26]. It also has been discussed that presence of glucose at high concentrations may inhibit xylose uptake due to competition for transporters [27,28]. Feeding of the liquid fraction from enzymatic hydrolysis was started after 24 h of prefermentation and was maintained for 24 h corresponding to a final WIS content of 7.5%. The glucose concentration gradually increased during the 24 h feeding phase until 48 h and thereafter was completely consumed. The xylose started to accumulate when glucose concentration reached a peak of 10 g l⁻¹ and thereafter no xylose was consumed and no change in ethanol concentration was observed indicating the end of fermentation. The increase in xylose concentration after 50 h could be due to enzymatic hydrolysis of xylan. After 96 h, an ethanol concentration of 32 g l⁻¹ was produced corresponding to 77% of the theoretical yield based on available sugars. This ethanol yield is well in accordance with ethanol yields of model SSCF in lab scale. Evidences from model SSCF with prefermentation clearly suggest that fermentation of initial free glucose and thereafter, maintenance of glucose at low levels are crucial factors for efficient xylose consumption.

Fed-batch SSCF

PDU scale

It was also possible to achieve similar ethanol yields in a fed-batch SSCF as that in the model SSCF. Fed-batch SSCF in PDU was carried out using the whole slurry with a total WIS of 7.9%. Initially, prefermentation was carried out for 2 h by adding 6 g dry cell weight l⁻¹ of yeast from cell suspension. To maintain glucose concentrations at a minimum level in the reactor and thereby facilitate effective xylose consumption, a strategy to add enzyme solution at multiple time points to ensure controlled release of glucose was investigated. An enzyme solution corresponding to 3 FPU gWIS⁻¹ was added at 2 h, 24 h, and 48 h. The glucose concentration was maintained around 5 g l⁻¹ until 72 h before it was completely consumed at 96 h (Figure 4). A steady co-consumption of glucose and xylose was observed throughout the SSCF. After 96 h, 50% of the available xylose was consumed producing xylitol with a concentration of only 1.5 g l⁻¹. An ethanol concentration of 32 g l⁻¹ was achieved corresponding to 76% of the theoretical ethanol yield based on available sugars.

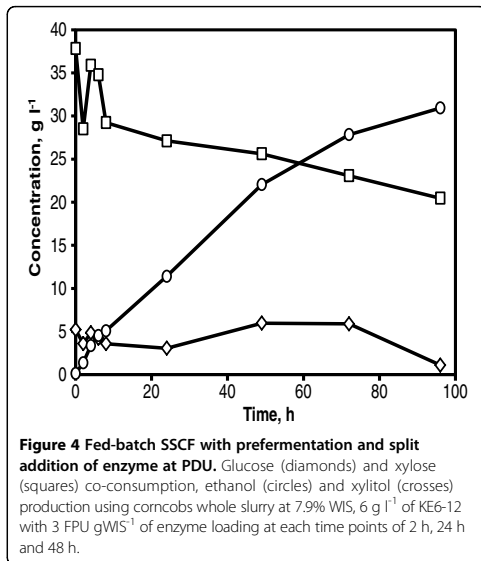


Figure 4 Fed-batch SSCF with prefermentation and split addition of enzyme at PDU. Glucose (diamonds) and xylose (squares) co-consumption, ethanol (circles) and xylitol (crosses) production using corncobs whole slurry at 7.9% WIS, 6 g l⁻¹ of KE6-12 with 3 FPU gWIS⁻¹ of enzyme loading at each time points of 2 h, 24 h and 48 h.

In a commercial bioethanol production process it is desirable that the substrate load is higher than 7% WIS to achieve 4% (w/v) ethanol concentration to yield a subsequent economical distillation process [29]. It has been shown that working at high WIS content increases the concentration of inhibitors and results in inhibition of yeast and lower ethanol yields [26]. Therefore, along with split addition of enzymes, fed-batch SSCF at higher WIS was investigated with split addition of substrate resulting in lower amount of inhibitors for each addition. Fed-batch SSCF experiment was performed with a corncobs slurry addition at 0 h, 5 h, 27 h and 49 h to a total final WIS of 10%. Enzyme solution was added at multiple time points of 2 h, 24 h, 48 h, 72 h and 96 h to a total of 15 FPU gWIS⁻¹. During the first 2 h of prefermentation, the glucose concentration was reduced to nearly 0 g l⁻¹ and reached around 5 g l⁻¹ after the first addition of enzyme (Figure 5a). The glucose concentration was then maintained below 5 g l⁻¹ throughout the SSCF process. The xylose was co-consumed along with glucose for more than 100 h. At the end of fed-batch SSCF, 55% of the available xylose was consumed and 11% of the consumed xylose was converted to xylitol (3.4 g l⁻¹). An ethanol concentration of 47 g l⁻¹ was achieved corresponding to a yield of 0.35 g g⁻¹ based on total available sugars (69% of the theoretical yield). Higher xylose consumption and ethanol yield at 10% WIS clearly suggest that the combination of prefermentation and a feed of enzymes and substrates as one of the possible SSCF strategies for demo scale execution.

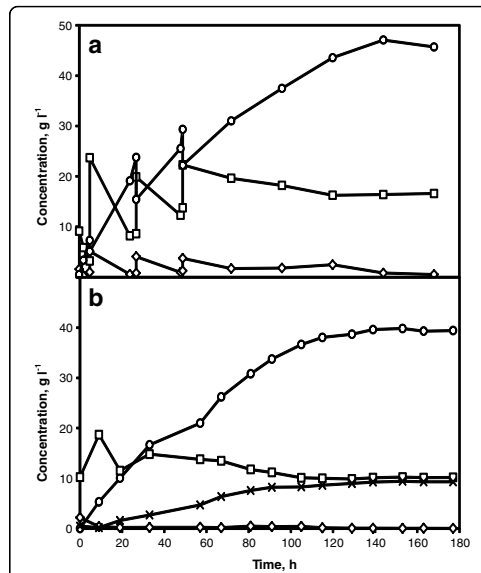


Figure 5 SSCF with prefermentation and fed-batch addition of substrate and enzyme. Glucose (diamonds) and xylose (squares) co-consumption, ethanol (circles) and xylitol (crosses) production using corncobs whole slurry at 10.5% WIS, 5 g l⁻¹ of KE6-12 and 15 FPU gWIS⁻¹ of enzyme loading. Split addition of substrate at 0 h, 5 h, 27 h, 49 h and enzyme solution at 2 h, 24 h, 48 h, 72 h, 96 h in PDU (a). Fed-batch addition of substrate for 48 h and split addition of enzyme solution at 2 h, 24 h, 48 h, 72 h, 96 h in demo scale (b).

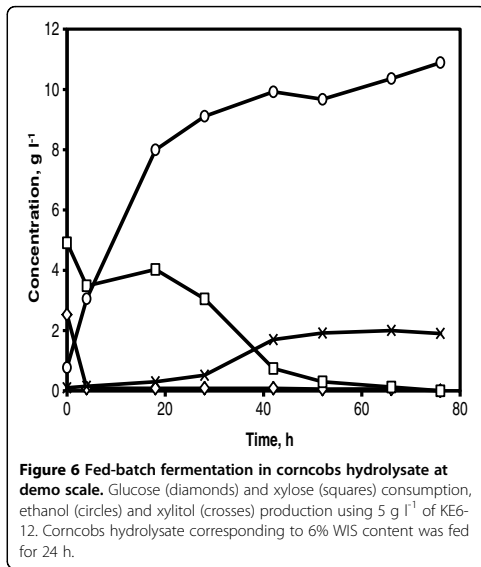
Demo scale

Xylose fermentation in hydrolysate

A time span of 24 to 48 h was used to pump a substrate in to the demo scale reactor of 10 m³. In order to address the potential of the strain KE6-12 on xylose consumption, fed-batch fermentation of corncobs hydrolysate corresponding to a WIS content of 6% was evaluated. The corncobs hydrolysate was fed into the reactor for 24 h. The glucose concentration was reduced to nearly 0 g l⁻¹ within 5 h and all xylose was consumed in 76 h (Figure 6). Only 10.6% of the consumed xylose was converted to xylitol (2.0 g l⁻¹). An ethanol concentration of 10.9 g l⁻¹ was achieved corresponding to a yield of 0.46 g g⁻¹ on total available sugars (90% of the theoretical yield).

Fed-batch SSCF

A fed-batch SSCF with substrate and enzyme feed and prefermentation for 2 h similar to the one performed at PDU scale was carried out in the demo scale. The corncobs slurry was fed into the reactor for 48 h resulting in a total WIS of 10.5%. Enzyme solution was added at five different time points, 2 h, 24 h, 48 h, 72 h and 96 h corresponding to



a total of 15 FPU gWIS⁻¹. The glucose concentration was quickly reduced to nearly 0 g l⁻¹ within 10 h and thereafter, it was maintained at low concentration throughout the process (Figure 5b). Co-consumption of xylose and glucose was observed for more than 100 h similar to the fed-batch SSCF at PDU scale. After 150 h, 65% of the available xylose was consumed and 24.7% of the consumed xylose was converted to xylitol (9.3 g l⁻¹). Surprisingly, in comparison to fed-batch SSCF at PDU scale, higher amount of xylose was consumed in demo scale, however, also higher amount of xylitol was produced. An ethanol concentration of 39.8 g l⁻¹ was achieved corresponding to a yield of 0.29 g g⁻¹ based on total available sugars (58% of the theoretical yield). More controlled conditions of temperature, pH and homogenous mixing in PDU scale resulted in higher final ethanol concentration and yield compared to demo scale conditions with higher mass transfer limitations.

Conclusion

The performance of recombinant xylose utilizing *S. cerevisiae* strains varied in two different screening experiments, which highlights the importance of experimental setup and conditions for screening of strains to be highly similar to that of the actual experiments. The choice of the strain KE6-12 seems well justified when xylose was completely consumed at demo scale during the fermentation of hydrolysate with 90% of the theoretical ethanol yield. Different feeding profiles of glucose and its influence on xylose consumption was studied using model SSCF and it proved to be a valuable tool to optimally design a

SSCF process. The potential of the fed-batch SSCF process is more vivid and we demonstrated that with prefermentation and substrate and enzyme feed it is possible to produce ethanol from corncoobs as high as 40 g l⁻¹ and more, with relatively high WIS content at both 30 l (PDU scale) and 10 m³ (demo scale). Using such a strategy it was possible to maintain low levels of glucose concentration, which facilitated co-consumption of glucose and xylose. We also confirmed that the results of fed-batch SSCF were similar at PDU and demo scales and the experimental system was reproducible at both the scales. However, at higher WIS content an optimal feeding strategy is required to ferment all xylose and avoid glucose accumulation.

Materials and methods

Saccharomyces cerevisiae strains

The seven *S. cerevisiae* strains used in this study (Table 1) were developed by a combination of different evolutionary engineering strategies and random mutagenesis (Albers *et al.*, manuscript in preparation) on *S. cerevisiae* TMB 3400 [30] that harbours the xylose reductase gene and xylitol dehydrogenase from *Scheffersomyces stipitidis* (formerly known as *Pichia stipitidis*) and endogenous xylulokinase overexpressed. All the strains were stored at -80°C in culture aliquots containing 20% sterile glycerol. Volumes of 100 µl from the vials were used to inoculate precultures.

Media

Corncoobs slurry

Corncoobs slurry with a water-insoluble-solids (WIS) content of 15% was received from SEKAB-E-Technology AB (Örnsköldsvik, Sweden) and was stored at -20°C. The corncoobs were pretreated at 185°C for 5 min with 0.6% dilute sulfurous acid (SO₂ in water). Two batches were pretreated and the composition of which are presented in the Table 2. Batch 1 was used for screening and selection experiments. Batch 2 was used in the

Table 2 Composition of the pretreated corncoobs

| | Content in solid fraction (% of WIS) | | Content in liquid fraction (g l ⁻¹) | | |
|----------|--------------------------------------|---------|---|---------|------|
| | Batch 1 | Batch 2 | Batch 1 | Batch 2 | |
| Glucan | 66.9 | 61.4 | Glucose* | 17.0 | 15.0 |
| Mannan | 0 | 0 | Mannose* | 0 | 0 |
| Galactan | 0 | 2.9 | Galactose* | 6.5 | 0 |
| Xylan | 5.8 | 8.2 | Xylose* | 79.4 | 74.4 |
| Arabinan | 1.0 | 1.4 | Arabinose* | 11.8 | 14.0 |
| Lignin | 27.6 | 28.9 | HMF | 2.0 | 1.9 |
| | | | Furfural | 3.8 | 4.0 |
| | | | Acetic acid | 10.4 | 8.3 |

*Both monomeric and oligomeric form is included.

demo scale experiments. The corncobs hydrolysate (liquid fraction of corncobs slurry), pH adjusted to 5.0 with 10 M NaOH, was used in yeast cell cultivations when required.

Molasses

Molasses was obtained from SEKAB-E-Technology AB (Örnsköldsvik, Sweden) and was either used alone or mixed with liquid fraction of corncobs slurry for cultivating yeast cells that was then used for SSCF experiments.

Minimal medium

The initial inoculum for screening yeast strains and SSCF experiments were cultivated in minimal medium containing 20 g l⁻¹ glucose and xylose, respectively and enriched with salts, two folds of vitamins and trace elements according to Verduyn *et al.* [31]. The pH of the medium was set to 6.0 with 1 M NaOH for all shake flask cultivations.

Cultivation of yeast

In order to improve inhibitor tolerance by adaptation, yeast cells were grown briefly in presence of corncobs hydrolysate during the cultivation for screening and SSCF experiments (as described below). It has been previously shown that the cultivation procedure of yeast significantly influences the performance in SSF and small-scale fermentations of hydrolysate liquor [32].

The precultures for screening *S. cerevisiae* strains for ethanol production were cultivated in 150 ml shake flasks with 50 ml of minimal medium. The cultures were inoculated to an initial OD₆₅₀ of 0.005, incubated at 30°C on an orbital shaker at 180 rpm. After 18 h of incubation, corncobs hydrolysate supplemented with 23.5 g l⁻¹ (NH₄)₂SO₄, 3.0 g l⁻¹ KH₂PO₄ and 2.25 g l⁻¹ MgSO₄ · 7H₂O was added to the preculture cultivation flask to a final volume of 35% (v/v) and incubated for another 24 h.

The yeast cells for SSCF experiments in lab and PDU scales were cultivated in aerobic batch on molasses,

followed by an aerobic fed batch on corncobs hydrolysate and molasses. In the demo scale molasses was used as the medium in aerobic batch and fed batch cultivation. The yeast strain was inoculated in to 50 ml (lab scale), 150 ml (PDU) of minimal medium contained in a 150 ml (lab scale) and 300 ml (PDU) shake flasks, respectively; incubated at 30°C on an orbital shaker at 180 rpm for 24 h. Aerobic batch cultivation was performed in 50 g l⁻¹ molasses supplemented with 23.5 g l⁻¹ (NH₄)₂SO₄, 3.0 g l⁻¹ KH₂PO₄, 2.25 g l⁻¹ MgSO₄ · 7H₂O, 33 µg l⁻¹ biotin, 125 ppm vitahop (Betatech GmbH, Schwabach, Germany) (to suppress bacterial growth) and 0.5 ml l⁻¹ antifoam. The yeast cultivation was carried out in 3.6 l Infors HT-Labfors bioreactor (lab scale), 30 l bioreactor (PDU) and 10 m³ bioreactor (demo scale). The cultivation was initiated in the bioreactors by adding 50 ml or 150 ml of minimal medium culture to a working volume of 500 ml (lab scale) or 1.5 l (PDU) of molasses medium, respectively. The cultivation was carried out until all sugars are consumed which was indicated by CO₂ evolution in the gas-out and dissolved oxygen concentration in the culture. Upon depletion of sugars in batch phase, a feed solution containing corncobs hydrolysate and molasses was fed linearly for 20 h to a final volume of 1.5 l (lab scale) or 4.5 l (PDU). The concentration of corncobs hydrolysate in the feed solution was same as that of concentration of corncobs slurry in the SSCF experiments. Molasses concentration was 100 g l⁻¹ in the feed solution. The stirrer speed during the batch phase in lab scale was 700 rpm and increased linearly to 1000 rpm during the fed batch phase; whereas, the stirrer speed was maintained at 700 rpm throughout the cultivation in PDU scale; aeration rate was maintained at 1 vvm and the pH was maintained at 5.0 by automatic addition of 2 M NaOH.

After the cultivation, cells were harvested by centrifugation for 8 min at 4°C, 1800 g and the cell pellet was resuspended in 0.9 % sterile NaCl solution to yield a cell suspension with a dry weight of 80 g l⁻¹.

Table 3 Brief list of SSCF experiments carried out in lab, PDU and demo scales

| Mode of operation_Scale | Initial Pre-fermentation time, h | Amount of solids, %WIS | Strain | Total cell amount, g l ⁻¹ | Total enzyme amount, FPU gWIS ⁻¹ |
|---------------------------------------|----------------------------------|------------------------|--------|--------------------------------------|---|
| Batch SSCF_Lab | None | 7.5 | RHD-15 | 5 | 5 |
| Batch SSCF_Lab | None | 7.5 | KE6-12 | 5 | 5 |
| Fed-batch Model SSCF_Lab ¹ | 2 | 7.5 | KE6-12 | 5 | None |
| Fed-batch Model SSCF_PDU ² | 24 | 7.5 | KE6-12 | 5 | None* |
| Fed-batch SSCF_PDU | 2 | 7.9 | KE6-12 | 6 | 9 |
| Fed-batch SSCF_PDU | 2 | 10 | KE6-12 | 5 | 15 |
| Fed-batch SSCF_Demo | 2 | 10.5 | KE6-12 | 5 | 15 |

¹Model SSCF in lab scale with a feed of glucose solution with glucose amounts corresponding to 7.5%WIS.

²Model SSCF in PDU scale with a feed of filtered hydrolysate from enzymatic hydrolysis of whole slurry at 7.5% WIS.

*No enzyme added during the model SSCF. However, filtered hydrolysate from enzymatic hydrolysis of whole slurry using an enzyme solution of 6 FPU gWIS⁻¹ was used as a feed solution.

Anaerobic fermentation in shake flasks

The pH of corncobs hydrolysate was set to 6.0, supplemented with 0.5 g l^{-1} $(\text{NH}_4)_2\text{HPO}_4$, 125 ppm vitahop and filter sterilized using $0.45 \mu\text{m}$ cellulose acetate filter. This fermentation medium was inoculated using the cell suspension to reach a yeast concentration of $3 \text{ g dry cell weight l}^{-1}$. The fermentations were carried out in 50 ml working volume in 100 ml shake flasks fitted with glycerol loops providing anaerobic condition. The flasks were incubated at 30°C on an orbital shaker at 180 rpm for 96 h and samples were withdrawn for OD_{650} measurement and extracellular metabolite analysis. Possible contamination during the shake flask fermentation was checked by ocular inspection in microscope.

SSCF

The SSCF experiments were carried out in lab scale (3.6 l Infors HT-Labfors), PDU scale (30 l), and demo scale (10 m^3) bioreactors with a total working weight of 1.5 kg, 20 kg and 4000 kg, respectively. In the lab and PDU scale experiments the corncobs slurry was pH adjusted to 5.0 with 10 M NaOH and supplemented with 0.5 g l^{-1} $(\text{NH}_4)_2\text{HPO}_4$. In the demo scale the pH was adjusted using NH_3 solution and supplemented with 0.25 g l^{-1} H_3PO_4 . To avoid possible contamination and foam formation 125 ppm of Vitahop solution and 0.5 ml l^{-1} antifoam, respectively were added to the medium. In order to obtain the desired WIS content the supplemented medium was diluted with water and used for SSCF experiments. Unless otherwise stated, all the experiments were initiated by adding $5 \text{ g dry cell weight l}^{-1}$ of yeast from cell suspension. An enzyme preparation, Cellic Ctec-2 from Novozymes A/S, Denmark with filter paper activity of 95 FPU g^{-1} enzyme, β -glucosidase activity of 590 IU g^{-1} enzyme was added to SSCF experiments corresponding to the desired cellulase activity. All SSCF experiments were carried out at 35°C ; pH was maintained at 5.0 by automatic addition of 3 M NaOH and the stirrer speed was maintained at 400 rpm in lab and PDU scales, respectively. A brief summary of all SSCF experiments carried out is listed in the Table 3. All SSCF experiments performed in duplicates in lab scale and one of them is represented in the results and discussion section.

Analysis of metabolites

Samples for extracellular metabolites were analyzed by high performance liquid chromatography using Aminex HPX-87H column with $30 \times 4.6 \text{ mm}$ Cation-H Biorad micro-guard column maintained at 45°C . $5 \text{ mM H}_2\text{SO}_4$ was used as an eluent at a flow rate of 0.6 ml min^{-1} . Ethanol, xylitol, and acetic acid were detected using RI detector maintained at 35°C and HMF, furfural and lactic acid were detected using UV detector at 210 nm. The sugars in corncobs hydrolysate and samples from shake

flasks and SSCF experiments were analyzed by high performance anion exchange chromatography using $4 \times 250 \text{ mm}$ Dionex CarboPac PA1 column with $4 \times 50 \text{ mm}$ guard column maintained at 30°C . Eluent A: 300 mM NaOH, eluent B: 100 mM NaOH + 85 mM sodium acetate were used for elution at a flow rate of 1 ml min^{-1} . Monosaccharides including arabinose, galactose, glucose, xylose and mannose were detected using pulsed amperometric detector. Optical density (OD) was used as an estimate of cell concentration in shake flask experiments. OD was measured at 650 nm using the cell free medium at the point of sampling as background.

Yield calculations

Ethanol yield (% of maximum theoretical yield)

The sum of available fermentable sugars including glucose, mannose, galactose, and xylose in liquid fraction and glucan and xylan fibers in the WIS was calculated. Due to the addition of water during hydrolysis, the theoretical weight of glucose and xylose released are 1.11 and 1.13 times the weight of glucan and xylan, respectively. By using the maximum theoretical ethanol yield of 0.51 g g^{-1} sugar, the maximum ethanol that can be produced from total available sugars was calculated. The percentage of the theoretical ethanol yield is defined as $Y_{SE} = 100 \times \text{produced amount of ethanol (g)}/\text{maximum theoretical amount of ethanol (g)}$.

Xylose consumed (%)

The percentage xylose consumed = $100 \times \text{amount of xylose consumed (g)}/\text{total amount of available xylose in liquid and WIS fraction (g)}$.

Xylitol yield (%)

The percentage xylitol yield = $100 \times \text{amount of xylitol produced (g)}/\text{amount of xylose consumed (g)}$.

Abbreviations

SSCF: Simultaneous saccharification and co-fermentation; SSF: Simultaneous saccharification and fermentation; WIS: Water insoluble solids; PDU: Process development unit; FPU: Filter paper unit; OD: Optical density; HPLC: High performance liquid chromatography; HPAEC: High performance anion exchange chromatography.

Competing interests

SW, AL were employed by SEKAB E-Technology during the time of this work. EA was employed by Taurus Energy AB during the time of this work. LW is employed by Taurus Energy AB and LO does consultancy for Taurus Energy AB.

Authors' contribution

RK, EA, AL, SW, LW, GZ and LO participated in the conception and design of the study. RK, AL and FN performed the experimental work. RK wrote the manuscript. All the authors commented on the manuscript, read and approved the final manuscript.

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Author details

¹Department of Chemical and Biological Engineering, Industrial Biotechnology, Chalmers University of Technology, Göteborg SE-412 96, Sweden. ²Department of Chemical Engineering, Lund University, P.O. Box 124, Lund SE-221 00, Sweden. ³Taurus Energy AB, Ideon, Ole Römers väg 12, Lund SE-223 70, Sweden. ⁴SEKAB E-Technology AB, P.O. Box 286, Örnsköldsvik SE-891 26, Sweden.

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Paper V

Evaluation of the effect of mixed agricultural feedstocks on pretreatment, enzymatic hydrolysis and cofermentation efficacy

Fredrik Nielsen, Mats Galbe, Guido Zacchi, and Ola Wallberg

Department of Chemical Engineering, Lund University
P.O. Box 124, SE-221 00, Lund, Sweden

Abstract

Traditionally second-generation fuel ethanol is produced from a single raw material, but the variability in supply and quality of biomass feedstocks calls for an expansion of the feedstock base. By blending different feedstocks, improved supply efficiency and consistent input quality can be attained, and economical risks can be hedged. The heterogeneity of feedstock blends requires the development of conversion strategies with minimal negative impact on the performance of pretreatment, saccharification, and fermentation to reach industrially relevant ethanol yields and titers. In this study the impact of blending of wheat straw and corn stover in various ratios on process ethanol yield was investigated. The single feedstocks and blends thereof were pretreated under uniform conditions with dilute-acid catalyzed steam-pretreatment and subsequently converted to ethanol with different simultaneous saccharification and cofermentation (SSCF) strategies, employing commercial cellulolytic enzymes and xylose-fermenting *Saccharomyces cerevisiae*.

The uniform pretreatment conditions imposed restrictions on the process ethanol yield, and trends of decreasing hydrolysability and xylose recovery were obtained with increasing ratios of corn stover in the feedstock blend. Process ethanol yields for single feedstocks and feedstock blends were maximized by SSCF coupled with prehydrolysis, which promoted enzymatic hydrolysis efficacy. Ethanol titers exceeding 50 g·L⁻¹ and process ethanol yields in the range of 74-78% of theoretical maximum, based on available of glucose and xylose in the raw material, were attained across the range of feedstock blends.

This study showed that the choice of SSCF strategy is highly intertwined with the employed pretreatment method and conditions. The SSCF strategy must be matched to the pretreatment conditions to maximize the process ethanol yield. The attained narrow range of process ethanol yields suggests that wheat straw and corn stover could be blended in various ratios and used interchangeably in combined processing.

Keywords: Mixed feedstock, Blending, SSCF, Cofermentation, Prefermentation, Prehydrolysis, Xylose fermentation, *Saccharomyces cerevisiae*, Lignocellulose

1. Introduction

To produce second-generation bioethanol at commercial-scale from agricultural residues considerable amounts of lignocellulosic feedstock have to be provided; ideally a consistent year-round supply of a single type with predictable composition and quality and at affordable cost. However, in reality, the supply and quality of agricultural feedstocks exhibits diversity across regions, seasons, and years [1, 2] and vary with environmental factors [3], and so do the prices of the feedstocks. In relying on a single feedstock, this can create supply barriers and incur prohibitive costs for raw material. Expanding the feedstock base to encompass multiple feedstock sources can aid in ensuring adequate supply to realize economies of scale [4], hedge risks of crop failure, and minimize seasonality constraints and storage requirements [5]. Furthermore, it can be used to manage economical risk. The cost of raw material constitutes a significant fraction of the total production cost [5, 6], and an expanded feedstock base can be used to minimize that cost and hedge the sensitivity to price volatility.

Traditionally research on the conversion of lignocellulosic feedstocks to ethanol has been based on single feedstocks, and limited attention has been given to the efficiency of converting feedstock blends. One of the reasons for this is limitations in the pretreatment step. Not all pretreatment methods can be used with all types of feedstocks. Furthermore, the structural diversity of various biomass sources implies that the different feedstocks preferably are pretreated separately and processed in successive campaigns. However, there are advantages to feedstock blending as compared to processing feedstocks in campaigns. Feedstock blending can be performed based on availability to enhance supply-chain efficacy, but can also be formulated to stabilize compositional variance and to target specific physiochemical characteristics that are beneficial for the conversion process [7]. Blending different feedstocks and different anatomical parts of feedstocks, which have inherent variability in composition and quality [1, 8, 9], can provide a consistent input to the process [7]. Further, blending provides the opportunity to upgrade the process input with regard to e.g. hemicellulose, cellulose, and ash content [7], which allows feedstocks with lower potential process ethanol yields to enter the resource pool and to meet acceptable compositional quality. The improved quality and consistency of the input contribute to the robustness of the process and reduces technological risk [7].

The conversion of lignocellulose to ethanol is a multistep process with highly intertwined processing steps. A change in single process parameters can affect the entire downstream process in a multitude of ways. In spite of this, many studies exclude integrative process analyses and focus at optimization of either the

pretreatment or the bioconversion step [10]. In the integrated process the compositional heterogeneity of blended feedstock affects all process steps, starting with pretreatment and propagating from there. The commercially pursued autocatalyzed and dilute-acid catalyzed steam-pretreatments [11] have a proven broad range of application [12], although different feedstocks have different established optimal pretreatment conditions that are founded on the attributes of the feedstock [13]. However, feedstocks with similar attributes could be co-pretreated with dilute-acid catalyzed steam-pretreatment. Agricultural residues, e.g. wheat straw and corn stover, have similar attributes and optimal pretreatment conditions in dilute-acid catalyzed steam pretreatment [14-17], which could make them suitable candidates for feedstock blending. It has also been suggested that there could be synergistic effects in steam pretreatment of feedstock blends of species that are heterogeneous in physical and chemical nature [18].

The pretreatment is an integral part of the conversion process that has effect on the downstream process. Conversely, the pretreatment requirements are dependent on the design of the subsequent processing. High yield of fermentable sugars as well as efficient cofermentation are prerequisites to realize high ethanol yields and high ethanol titers, which are key factors for viable process economics [19]. However, a tradeoff exists in the pretreatment. Higher severity in the pretreatment, e.g. higher temperatures, longer duration and higher concentrations of catalyst, with dilute-acid catalyzed steam pretreatment increases the acid hydrolysis of the raw material during pretreatment and improve the susceptibility of pretreated biomass to enzymatic digestion [13]. However, increased severity also results in greater secondary degradation of sugars [13], which decreases the recovery of fermentable sugars and generates greater amounts of inhibitory compounds with detrimental effects on the fermentation performance [20]. The conditions that maximize individual sugar recoveries and yields in pretreatment are often neither the same as those that maximize total sugar yields in the process nor the conditions that promote the most efficient cofermentation of the sugars to ethanol.

Downstream of the pretreatment, simultaneous saccharification and cofermentation (SSCF) has been proposed as a feasible approach to attain high ethanol yield and titers from pretreated lignocellulosic biomass [21]. The integrated enzymatic hydrolysis and co-fermentation strategy cater to the sugar preferences of the fermenting microorganism to improve ethanol yield. In addition, the continuous removal of hydrolysis end-products alleviates the end-product inhibition of the cellulase system [21]. However, the suboptimal temperatures for enzymatic hydrolysis in SSCF, mandated by the fermenting microorganism, could impose hydrolysis limitations to the ethanol yield. The constraints entailed by suboptimal temperatures can be compensated for by increased enzyme loads in the conversion process [22], but it also implies greater conversion costs. Another approach is the introduction of a high temperature prehydrolysis step prior to SSCF [23, 24]. Elevated temperature

used in the prehydrolysis step promotes favorable enzyme kinetics [25] and can alleviate the hydrolysis constraints. The choice of integrated cofermentation strategy is strain and feedstock dependent and needs to be made in conjunction with the pretreatment conditions to maximize the ethanol yield and titers. With the heterogeneity of feedstock blends it is imperative to develop integrated conversion processes that can convert feedstock blends to ethanol with minimal negative impact on the performance of pretreatment, saccharification, and fermentation, as compared to processing of single feedstocks. To do so it is important that the efficacy of the entire integrated chain of unit operations is evaluated and optimized concurrently.

In this study we examined feedstock blends of wheat straw and corn stover in combined processing already from the pretreatment step. The aim was to evaluate the effect of blended feedstock inputs on pretreatment, hydrolysability, fermentability, and—in the end—ethanol yields and titers. Enzymatic hydrolysis and cofermentation was performed with commercial enzyme systems and xylose-fermenting *Saccharomyces cerevisiae*, respectively. The goal was to reach industrially relevant ethanol yields and titers in the biochemical conversion, preferably ethanol concentrations exceeding 50 g·L⁻¹ and ethanol yields above 80% of theoretical maximum, based on both glucose and xylose in the pretreated feedstocks. Pretreatment conditions for dilute-acid catalyzed steam-pretreatment were derived from reported pretreatment conditions that maximize the total sugar yield from wheat straw and corn stover [14, 17], with the aim to accommodate feedstock blends and enable the desired ethanol titers. Enzymatic hydrolysis and cofermentation of xylose and glucose was performed with 2 different SSCF strategies. The strategies either promoted cofermentation efficacy [26] or provided advantageous conditions for enzymatic hydrolysis [23, 24]. The investigated processes were evaluated with regard to conversion efficacy across different wheat straw and corn stover blends and SSCF configurations.

2. Materials and Methods

2.1. Raw material and pretreatment

Wheat straw (Johan Håkansson Lantbruksprodukter), corn stover (provided by State Grid Corporation of China, Handan City, Hebei Province, China), and blends thereof (ratio 3:1, 1:1, and 1:3, based on dry weight) were pretreated with acid-catalyzed steam pretreatment. The feedstocks and feedstock blends are denoted WS100 to WS0, based on the percentage of wheat straw dry matter in the raw material used in the pretreatment. The compositions of the raw materials are listed in Table 1.

The applied pretreatment conditions for the feedstocks and feedstock blends were based on reported optima [14, 17]. The feedstock was soaked for 20 min at room temperature in 0.2 wt% aqueous H₂SO₄ solution. The liquid-solid ratio was 20 kg·kg⁻¹ of dry matter (DM). The soaked feedstock was dewatered by filtration with a filter press (HP5M, Fischer Maschinenfabrik GmbH), yielding a DM content of 52±2 wt%. The dewatered feedstock was steam pretreated in batches of 500 g DM at 190°C for 5 min in a preheated 10L batch-pretreatment reactor, previously described by Bondesson et al. [27], and collected after 5 consecutive batches.

2.2. Microorganism

Fermentation was performed with the noncommercial xylose-fermenting *Saccharomyces cerevisiae* KE6-12 strain (Taurus Energy AB), which harbors genes from *Scheffersomyces stipitis* that encode for xylose reductase (XR) and xylitol dehydrogenase (XDH) and overexpressed endogenous xylulokinase (XK). The strain was developed by evolutionary engineering [28] of the industrial strain *S. cerevisiae* TMB3400 [29] to improve inhibitor tolerance and xylose-fermenting capacity. Stock culture aliquots contained 20 wt% of glycerol in water and were stored at -80°C.

2.3. Cultivation of yeast

The precultures were cultivated in 250 ml shake flasks with 150 ml of sterile minimal medium, containing 20 g·L⁻¹ glucose, 20 g·L⁻¹ xylose, 7.5 g·L⁻¹ (NH₄)₂SO₄, 3.75 g·L⁻¹ KH₂PO₄, 0.75 g·L⁻¹ MgSO₄, and supplemented with 1 mL·L⁻¹ vitamin solution and 10 mL·L⁻¹ trace element solution, per Taherzadeh et al. [30]. The pH of the medium was adjusted to 5.5 with 5 M NaOH. The precultures were inoculated with 300 µl of

Table 1. Composition of raw materials.

Composition of structural carbohydrates in wheat straw and corn stover expressed as wt% of dry weight.

| | Wheat straw | | Corn stover | |
|-------------------|-------------|----------|-------------|----------|
| | \bar{x} | <i>s</i> | \bar{x} | <i>s</i> |
| Glucan | 36.9 | 0.3 | 37.5 | 0.7 |
| Xylan | 23.6 | 0.2 | 22.7 | 0.3 |
| Galactan | 2.6 | 0.2 | 2.8 | 0.1 |
| Arabinan | 3.4 | 0.1 | 3.5 | 0.1 |
| Mannan | 1.0 | 0.1 | 0.8 | 0.3 |
| Lignin | 20.8 | 0.2 | 21.9 | 0.5 |
| Ash | 4.3 | 0.4 | 2.8 | <0.1 |
| Total determined: | 92.6 | | 91.9 | |

the stock cell aliquots and incubated at 30°C on an orbital shaker (Lab-Therm, Kühner) at 180 rpm for 24 h.

The yeast was propagated sequentially by aerobic batch cultivation on molasses and aerobic fed-batch cultivation on molasses and hydrolysate liquor from pretreated WS50 feedstock blend. The cultivations were performed in a sterilized 2 L Labfors bioreactor (Infors AG) equipped with two six-blade Rushton turbines. The reactor diameter:impeller diameter ratio was 3, and the reactor height:diameter ratio was 1.7. The batch cultivation was performed with 50 g·L⁻¹ molasses solution that was supplemented with 23.5 g·L⁻¹ (NH₄)₂SO₄, 3 g·L⁻¹ KH₂PO₄, 2.25 g·L⁻¹ MgSO₄, 33 µg·L⁻¹ biotin, and 120 ppm Vitahop (BetaTec). The molasses (Nordic Sugar A/S) contained 40 wt% of fermentable sugars, i.e. sucrose, fructose, and glucose. The cultivation was initiated by inoculation with the preculture. The batch cultivation was performed with a working volume of 0.5 L, a constant aeration rate of 1 vvm, and an agitation rate of 700 rpm. The pH was maintained at 5.2 automatically with sterile 2.5 M NaOH solution. The batch phase was concluded when all sugars were consumed, as indicated by the evolution of carbon dioxide and oxygen in the bioreactor gas effluent.

The fed-batch phase was initiated after fermentable sugars were depleted in the batch phase. The feed solution comprised diluted hydrolysate liquor from a pretreated blend of wheat straw and corn stover, ratio 1:1, that was supplemented with 150 g·L⁻¹ of molasses. The hydrolysate liquor in the feed solution brought about inhibitor concentrations in the final working volume that corresponded to the concentrations in a broth of pretreated WS50 with a 7.5 wt% WIS load. The purpose of the hydrolysate liquor in the fed-batch phase was to improve yeast tolerance by short-term adaptation of the cultivated yeast to the environmental conditions in the fermentation experiments, per Nielsen et al. [31]. The feed solution was pulse-fed to the bioreactor at a constant rate for 20 h to a final working volume of 1.5 L. The

reactor was aerated by sparging at a constant rate of 1 vvm, based on the final volume, and the pH was maintained at 5.2. The propagated yeast was harvested by centrifugation (3800×g, 10 min) and washed with 9 g·L⁻¹ sterile NaCl solution.

2.4. Enzymatic hydrolysis

Enzymatic hydrolyses were performed in sterilized 2 L Labfors bioreactors (Infors AG) equipped with an anchor impeller and a pitched 6-blade turbine. The reactor diameter:impeller diameter ratio was 1.5 for the anchor impeller and 1.7 for the pitched 6-blade turbine, and the reactor height:diameter ratio was 1.7. The enzymatic hydrolyses were performed with a working weight of 1 kg of pretreated feedstock. The hydrolysate liquor was separated from the solid fraction by filtration, and subsequently pH adjusted to pH 5.5 with 12.5 M NaOH solution and Cellic CTech3 enzyme preparation (Novozymes A/S) was dispersed therein. The hydrolysate liquor was subsequently added back to the solid fraction. The procedure promoted even enzyme dispersion in the bioreactor prior to liquefaction. Hydrolysis was performed at 45°C for 96 hours with an enzyme load of 10 FPU·g⁻¹ WIS. The pH was maintained at 5 by manual addition of 5 M sterile NaOH solution.

2.5. Simultaneous saccharification and cofermentation

SSCF was performed with two different strategies, batch SSCF with prehydrolysis and fed-batch SSCF with prefermentation (Figure 1), in sterilized 2 L Labfors bioreactors (Infors AG), described above. The SSCF experiments were performed with 1 kg working weight of pretreated feedstock. The pH of the slurry was adjusted to 5.5 with 12.5 M NaOH prior to fermentation, and was supplemented with 0.5 g·L⁻¹ (NH₄)₂HPO₄, 0.125 mL·L⁻¹ Vitahop (BetaTec), and 0.4 mL·L⁻¹ antifoam RD Emulsion (Dow Corning), based on the final volume. The fermentation was inoculated with a yeast load of 4 g·L⁻¹ of dry matter, based on the final volume, and an overall enzyme load (Cellic CTec3, Novozymes A/S) of 10 FPU·g⁻¹ WIS, based on total ingoing WIS. Total WIS loads close to those of the original slurries (Table 2) were obtained after addition of enzymes, yeast and supplements. The pH in the fermentation broth was maintained at 5.2 by automatic addition of sterile 2.5 M NaOH solution during fermentation. Agitation was maintained at 300 rpm throughout the fermentation.

2.5.1. SSCF with prehydrolysis

The utilized strategy for SSCF with prehydrolysis was modified from Öhgren et al. [23], and is outlined in Figure 1a. Pretreated feedstock, supplements and the full

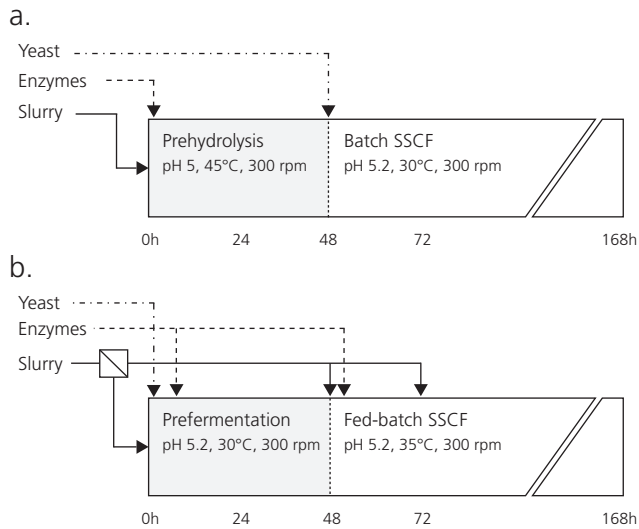


Figure 1. Schematics of SSCF strategies.

Schematic representation of (a) batch SSCF with prehydrolysis (*hybrid SSCF*) and (b) fed-batch SSCF with prefermentation (*fed-batch SSCF*).

enzyme load was added at outset and prehydrolysis was performed at 45°C for 48h, controlled during the first 12h by the heating jacket temperature and then on broth temperature. After 48 h the temperature was decreased to 30°C and the bioreactor was inoculated with the harvested yeast. The SSCF was terminated after 168h.

2.5.2. Fed-batch SSCF with prefermentation

Fed-batch SSCF with prefermentation of hydrolysate liquor was performed per Nielsen et al. [26], and is outlined in Figure 1b. The pretreatment hydrolysate liquor was separated from the solids by filtration using a hydraulic press (HP5M, Fischer Maschinenfabrik). The retained filter cake had a WIS content of 43 ± 2 wt%. The fermentation was performed sequentially by batch prefermentation of the separated hydrolysate liquor, followed by fed-batch SSCF of the solids. The hydrolysate liquor, supplements, and yeast were added at outset and prefermented at 30°C for 48 h. An enzyme load of $2 \text{ FPU} \cdot \text{g}^{-1}$ WIS, based on the total ingoing WIS, was added after 4 h to hydrolyze oligosaccharides in the hydrolysate liquor. After prefermentation, i.e. 48 h, half of the solids was added to the prefermented hydrolysate liquor together with remaining enzymes, yielding a total cellulolytic activity of $10 \text{ FPU} \cdot \text{g}^{-1}$ WIS, based on total ingoing WIS. The temperature was increased to 35°C to enhance enzyme activity. Remaining solids were added after 72 h. The SSCF was terminated after 168 h.

2.6. Analytical procedures

Dry matter (DM) content and water-insoluble solids (WIS) were measured per Sluiter et al. [32] and Sluiter et al. [33], respectively.

Extracellular metabolites, inhibitors, and sugars were quantified by high-performance liquid chromatography (HPLC) on a Shimadzu HPLC system that was equipped with an RID-10A refractive index detector (Shimadzu). Samples for carbohydrate analysis were pH adjusted to 5, if needed, with CaCO₃ and centrifuged in 10 mL tubes (960×g, 5 min). All samples were centrifuged (16000×g, 3 min), and the supernatants filtered through 0.20 μm syringe filters (GVS Filter Technology). Filtered samples were stored at -20°C until analysis. Extracellular metabolites, organic acids, and degradation products in the samples were analyzed by isocratic ion-exchange chromatography using an Aminex HPX-87H column (Bio-Rad Laboratories) at 50°C. The eluent was 5 mM H₂SO₄, applied at a flow rate of 0.5 mL·min⁻¹. Sugars and xylitol in the samples were quantified by isocratic ion-exchange chromatography on an Aminex HPX-87P column (Bio-Rad Laboratories) at 85°C. Deionized water was used as the eluent at a flow rate of 0.5 mL·min⁻¹.

Soluble carbohydrates and pretreatment degradation products in the hydrolysate liquor were quantified by acid hydrolysis and HPLC per Sluiter et al. [34]. Further, structural carbohydrates, lignin, and ash contents of the water-insoluble fraction of the raw material, the pretreated material, and the residues after enzymatic hydrolysis and cofermentation were measured by the 2-step hydrolysis method per Sluiter et al. [35]. The monomeric sugars from the assay were measured by high-performance anion-exchange chromatography coupled with pulsed amperometric detection on an ICS-3000 chromatography system (Dionex) using a Carbo Pac PA1 analytical column (Dionex). Deionized water was used as eluent at a flow rate of 1 mL·min⁻¹.

2.7. Calculations

The recoveries of glucose and xylose after pretreatment were calculated based on measured glucan and xylan in the WIS, before and after pretreatment, and monomeric and oligomeric glucose and xylose in the liquid phase.

The degree of hydrolysis in enzymatic hydrolysis and SSCF was calculated per Palmqvist and Lidén [24]. WIS content and the composition of structural carbohydrates in the WIS before and after hydrolysis were measured, and the degree of glucan and xylan hydrolysis was calculated based on the change in composition of the WIS.

Ethanol yields in the cofermentation experiments were calculated at 3 levels: metabolic ethanol yield, technical ethanol yield and process ethanol yield. The

metabolic ethanol yield was based on consumed glucose and xylose during SSCF. The technical ethanol yield was based on total supplied glucose and xylose in the SSCF; i.e. the sum of glucose and xylose present in the slurries after pretreatment, including monomers, oligomers, and polymers. The process ethanol yield was based on total ingoing glucose and xylose in the raw material. The ethanol yields were calculated based on compositional analyses, HPLC measurements, measured liquid densities, measured WIS content, and applied working weight, per Palmqvist and Lidén [24]. The percentage of maximum theoretical ethanol yield was based on a theoretical stoichiometric yield of $0.51 \text{ g}\cdot\text{g}^{-1}$ on glucose and xylose.

3. Results and discussion

3.1. Pretreatment of feedstock blends

Dilute-acid catalyzed steam-pretreatment was the first step for the decomposition of the lignocellulosic biomass. Pretreatment conditions optimized for one feedstock may not apply to other feedstocks, or blends of the two. The applied pretreatment conditions were chosen to meet the ends of process relevant ethanol yields and titres; preferably technical ethanol yields exceeding 80% of theoretical maximum, based on both glucose and xylose, and ethanol titers exceeding 50 g·L⁻¹.

3.1.1. Pretreatment conditions

The optimal pretreatment conditions for dilute-acid catalyzed steam-pretreatment of wheat straw in batch-pretreatment units have been established to be in the range of 190-200°C for 5-10 min using 0.2-1 wt% H₂SO₄ as catalyst [14, 15]. Optimal pretreatment conditions for corn stover have been established to be in the range of 160-200°C for 5-20 min using 0.5-3 wt% H₂SO₄ [16, 17, 22]. The different optima combine elevation of temperature with decreased holdup times. The reported optimal pretreatment conditions are in part dependent on the enzyme load and performance of the enzyme system in the enzymatic hydrolysis used to evaluate the pretreatment [25]. The use of low amounts of acid catalyst is favorable from an economic perspective because it reduces the cost of catalyst and the need for neutralizing agents.

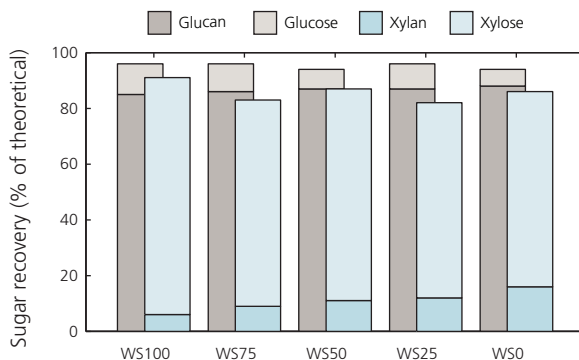
The applied pretreatment conditions in this study were based on reported pretreatment conditions for wheat straw that maximized the yield of glucose and xylose after enzymatic hydrolysis, 190°C for 10 min with 0.2 wt% of H₂SO₄ as catalyst [14]. These conditions are also within a range that has been reported to be suitable for corn stover [17]. However, these pretreatment conditions diluted the feedstock blends excessively, and consequently the desired ethanol titers could not be reached. To reduce the dilution during pretreatment and enable meeting both the desired targets simultaneously, the pretreatment time was shortened to 5 min and, thus, the injection of direct steam and the dilution decreased. The use of higher catalyst concentrations (0.5 wt% aqueous H₂SO₄) in the pretreatment to compensate for the shorter holdup time was investigated. However, it resulted in negligible differences in sugar yield after enzymatic hydrolysis as compared to 0.2 wt% aqueous H₂SO₄ with the same temperature and duration (data not shown). Based on the lower requirement of neutralization agent and the negligible differences in hydrolysability between pretreatments with different concentrations of catalyst,

Table 2. Composition of steam-pretreated wheat straw, corn stover and blends thereof.

Composition of structural carbohydrates in the WIS fraction and content of select sugars and inhibitors in the hydrolysate liquor from steam-pretreated feedstock blends.

| | WS100 | | WS75 | | WS50 | | WS25 | | WS0 | |
|---|-----------|------|-----------|------|-----------|------|-----------|------|-----------|------|
| | \bar{x} | s | \bar{x} | s | \bar{x} | s | \bar{x} | s | \bar{x} | s |
| WIS content (wt%) | 0.146 | | 0.143 | | 0.131 | | 0.152 | | 0.144 | |
| WIS (% of dry weight) | | | | | | | | | | |
| Glucan | 54.8 | 0.9 | 50.5 | 1.7 | 51.1 | 1.4 | 49.2 | 1.1 | 49.7 | 0.6 |
| Xylan | 2.6 | <0.1 | 3.3 | 0.1 | 3.9 | 0.2 | 4.3 | 0.1 | 5.3 | <0.1 |
| Galactan | 0.2 | <0.1 | 0.1 | <0.1 | 0.2 | <0.1 | 0.0 | <0.1 | 0.2 | <0.1 |
| Arabinan | 0.1 | <0.1 | 0.0 | <0.1 | 0.1 | <0.1 | 0.0 | <0.1 | 0.2 | <0.1 |
| Mannan | 0.5 | <0.1 | 0.6 | <0.1 | 0.5 | <0.1 | 0.5 | <0.1 | 0.6 | <0.1 |
| Lignin | 27.6 | 0.3 | 30.1 | 0.1 | 28.4 | 0.1 | 29.4 | 0.3 | 30.3 | 0.6 |
| Ash | 6.7 | <0.1 | 6.4 | 0.7 | 6.6 | <0.1 | 6.8 | 0.2 | 5.0 | 0.1 |
| Total determined: | 92.5 | | 91.0 | | 90.8 | | 90.2 | | 91.3 | |
| Hydrolysate liquor (g·L ⁻¹) | | | | | | | | | | |
| Glucose ^a | 11.1 | 0.3 | 9.4 | <0.1 | 5.9 | 0.1 | 8.0 | 0.1 | 5.0 | 0.3 |
| Xylose ^a | 54.3 | 0.6 | 41.7 | 0.2 | 37.6 | 0.8 | 39.5 | 0.1 | 36.4 | 0.5 |
| Galactose ^a | 3.1 | 0.1 | 2.9 | <0.1 | 2.6 | <0.1 | 3.4 | <0.1 | 3.1 | 0.2 |
| Arabinose ^a | 5.6 | 0.2 | 5.0 | <0.1 | 4.0 | 0.1 | 4.7 | <0.1 | 4.0 | 0.2 |
| Mannose ^a | 4.2 | 0.1 | 3.6 | 0.1 | 2.9 | 0.1 | 3.6 | 0.1 | 2.9 | 0.2 |
| Acetic acid | 3.5 | | 2.7 | | 2.0 | | 2.1 | | 1.8 | |
| HMF | 0.2 | | 0.3 | | 0.2 | | 0.3 | | 0.3 | |
| Furfural | 2.2 | | 2.4 | | 1.7 | | 2.0 | | 1.8 | |

^a Including both monomeric and oligomeric forms.

**Figure 2. Sugar recovery after the pretreatment step.**

Recovery of glucose and xylose equivalents in the WIS and hydrolysate liquor after dilute-acid catalyzed steam pretreatment of wheat straw, corn stover, and various blends thereof.

190°C for 5 min with 0.2 wt% of H₂SO₄ was chosen as pretreatment conditions for the feedstocks and feedstock blends.

3.1.2. Pretreatment results

The obtained WIS content, composition of the WIS and composition of hydrolysate liquor after pretreatment are listed in Table 2. The variation in WIS content in the pretreated material was mainly due to variation in dilution during the pretreatment (Table 2). The recoveries of glucose and xylose after pretreatment were in the range of 94-96% and 86-92%, respectively. The highest total recovery of glucose and xylose was attained with WS100, 95%, whereas recovery for the feedstock blends, WS25-75, and corn stover, WS0, were 90-91% (Figure 2). The difference in total recovery of glucose and xylose was mainly related to differences in the recovery of xylose. The incomplete recovery of glucose and xylose was due to mass losses in the retrieval of the pretreated biomass from the collection vessel and the formation of secondary degradation products [20] and pseudo-lignin [36]. The main part of the glucan remained in the lignocellulosic solids, whereas most of the hemicellulose was solubilized (Figure 2). There was a trend of increasing recovery of xylose bound in hemicellulose in the lignocellulosic solids with increasing ratios of corn stover in the feedstock blend (Figure 2). This was indicative of corn stover being less susceptible to the applied pretreatment.

3.1.3. Evaluation of hydrolysability

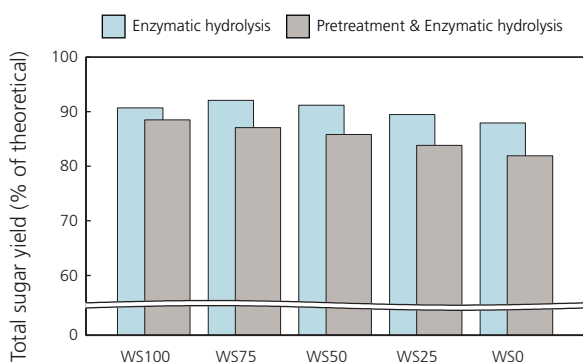
Enzymatic hydrolysis was performed on the unwashed pretreated biomass to evaluate the applicability of the uniform pretreatment conditions for single feedstocks and feedstock blends, as well as to assess the feasibility to reach the desired ethanol yields and titers in the fermentative conversion process.

The degrees of glucan and xylan hydrolysis in the enzymatic hydrolysis were in the range of 86-94% and 76-96%, respectively (Table 3). The total degree of glucan and xylan hydrolysis was in the range of 86-93%, and exhibited a weak decreasing trend with increasing ratios of corn stover in the pretreated raw material (Figure 3). The main contribution to the decreasing trend was lower degree of glucan hydrolysis with increasing ratios of corn stover in the blend (Table 3). The degree of xylan hydrolysis exhibits greater variability (Table 3), but was of less significance because of the low xylan content in the WIS (Table 2). The decreasing degree of hydrolysis indicates that the pretreatment improved the accessibility to the cellulosic structures for the enzymes to different extent for corn stover and wheat straw. This argument was supported by the increasing amounts of hemicellulose and lignin retained in the solid fractions after pretreatment in blends that contained increasing ratios of corn stover (Table 2).

Table 3. Summary of hydrolysis performance and end-results.

Hydrolysis and process performance for enzymatic hydrolysis feasibility trials.

| | WS100 | WS75 | WS50 | WS25 | WS0 |
|---|-------|------|------|------|------|
| Degree of hydrolysis in enzymatic hydrolysis (%) ^a | | | | | |
| Glucan | 0.90 | 0.94 | 0.88 | 0.89 | 0.86 |
| Xylan | 0.76 | 0.80 | 0.96 | 0.91 | 0.83 |
| Total sugar yields (% theoretical maximum) ^b | | | | | |
| Glucose | 88 | 91 | 84 | 86 | 82 |
| Xylose | 90 | 81 | 86 | 82 | 83 |
| Total | 89 | 87 | 86 | 84 | 82 |
| Final sugar concentrations (g·L ⁻¹) | | | | | |
| Glucose | 101 | 93 | 78 | 92 | 82 |
| Xylose | 58 | 46 | 45 | 44 | 45 |
| Total | 159 | 139 | 124 | 135 | 127 |

^a Base on available glucose and xylose in the WIS.^b Based on available glucose and xylose in the raw material.**Figure 3. Total yield of glucose and xylose in enzymatic hydrolysis.**

Total yields of glucose and xylose after enzymatic hydrolysis of dilute-acid catalyzed steam pretreatment of wheat straw, corn stover, and various blends thereof, expressed as percentage of the maximum theoretical yield from the pretreated feedstocks and the raw material.

These can shield the cellulose structures and make them less susceptible to enzymatic hydrolysis [12].

The yield of glucose and xylose after pretreatment and enzymatic hydrolysis was in the range 82-91% and 81-90%, respectively, based on glucose and xylose in the ingoing raw material (Table 3). The total yield of glucose and xylose was in the range of 82-89% of total ingoing glucose and xylose in the raw material, and reinforced the trend of decreasing yield with increasing ratios of corn stover in the feedstock blend (Figure 3). The overall glucose and xylose yields attained for wheat straw was in the

same range as previously reported for pretreatment conditions within the optimal range (190°C for 10 min with 0.2 wt% H₂SO₄, 200°C for 5 min with 0.9 wt% H₂SO₄) [14, 15]. The attained overall glucose yield for corn stover was higher than the reported glucose yield at pretreatment conditions that maximized total sugar yield after enzymatic hydrolysis (200°C for 10 min with 0.2 wt% H₂SO₄ as catalyst). Meanwhile, the xylose yield was significantly lower [17], due to extensive xylose degradation during pretreatment. Sufficiently high total glucose and xylose concentration (>123 g·L⁻¹) were attained across the whole range of feedstocks and feedstock blends to be able to meet the desired ethanol titers and technical ethanol yields simultaneously at minimum requirements (Table 3).

3.2 Simultaneous saccharification and cofermentation

The fermentative conversions of glucose and xylose derived from the pretreated feedstock blends to ethanol were performed with two 2-step SSCF strategies: fed-batch SSCF with prefermentation of the hydrolysate liquor and batch SSCF with prehydrolysis of the lignocellulosic solids (Figure 1). These are henceforth denoted fed-batch SSCF and hybrid SSCF, respectively. The fed-batch SSCF strategy has previously been employed for cofermentation of glucose and xylose derived from steam-pretreated wheat straw [26]. Technical ethanol yields that exceeded 90% of theoretical maximum and xylose utilizations that exceeded 90% were achieved in that study. The hybrid SSCF strategy has previously been applied to improve enzymatic hydrolysis and ethanol yields in SSCF with various substrates [23, 24].

3.2.1 Fed-batch SSCF - with prefermentation

Fed-batch SSCF of all pretreated feedstocks and feedstock blends elicited ethanol titers that exceeded the desired 50 g·L⁻¹ (Figure 4). The higher ethanol titer of WS100 reflected the higher recovery of glucose and xylose after pretreatment, as compared with feedstock blends that contained corn stover. The deviating ethanol concentration for WS50 (Figure 4) was an effect of the higher dilution in the pretreatment, as compared with the other blends (Table 2).

The attained metabolic ethanol yields in fed-batch SSCF were in the range of 0.424-0.460 g·g⁻¹ (83-90% of theoretical maximum), with a trend of increasing metabolic ethanol yields with increasing amounts of corn stover in the feedstock blend (Table 4). Glucose liberated by pretreatment and enzymatic hydrolysis was depleted during cofermentation. In contrast, noteworthy residual fractions of xylose were present in the fermentation broth upon conclusion of the SSCF (Figure 6). The attained xylose utilizations were in the range of 86-92% of the xylose liberated from the raw material in pretreatment and enzymatic hydrolysis.

The technical ethanol yields were in the range of 0.382-0.405 g·g⁻¹ (75-79% of theoretical maximum) with a uniform distribution across the range of feedstock blends (Figure 5). The deviating higher technical ethanol yield of WS50 was attributed to the distinguishable lower WIS content of the pretreated material, which have implications on the enzymatic hydrolysis and cofermentation. Limitations to the technical ethanol yields were entailed by the degree of glucan hydrolysis, incomplete utilization of xylose, and by-product formation.

The degree of hydrolysis of glucan and xylan after fed-batch SSCF was in the range of 83-93% and 69-84% (Table 4), respectively, which was comparable with those of the hydrolysability evaluations (Table 3). Similar to the hydrolysability evaluations, the degree of glucan hydrolysis exhibited a decreasing trend with increasing ratios of corn stover in the blend. The limited degree of xylan hydrolysis was of less importance for the technical ethanol yield, because most of the xylan was solubilized during the pretreatment for all feedstock blends (Figure 2). In addition, considerable amounts of undesirable by-products were formed. On average 0.198 g·g⁻¹ of xylitol, based on consumed xylose, and 0.04 g·g⁻¹ of glycerol, based on consumed glucose and xylose, were produced (Table 4). By-product formation was similar across the range of feedstocks and feedstock blends. The similar technical ethanol yields attained across the range of feedstock blends with fed-batch SSCF appeared to be the result of a tradeoff between decreasing hydrolysability and increasing fermentability of the pretreated feedstock with increasing amounts of corn stover in the feedstock blend.

3.2.2 Hybrid SSCF - with prehydrolysis

In hybrid SSCF all pretreated feedstocks and feedstock blends elicited ethanol titers that exceeded the desired 50 g·L⁻¹ (Figure 4). Glucose and xylose liberated during hybrid SSCF were converted at metabolic ethanol yields in the range of 0.443-0.457 g·g⁻¹ (87-90 % of theoretical maximum), and with greater consistency across the range of blends than with fed-batch SSCF (Table 4). Glucose liberated by pretreatment and enzymatic hydrolysis was depleted, but low residual xylose concentrations were present upon conclusion of the cofermentations (Figure 6). The xylose utilizations were in the range of 95-98% of total liberated xylose.

The attained technical ethanol yields were in the range of 0.414-0.433 g·g⁻¹ (81-85% of theoretical maximum), and consistent across the full range of feedstock blends (Figure 5). The technical ethanol yields were mainly limited by incomplete glucan hydrolysis and by-product formation. The degree of hydrolysis of glucan and xylan after fed-batch SSCF with prefermentation was in the range of 91-95% and 77-85% (Table 4), respectively; a significant improvement compared to the hydrolysability evaluations (Table 3) and fed-batch SSCF (Table 4). Similar to fed-batch SSCF, a decreasing trend in degree of glucan hydrolysis was obtained with increasing amounts of corn stover in the feedstock, effecting increasing amounts of residual glucan after

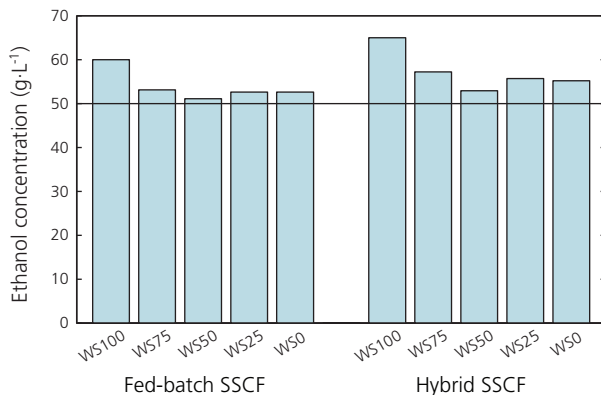


Figure 4. Ethanol titers after SSCF.

Attained ethanol concentrations in *fed-batch SSCF* and *hybrid SSCF* of dilute-acid catalyzed steam pretreatment of wheat straw, corn stover, and various blends thereof.

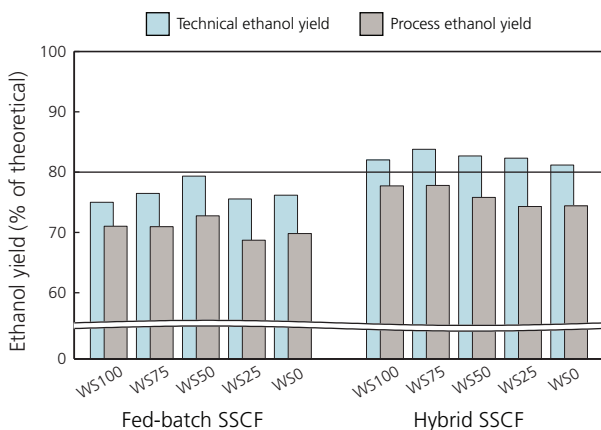


Figure 5. Technical and process ethanol yields after SSCF.

Attained technical and process ethanol yields after *fed-batch SSCF* and *hybrid SSCF* of dilute-acid catalyzed steam pretreatment of wheat straw, corn stover, and various blends thereof.

SSCF (Figure 6). Furthermore, potential ethanol yield was lost to by-products. Xylitol and glycerol production were uniform around $0.185 \text{ g}\cdot\text{g}^{-1}$ and $0.06 \text{ g}\cdot\text{g}^{-1}$, respectively. Although there were slight differences in xylitol and glycerol production compared to fed-batch SSCF, overall no significant differences in carbons diverted to the major by-products between the two strategies were obtained.

Ethanol titers and technical ethanol yield were higher across the range of feedstock blends with hybrid SSCF as compared to fed-batch SSCF (Figure 4 and Figure 5). The improvements were attributed to a combination of the improved efficacy of the

enzymatic hydrolysis and more consistent cofermentation performance. Whereas the metabolic ethanol yields and by-product formation were similar across the range of feedstock blends between the two cofermentation strategies, the degree of glucan and xylan hydrolysis and xylose utilization was significantly improved by the hybrid SSCF strategy (Table 4). The advantageous conditions for enzymatic hydrolysis, especially with regard to temperature, during the prehydrolysis step overcame the implied hydrolysis limitations entailed by the sub-optimal temperatures for enzymatic hydrolysis during SSCF. The degrees of glucan and xylan hydrolysis were more uniform across the range of feedstock blends and were improved by on average 5% and 10%, respectively, as compared with fed-batch SSCF (Table 4), thus eliciting lower residual glucan and xylan fractions after SSCF (Figure 6). The other major contributor to the increased technical ethanol yields was the improved utilization of liberated xylose (Table 4), which was improved by 7.5% on average.

3.3 Integration of process steps and process analysis

The processing steps for decomposition and conversion of lignocellulose to ethanol are often strongly interconnected. Changes in process parameters in one step can impact the overall process efficacy through indirect effects in the downstream process, potentially preventing the realization of industrially relevant ethanol titers and yields. Studies, therefore, must account for the dependencies within the process—from pretreatment to product.

The evaluation of the individual processing steps in the conversion chain showed that the efficacy of the pretreatment and the two different SSCF configurations was similar for wheat straw, corn stover, and blends thereof. The total yield of glucose and xylose obtained after pretreatment and enzymatic hydrolysis in our study were congruent with previously reported maximum sugar yields from dilute-acid catalyzed steam pretreated wheat straw and corn stover [14, 15, 17], which suggests that the applied uniform pretreatment condition were compatible with wheat straw, corn stover, and the blends thereof. In addition, comparable technical ethanol yields were attained across the full range of blends for each strategy (Figure 5). In despite, differences between different blends and SSCF strategies were obtained, which were mandated by the applied pretreatment conditions.

The pretreatment reduces the potential process ethanol yield that can be derived from the raw material, both directly by degrading sugars and indirectly by the attributes of the pretreated feedstock. Decreasing sugar recovery after pretreatment (Figure 2) and decreasing hydrolysability in SSCF (Figure 6) with increasing ratios of corn stover in the blends reduced the process ethanol yields systematically with either SSCF configuration (Figure 5). The process ethanol yields in fed-batch and hybrid SSCF were in the range of 187-195 g·kg⁻¹ and 202-213 g·kg⁻¹ of feedstock DM (69-72%

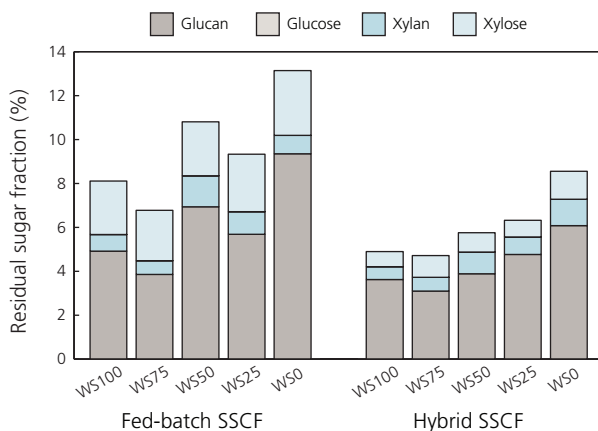


Figure 6. Residual glucose and xylose equivalents after SSCF.

Residual glucose and xylose equivalents in the WS and fermentation liquid after *fed-batch SSCF* and *hybrid SSCF*, expressed as percentage of total ingoing glucose and xylose equivalents in the pretreated feedstock in the SSCF.

Table 4. Summary of the SSCF results.

Hydrolysis and fermentation performance for the different SSCF configurations with various feedstock blends.

| | Fed-batch SSCF | | | | Hybrid SSCF | | | | | |
|---|----------------|-------|-------|-------|-------------|-------|-------|-------|-------|-------|
| | WS100 | WS75 | WS50 | WS25 | WS0 | WS100 | WS75 | WS50 | WS25 | WS0 |
| Hydrolysis Performance | | | | | | | | | | |
| Degree of glucan hydrolysis (%) | 0.90 | 0.93 | 0.88 | 0.88 | 0.83 | 0.93 | 0.95 | 0.94 | 0.91 | 0.91 |
| Degree of xylan hydrolysis (%) | 0.69 | 0.84 | 0.69 | 0.76 | 0.71 | 0.77 | 0.85 | 0.80 | 0.83 | 0.77 |
| Fermentation performance | | | | | | | | | | |
| Metabolic ethanol yield (g·g ⁻¹) ¹ | 0.424 | 0.430 | 0.456 | 0.443 | 0.460 | 0.443 | 0.457 | 0.447 | 0.455 | 0.451 |
| Glycerol production (g·g ⁻¹) ¹ | 0.04 | 0.05 | 0.04 | 0.04 | 0.04 | 0.05 | 0.05 | 0.06 | 0.05 | 0.06 |
| Xylitol production (g·g ⁻¹) ² | 0.21 | 0.19 | 0.18 | 0.20 | 0.21 | 0.18 | 0.18 | 0.19 | 0.17 | 0.20 |
| Xylose utilization (%) ³ | 92.5 | 89.2 | 91.6 | 86.1 | 88.8 | 97.9 | 94.8 | 97.0 | 95.2 | 96.6 |
| Technical ethanol yield (g·g ⁻¹) ⁴ | 0.382 | 0.395 | 0.405 | 0.385 | 0.388 | 0.418 | 0.433 | 0.422 | 0.416 | 0.414 |
| Process ethanol yield (g·kg ⁻¹) ⁵ | 195 | 194 | 199 | 187 | 190 | 213 | 213 | 207 | 202 | 202 |

¹ Based on consumed glucose and xylose

² Based on consumed xylose

³ Based on xylose liberated in pretreatment and enzymatic hydrolysis

⁴ Based on supplied glucose and xylose in the pretreated material

⁵ Based on supplied raw material.

and 74-78% of theoretical maximum), respectively, based on total glucose and xylose in the raw materials. The process ethanol yields in fed-batch SSCF were uniformly distributed (Figure 5), since the effect of decreasing recovery and hydrolysability was offset by increased fermentability (Table 4). In contrast, the process ethanol yields in hybrid SSCF exhibited a decreasing trend with increasing ratios of corn stover in the blend (Figure 5). Because of the uniform co-fermentation and hydrolysis

performance, the decreasing sugar recoveries after pretreatment were reflected in the process ethanol yield. Despite the detrimental effects on process ethanol yield entailed by the pretreatment, the process ethanol yields were surprisingly robust across the whole range of feedstocks and feedstock blends with either strategy. This suggests that wheat straw and corn stover could be used interchangeably, although the pretreatment conditions for corn stover and the blends might require fine tuning to maximize the liberation of fermentable sugars in SSCF. However, the use of wheat straw as single feedstock was favorable with either strategy because it elicited higher ethanol titers compared to the blends (Figure 4), which lower the energy demand in the product recovery step.

Despite the consistency in both technical and process ethanol yields with either strategy, the efficacy of the pretreatment had profound effect on the choice of SSCF configuration in the further processing steps. The attributes of the pretreated feedstock clearly favored hybrid SSCF, eliciting higher process ethanol yields. The restrictions entailed by the pretreatment prevented the realization of the desired technical ethanol yield with the fed-batch SSCF strategy (Figure 5). The technical ethanol yield with fed-batch SSCF was curtailed by inferior and variable degree of glucan and xylan hydrolysis (Figure 6). In contrast, the hybrid SSCF strategy could compensate for the tradeoffs made in the pretreatment conditions to allow for feedstock blends and reaching the desired ethanol titer and yields, making hybrid SSCF the preferred configuration for further processing.

Higher degree of hydrolysis in SSCF configurations employing prehydrolysis than in more conventional SSCF configurations has previously been observed for cofermentation of steam-pretreated *Arundo Donax* [24]. This indicates that strategies employing prehydrolysis are more robust with regard to enzymatic hydrolysis than conventional SSCF designs. These strategies combine the beneficial properties of a separate hydrolysis step, with reduced end-product inhibition during the subsequent SSCF step.

The applied pretreatment might have limited the possibilities to promote the cofermentation efficacy with the fed-batch SSCF strategy. The hydrolysis constraints to higher process ethanol yield with fed-batch SSCF could likely have been alleviated with higher severity in the pretreatment. Higher severity implies improved hydrolysability, but also lowers recovery of sugars and higher inhibitor concentrations in the pretreated feedstock. Technical ethanol yields exceeding 90% of theoretical maximum have previously been achieved with more severely steam-pretreated wheat straw with the same SSCF strategy and fermenting microorganism [26]. Technical ethanol yield of that magnitude could more than offset the decreased recovery of sugars, and, thus, increase the process ethanol yields. Notable is the differences in xylitol production between the studies, where 21% of consumed xylose was diverted away from ethanol production in our study versus 4.4% in the earlier study [26]. This

suggests that the pretreatment conditions that maximize the efficiency of the cofermentation and the process ethanol yield might be different from those that maximize recovery of fermentable sugars after pretreatment and enzymatic hydrolysis. It puts emphasis on the importance of clearly defining pretreatment goals when optimizing the process.

4. Conclusions

Ethanol titers and technical ethanol yields exceeding 50 g·L⁻¹ and 80% of theoretical maximum, respectively, were attained across the range of uniformly pretreated feedstocks and feedstock blends, and the process ethanol yields reached between 202 and 213 g·kg⁻¹ of feedstock DM (74-78% of theoretical maximum). Ethanol yields were maximized with a hybrid SSCF configuration that promoted enzymatic hydrolysis efficacy, thus overcoming the hydrolysis limitations imposed by the applied pretreatment. This study showed that the appropriate choice of SSCF configuration is heavily intertwined with the attributes of the pretreated material, which are mandated by the applied pretreatment method and conditions. This indicates that the biochemical conversion strategy needs to be matched with the pretreatment to maximize the liberation of fermentable sugars and process ethanol yield. The narrow range of ethanol yields attained with the different feedstock blends suggests that wheat straw and corn stover can be used interchangeably in various ratios with maintained performance, thus enabling hedging economic and supply chain risks.

List of abbreviations

DM: dry matter; FPU: filter paper unit; HMF: 5-hydroxymethylfurfural; HPLC: high-performance liquid chromatography; SSCF: simultaneous saccharification and cofermentation; vvm: gas volume flow per unit of liquid volume per minute; WIS: water-insoluble solids; XDH: xylitol dehydrogenase; XK: xylulokinase; XR: xylose reductase.

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One of the greater challenges that our society is facing today is the transition from an economy that is based largely on fossil raw materials towards a sustainable bio-based economy that relies on renewable raw materials. Second-generation bioethanol can have a role to play in this transition, reducing the fossil fuel dependence of the energy-intensive transportation sector. However, although second-generation bioethanol is recognized as a transportation fuel with important economic, environmental, and strategic attributes, it has not been widely commercialized. Several technological barriers remain for second-generation biofuel production, preventing it from being competitive with fossil fuels and first-generation bioethanol from sugar and starch crops.

This dissertation presents process developments for intensification of the lignocellulose-to-ethanol process. The dissertation addresses the need for improved conversion efficiency and an expanded feedstock base. It focuses on improving xylose utilization of xylose-fermenting *Saccharomyces cerevisiae* in cofermentation by improving the yeast phenotype during propagation and by applying novel feeding and fermentation strategies. Further, it delves into the feasibility of scaling up and employing feedstock blends with the devised strategies.