0273-2289/06/129–132/117–129/$30.00

Separate and Simultaneous Enzymatic Hydrolysis and Fermentation of Wheat Hemicellulose With Recombinant Xylose Utilizing *Saccharomyces cerevisiae*

L. Olsson,*[,1] H. R. Soerensen,[2] B. P. Dam,[1] H. Christensen,[1] K. M. Krogh,[1] and A. S. Meyer[1]

[1]*BioCentrum-DTU, Technical University of Denmark, DK-2800 Lyngby, Denmark, E-mail: lo@biocentrum.dtu.dk; and* [2]*Novozymes A/S, Krogshoejvej 36, DK-2880 Bagsvaerd, Denmark*

Abstract

Fermentations with three different xylose-utilizing recombinant *Saccharomyces cerevisiae* strains (F12, CR4, and CB4) were performed using two different wheat hemicellulose substrates, unfermented starch free fibers, and an industrial ethanol fermentation residue, vinasse. With CR4 and F12, the maximum ethanol concentrations obtained were 4.3 and 4 g/L, respectively, but F12 converted xylose 15% faster than CR4 during the first 24 h. The comparison of separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) with F12 showed that the highest, maximum ethanol concentrations were obtained with SSF. In general, the volumetric ethanol productivity was initially, highest in the SHF, but the overall volumetric ethanol productivity ended up being maximal in the SSF, at 0.013 and 0.010 g/Lh, with starch free fibers and vinasse, respectively.

Index Entries: Xylose conversion; ethanol; starch free fibers; vinasse.

Introduction

In Europe, wheat is one of the major feedstocks employed in the industrial production of ethanol for potable spirits, technical alcohol, and fuel ethanol. This production of ethanol is based on enzyme-catalyzed conversion of wheat endosperm starch to glucose with subsequent fermentation of glucose to ethanol by the yeast *Saccharomyces cerevisiae (1).* The wheat endosperm cell wall material is currently left behind as an unhydrolyzed, unfermented residue.

Wheat endosperm cell walls comprise various nonstarch polysaccharides notably (1→3)(1→4)-β-D-glucans, glucomannans, cellulose, and arabinoxylans as well as some protein. Arabinoxylans make up approx 70% by

*Author to whom all correspondence and reprint requests should be addressed.

weight of the wheat endosperm polysaccharides (2). Arabinoxylans are pentose polymers that consist of a backbone of (1→4)-linked β-D-xylopyranosyl residues with single α-arabinofuranosyl substituents attached to the C(*O*)-2, C(*O*)-3 or to both C(*O*)-2,3 of the xylose residues with the A : X ratio typically being approx 0.5 : 1 (3). The xylan backbone may additionally be substituted with α-D-glucopyranosyl uronic acid, its 4-*O*-methyl derivative, and/or acetyl groups (4). The arabinofuranosyl units may be esterified with ferulic acid and/or *p*-coumaric acid (5).

Utilization of the wheat endosperm cell wall polysaccharides in ethanol production will require both, (1) generation of a fermentable hydrolysate from the cell wall polysaccharides, notably from the arabinoxylan because of its dominance in these cell walls, (2) a microorganism able to utilize the resulting pentoses for ethanol production. Arabinoxylans are readily hydrolyzed to monosaccharides by acid treatment. However, acid hydrolysis unavoidably generates byproducts that inhibit the subsequent microbial fermentation (6). For this reason enzymatic hydrolysis is preferable. Furthermore, this method is considered to be a more economically viable in future bioethanol processes (7,8). Hence, production of ethanol from wheat endosperm cell walls using biological conversion includes both degradation of the polysaccharides by enzymes and microbial conversion of the monomeric sugars (hexoses and pentoses) to ethanol.

As a result of the complexity and heterogeneity of the arabinoxylan structure, complete enzymatic degradation into monosaccharides requires both side-group cleaving and depolymerizing enzyme activities (9). A synergistic interaction during degradation of soluble arabinoxylan using enzyme side-activities in a cellulase preparation from *Trichoderma reesei* (Celluclast 1.5 L) and a hemicellulosic enzyme preparation produced by *Humicola insolens* (Ultraflo L) has recently been identified (10,11). Depending on the degree of entanglement among the polysaccharide structures in the substrate, endoglucanases (EC 3.2.1.4) and β-glucanases (EC 3.2.1.6) may also be required in enzymatic degradation of wheat endosperm cell walls.

Traditionally, yeast, *S. cerevisiae* is widely used for ethanol production, however, it cannot ferment pentoses naturally. A number of natural pentose fermenting microorganisms have been identified and many efforts have been made to genetically engineer different microorganisms, for example, *S. cerevisiae*, *Zymomonas mobilis* and *Escherichia coli* to efficiently produce ethanol from both hexoses and pentoses (for recent reviews *see* refs. *[12–15]*). Because xylose is the dominant pentose monosaccharide in most lignocellulosic and hemicellulosic hydrolysates, identification and development of efficient xylose fermenting microorganisms have received most attention. Several recombinant, mutated, and evolved xylose-fermenting *S. cerevisiae* strains have thus been developed during the past 10–15 yr. In a recent report, *S. cerevisiae* F12 was identified as being attractive

for further investigation as a result of its high robustness to inhibitors in lignocellulosic hydrolysates *(16)*. *S. cerevisiae* F12 is an industrial strain engineered to use xylose for ethanol production by integration of the xylose reductase (XR) and xylitol dehydrogenase (XDH) encoding genes from *Pichia stipitis*, and by over expression of the endogenous xylulokinase (XKS) gene *(16)*. A major problem of recombinant, xylose fermenting *S. cerevisiae* is that the ethanol yield on xylose in many constructs is sub-optimal. Among many attempts to improve the xylose utilization in recombinant *S. cerevisiae*, two redox manipulated strains, *S. cerevisiae* CR4 (redox manipulated) *(17)* and CB4 (redox and ATP manipulated) *(18)* have been developed. These two strains were selected in addition to *S. cerevisiae* F12 for evaluation in the present study.

Two different process strategies can be taken in order to accomplish the enzymatic degradation of polysaccharides and the microbial conversion of the resulting monosaccharides to ethanol, either the process may be performed as a separate hydrolysis and fermentation (SHF) process or it may be performed as a simultaneous saccharification and fermentation (SSF) process. The main advantage of the SHF process is that both process steps can be carried out at their individual, optimal process conditions, whereas in the SSF process a compromise with respect to reaction conditions must be made. However, a main advantage of the SSF process is that the end products from the enzymatic hydrolysis are not building up as they are further converted by the microorganism. This conversion diminishes the effect of any eventual end-product inhibition in the enzymatic catalysis. Additionally, the SSF process can be accomplished in one process step.

The aim of the present study was to determine the efficiency of ethanol production from xylose in the two different processing strategies, SHF and SSF, on (1) starch free fibers in the stream before industrial ethanol fermentation and on (2) the wheat endosperm residue left behind after the industrial fermentation. A main objective was to assess these two process strategies and substrates, and to unravel any potential differences in the ethanol productivity and yield. A second objective was to compare the xylose conversion efficiency of three genetically engineered *S. cerevisiae* strains: CR4, CB4, and F12.

Materials and Methods

All chemicals were standard, analytical grade chemicals unless otherwise stated.

Strains

The strains *S. cerevisiae* CR4 (*MATαSUC2 MAL2-8c pADH-XYL1 pPGK-XYL2 pPGK-XKS1 gdh1ΔpPGK-GDH2*) *(17)* and *S. cerevisiae CB4* (*MATa SUC2 MAL2-8 pADH-XYL1 pPGK-XYL2 pPGK-XKS1 pTPIp-gapN) (18)* has been constructed in a laboratory strain background (*S. cerevisiae*

CEN.PK), whereas *S. cerevisiae* F12 (*HIS3::YIploxZEO overexpressing XR, XDH, and XKS)* was of an industrial strain background *(16)*.

Preparation of the Precultures

The *S. cerevisiae* strains were stored at –80°C in yeast peptone dextrose (YPD)-glycerol solution. The strain from the frozen stock was streaked on to YPD-agar plates and grown at 30°C. The agar plates were kept at 4°C.

A defined medium containing trace metal elements and vitamins was used for the precultures prepared according to *(19)*. The trace element solution and vitamin solution had the following compositions: Trace element solution: 15 g/L ethylenediaminetetraacetic acid (EDTA); 4.5 g/L $CaCl_2 \cdot 2H_2O$; 4.5 g/L $ZnSO_4 \cdot 7H_2O$; 3 g/L $FeSO_4 \cdot 7H_2O$; 1 g/L H_3BO_3; 0.84 g/L $MnCl_2 \cdot 2H_2O$; 0.3 g/L $CoCl_2 \cdot 6H_2O$; 0.3 g/L $CuSO_4 \cdot 5H_2O$; 0.4 g/L $Na_2MoO_4 \cdot 2H_2O$ and 0.1 g/L KI. The pH of the trace element solution was adjusted to 4.0 with NaOH and autoclaved. Vitamin solution: 50 mg/L d-biotin; 200 mg/L *para*-amino benzoic acid; 1 g/L nicotinic acid; 1 g/L Ca-pentothenate; 1 g/L pyridoxine· HCl; 1 g/L thiamine HCl and 25 g/L m-inositol. The pH was adjusted to 6.5 and the solution was stored at 4°C after sterile filtration. The medium used for the precultures had the following composition: 7.5 g/L $(NH_4)_2SO_4$; 14.4 g/L KH_2PO_4; 0.5 g/L $MgSO_4 \cdot 7H_2O$; 2 mL/L trace element solution; 1 mL/L vitamins solution and 0.05 mL/L antifoam 289 (Sigma A-8436, St.Louis, MO). For cultivations on glucose, the concentration was 10 g/L. A colony taken from an YPD-agar plate was inoculated into a 500 mL baffled Erlenmeyer flask containing 100 mL medium. The preculture was placed at 30°C and grown on a rotary shaker at 150 rpm for 24–36 h. The cells were harvested in the late exponential growth phase. Two shake flasks supplied enough cell mass for inoculation of one fermentor.

At harvest, the preculture was transferred to sterile centrifuge glasses and centrifuged at 18,000g for 5 min at 4°C and the supernatant was discarded. The cells were washed with sterile 0.9% sodium chloride solution and centrifuged again. The supernatant was removed and the cells were diluted into 50 mL media used for the fermentation (vinasse or starch free fiber hydrolysate).

Raw Materials

Both substrates, i.e., the starch free fibers and the still bottoms fermentation residue (vinasse), were sampled from an industrial starch processing plant (Tate & Lyle, Amylum, UK). The starch free fiber fraction was extracted from a sample of liquified wheat endosperm stream (WES) entering the conventional, industrial ethanol fermentation. During the starch process at the factory, the WES had undergone a brief hemicellulytic enzyme treatment (to lower the viscosity and ease the early starch separation), and an enzymatic liquifaction by treatment with α-amylase. Before

Table 1
Monosaccharide Composition of the Starch Free Fiber Hydrolysate and the Still Bottoms Fermentation Residue "Vinasse" Employed As Substrates in This Study[a]

Monosaccharide[a]	Starch-free-fibers g/kg DM[b]	Vinasse g/kg DM[b]
Arabinose	147 ± 2	91.4 ± 4.5
Galactose	17.6 ± 0.3	25.4 ± 1.5
Glucose	31.4 ± 0.4	76.9 ± 1.3
Xylose	213 ± 2	168 ± 6

Data are shown as the average of three determinations ± the standard deviation.
[a]The monosaccharide compostions were determined by HPAEC analysis after standardized acid hydrolysis of the substrates (at 0.4 *M* HCl for 2 h at 100°C). Data are shown as the average of three determinations ± the standard deviation.
[b]DM is dry matter.

use in our study, the WES was subjected to enzymatic saccharification in the form of a treatment with amyloglucosidase for 48 h at 50°C in order to hydrolyze the remaining starch and dextrins to glucose. The starch free fiber fraction was collected as the solid residue after separation of the saccharified material by filtration through a Munktell filter paper (quality 5). Immediately after this filtration, the solid starch free fiber residue was washed with four times its own volume of deionized water in order to remove remaining glucose. The vinasse constituted a sample of the ethanol fermentation residue left behind after the industrial fermentation of the WES. The dry matter content of the starch free fibers and vinasse were 18.8 and 9% by weight, respectively. Monosaccharide compositions are given in Table 1.

Separate Hydrolysis and Fermentation

In SHF, a complete enzymatic hydrolysis was performed before fermentation. In order to achieve a dry matter content of 5% (w/w), the raw materials were diluted with 0.025 *M* KH_2PO_4 buffer and pH was adjusted to 5.0 with NaOH. Each of the two substrates were then enzymatically hydrolysed for 24 h at 50°C, pH 5.0 with a 50:50 mixture of Ultraflo L (*Humicola insolens*, Novozymes A/S) and Celluclast 1.5 L (*Trichoderma reesei*, Novozymes A/S) at a dosage of 15% w/w enzyme/substrate dry matter (E/S). The enzymatic hydrolysis was stopped by boiling for 10 min. After this, the hydrolysates were cooled on ice until the fermentation was started. These hydrolysates were used for the experiments in which the SHF process was compared with the SSF process using *S. cerevisiae* F12. In the SHF experiments in which the performance of the three *S. cerevisiae* strains were compared using vinasse, a higher dry matter content 9.0% (w/w) and a lower enzyme loading 10% (w/w) were used for the enzymatic hydrolysis step.

Anaerobic batch cultivations were carried out in in-house manufactured 1 L fermenters. The fermentation substrate was added into a sterile fermenter to a volume of 800 mL and to prevent contamination 80 mg Penicillium V (Novo Nordisk) and 80 mg streptomycin sulfate (Sigma) were added to the substrates. The fermentations comparing the different strains were inoculated with 5 g/L dry weight and they were run for 45 h. For the experiments comparing *S. cerevisiae* F12 during SSF and SHF, the fermenter was inoculated to a cell mass concentration of 0.5–1 g biomass/L. These fermentations were run for 185 h.

The fermentations were run at 30°C, at a stirring speed of 300 rpm and with pH maintained at pH 5.0 with 2 *M* NaOH. The fermenter was continuously sparged with nitrogen at 0.02 L/min to maintain an anaerobic environment. The exhaust gas was led through a condenser.

Simultaneous Saccharification and Fermentation

Before the SSF, the substrates were heated to 95°C for 10 min. Because the enzymes were most efficient at 50°C, and the yeast is most efficient at 30°C, the process was started with a 2 h enzymatic hydrolysis at 50°C with the purpose of ensuring a rapid initial release of fermentable sugars. After 2 h of prehydrolysis, the temperature was lowered to 30°C, and the hydrolysate (including the enzymes) was transferred to the fermenter, whereafter the cell mass was added as described earlier. The SSF was performed at 30°C, pH 5.0 and with an enzyme dosage of (50:50 mixture of Ultraflo L and Celluclast 1.5 L) 15% w/w E/S dry matter.

Sampling and Analysis

Samples were taken regularly from the fermenter. SHF samples were centrifuged at 10,000g for 1 min before filtration through a 0.45-μm filter. SSF samples were treated in two different manners, the samples, which were to be analysed by high performance anonic exchange chromatography (HPAEC) (*see* later) were centrifuged at 10,000g for 1 min, filtered, placed in boiling water for 10 min to inactivate the enzymes in the samples. Samples for high performance liquid chromatography (HPLC) analysis (described later) were centrifuged at 10,000g for 1 min, filtered, and diluted 1:1 with a 10 m*M* H_2SO_4 solution. All samples were stored at –20°C until analysis.

The monosaccharide composition and concentration were determined by HPAEC as described previously (*see* ref. *10*). The content of xylitol, lactate, acetate, glycerol, and ethanol were determined by HPLC on a Waters (Milford, MA) HPLC system that encompassed a Waters 717 Plus autosampler, a Waters 515 pump, a Waters 2410 RI detector, and was equipped with a precolumn, cation-H 30 × 4.6 mm from Bio-Rad (Hercules, CA), and two serially placed Bio-Rad Aminex HPX 87-H columns (held at 60°C). The eluent used was 5 m*M* H_2SO_4 at 0.40 mL/min.

Results

Comparison of the Performance of Three Recombinant Xylose Fermenting S. cerevisiae *Strains*

The performance of the three, metabolically engineered, xylose fermenting *S. cerevisiae* strains, CB4, CR4, and F12 were followed during fermentation of enzymatically prehydrolyzed vinasse. All three strains converted all the present glucose within the first few hours of fermentation, and as a result, the main ethanol production took place during this period (Fig. 1A–C). All three strains were able to convert some of the xylose, but at varying xylose consumption rates and with different ethanol productivities: the CB4 strain converted the xylose very slowly, and the conversion of xylose ceased after approx 24 h of fermentation. As a result, only about one third of the xylose was utilized, during the 45 h of fermentation. Only limited amounts of xylitol were produced by this strain, and the maximum ethanol concentration achieved was 3.1 g/L (Fig. 1A). In contrast, both the CR4 and the F12 strain converted almost 90% of the xylose during the 45 h of fermentation, and maximum ethanol concentrations of 4.3 g/L and 4.0 g/L, respectively, were achieved. The volumetric ethanol productivities achieved during the first 24 h of the fermentations were 0.17 and 0.16 g/Lh for CR4 and F12, respectively. The F12 strain produced higher xylitol levels than the CR4 (final concentration of 1.1 g/L vs 0.7 g/L, respectively Fig. 1B and C). However, a comparison of the rate of xylose consumption revealed that the F12 converted the xylose about 15% faster than the CR4. Thus, assuming a linear conversion rate during the first 24 h of the fermentations, the rates of xylose consumption, (from linear regression of the xylose data) gave 0.17 g/Lh for F12 and 0.15 g/Lh for CR4. This slightly better performance in xylose conversion by F12, coupled with its industrial strain background and previously demonstrated high robustness, led us to choose this strain for further study in the different SHF and SSF fermentations.

Comparison of SHF and SSF in Vinasse and Starch Free Fibers Using S. cerevisiae *F12*

Initial Glucose and Xylose levels

The initial glucose levels in the fermentation substrates ranged from 1.1 to 4 g/L, whereas the initial xylose levels were higher, and ranged from 2.7 to 6.2 g/L in the different fermentation substrates (Table 2). Irrespective of enzymatic prehydrolysis, the initial glucose concentrations were generally twofold higher in the starch free fibers than in the vinasse (Table 2). The higher glucose level in the starch free fibers could be a result of incomplete glucose removal by filtration and washing after the saccharification treatment during their preparation (*see* Materials and Methods). In accordance with the expectation that both some β-glucan and cellulose

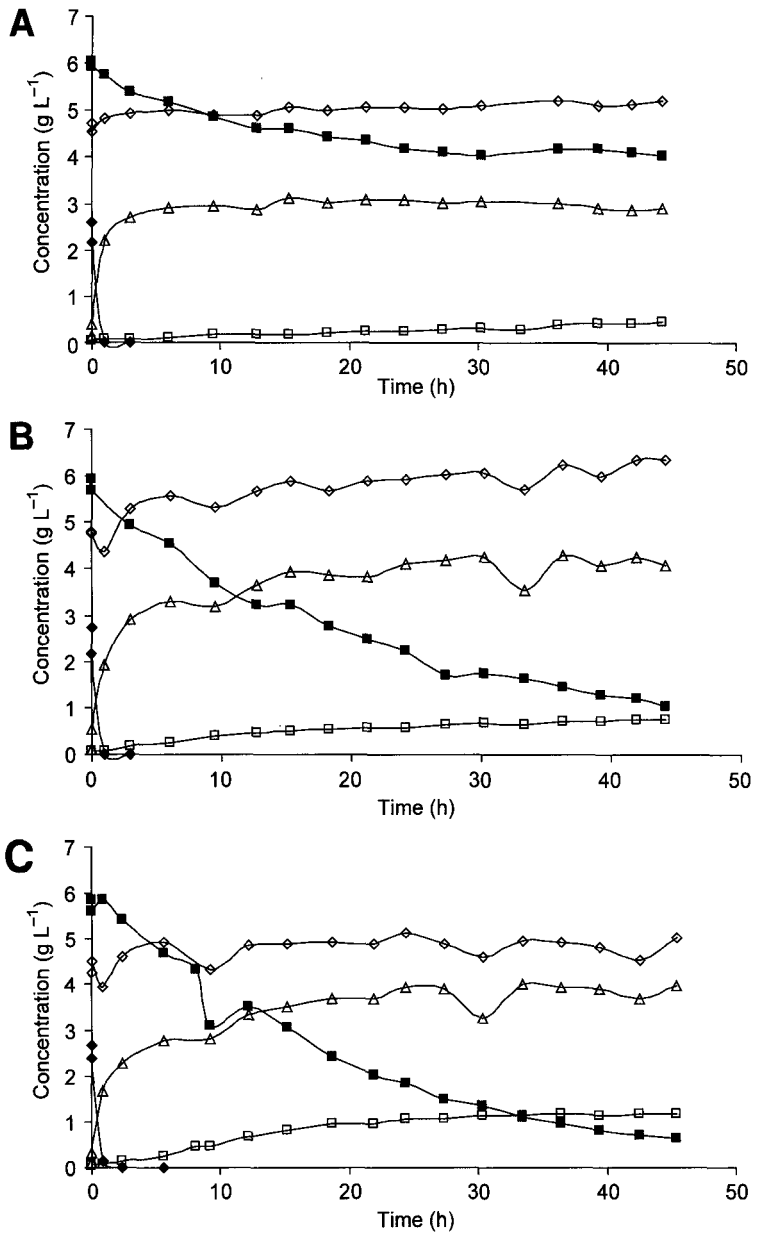

Fig. 1. Time courses for SHF fermentations on vinasse with different recombinant, xylose fermenting *S. cerevisiae* strains. **(A)** *S. cerevisiae* CB4 **(B)** *S. cerevisiae* CR4 **(C)** *S. cerevisiae* F12. Symbols used: ◆ glucose; ■ xylose; △ ethanol; □ xylitol; ◇ glycerol. Before fermentation the vinasse substrate had been enzymatically treated with a 50 : 50 mixture of Ultraflo L and Celluclast added at a dosage of 10% w/w E/S dry matter.

would be hydrolysed during the enzymatic treatment in the separately hydrolyzed samples, the initial glucose levels were higher in both types of the SHF samples than in the SSF substrates at the start of the fermentation (Table 2). The xylose levels in the SHF substrates were similar, but twofold

Table 2
Summary of Fermentation Performance Results Using *S. cerevisiae* F12

		Vinasse SHF	Vinasse SSF	Starch free fibers SHF	Starch free fibers SSF
Glucose concentration	at start[a]	2.3	1.1	4.0	2.0
Xylose concentration	at start	5.6	2.7	6.2	3.3
	at end	0.9	1.4	1.9	2.2
Ethanol concentration	at start[b]	0	0	0.14	0
	at max.	2.0 (after 88 h)	2.1 (after 88 h)	2.0 (after 40 h)	2.8 (after 185 h)
Xylitol	at start	0	0	0	0
	at end	0.5	1.0	1.2	0.8
Glycerol	at start	3.8	3.7	0.7	0.6
	at end	4.1	4.4	1.2	0.9
Acetate	at start	1.2	1.2	0.9	0.9
	at end	1.3	1.2	1.0	0.9
Lactate	at start	6.0	6.0	0.4	0.3
	at end	5.5	5.7	0.1	0.1
Volumetric ethanol production g/Lh	Phase 1 (0–3 h)	0.40	0.28	0.63	0.35
	Phase 2 (3–88 h)	0.009	0.015	0.003	0.01
Specific ethanol productivity[c] g/gh	Phase 1 (0–3 h)	0.24	0.18	0.74	0.44
	Phase 2 (3–88 h)	0.005	0.010	0.003	0.013

Concentrations are given as g/L and the fermentation were run for 185 h.
[a]The final glucose concentration at the end of the fermentation was 0.0 g/L in all cases; glucose was used up within the first 3 h of the fermentation.
[b]The first ethanol measurement was made 5 min. after inoculation.
[c]Productivity: calculations for the specific ethanol productivities were based on the amount of final cell mass concentration.

higher than the levels of those in the SSF substrates (Table 2). During the fermentations, the glucose consumption rates were high and no glucose was measurable after 3 h of fermentation. In contrast, the xylose was consumed more slowly in all the four fermentations, and xylose was not fully consumed at the end of the fermentations in either of the cases (Table 2).

Ethanol Production Levels and Total Specific Ethanol Productivities

The maximal ethanol level obtained was 2–2.1 g/L except in the simultanously saccharified and fermented starch free fibers, in which the maximal ethanol level reached 2.8 g/L (after 185 h) (Table 2). As expected, the total volumetric ethanol productivity was in all four cases highest in the

first few hours of the fermentation, when the glucose was consumed. The volumetric ethanol productivities during the first few hours of fermentation ranged from 0.28 to 0.63 g/L·h and were highest in the separately hydrolyzed starch free fibers fermentation (starch free fibers SHF, Table 2). These rates corresponded to specific ethanol productivity rates of 0.18–0.74 g/g·h (when using the final level of cell mass for the calculations). The specific ethanol productivity rates were consistently higher in the starch free fibers fermentations than in the vinasse fermentations, with the rate in the starch free fibers SHF being the highest (Table 2). In all cases, the initial glucose was consumed during the first 3 h of the fermentation, and after this period, the volumetric ethanol productivities fell drastically to 0.003–0.015 g/L·h in all four types of fermentation (Table 2). The rates in phase 2 allowed a comparison of the ethanol production from xylose, and because the volumetric ethanol productivity was highest in phase 2 in both vinasse fermentations, the data indicated that xylose was better fermented to ethanol in the vinasse than in the starch free fibers substrate.

Production of Byproducts

Not surprisingly, the initial glycerol levels, relating to microbial biomass production, were much higher in the vinasse fermentations than in the starch free fibers fermentations, as vinasse is a post fermentation waste stream. In all four fermentations, the glycerol levels increased marginally from 0.3 to 0.7 g/L, from start to end of the fermentation. As discussed earlier, xylitol was also produced as a byproduct during the fermentation, and the xylitol concentrations reached levels of 0.5–1.2 g/L with no clear dependence of the substrate or process type (Table 2). The initial lactate levels were relatively high in the vinasse samples (6 g/L), but modest in the starch free fibers (approx 0.3 g/L). The higher levels of lactate found in the vinasse showed that lactate production had taken place before recovering the vinasse residue. This lactate production was probably as a result of growth of lactic acid bacteria as contaminants immediately before the industrial ethanol fermentation or during the industrial fermentation process itself, which is common in industrial ethanol fermentations *(20)*. Small decreases in the lactate concentration were observed (Table 2). The initial acetate levels were also slightly higher in the vinasse than in the starch free fibers substrate samples. However, the acetate levels remained constant throughout all fermentations.

Discussion

Components other than fermentable monosaccharides are often present during fermentation of industrial substrates, originating as residual products from food and agroindustrial processes, such as in vinasse. The presence of these components may negatively influence the fermentation yield and productivity. The three *S. cerevisiae* strains F12, CB4, and CR4, which all perform well in a laboratory medium *(16–18)*, could ferment in

vinasse. However, the performance of the strain *S. cerevisiae* CB4 during xylose utilization was poor and the utilization of xylose completely stopped before the fermentation was terminated. As also indicated in an earlier study *(17)*, recombinant strains with decreased ATP yield, such as the *S. cerevisiae* CB4 strain, in which the adenosine triphosphate (ATP) generation has been weakened with a nonATP generating step, encounter problems during xylose fermenting. These problems can get severe, as illustrated in the present study, when fermenting industrial substrates containing components other than monosaccharides, which put further demands on the cellular metabolism. Vinasse contains components like acetate, glycerol, and lactate (Table 2) in addition to monosaccharides. Acetic acid, and to a lesser extent lactic acid, inhibits *S. cerevisiae* because they can diffuse into the cells in their undissociated form, thereby lowering the intracellular pH, which in turn leads to higher ATP requirement for maintenance *(21,22)*. The occurrence of this series of events can explain the bad performance of *S. cerevisiae* CB4.

Comparing the fermentation performance of the *S. cerevisiae* strain F12 with CR4, showed that the redox modulated strain had the lowest production of xylitol resulting in a slightly higher maximum ethanol concentration. Several other studies have also shown that redox modulation of strains can improve the ethanol production from xylose, when the XR-XDH-XKS system is used for introducing xylose utilization in *S. cerevisiae* *(23–25)*.

During the fermentation process glucose was taken up preferentially before xylose, and furthermore, the glucose uptake was a magnitude higher than the xylose uptake both during fermentation of vinasse (Fig. 1), and during fermentation of laboratory medium *(26)*. It has been found that during fermentation of recombinant xylose utilizing *S. cerevisiae* that the specific xylose consumption rate is highest during coconsumption of glucose, after which the rate declines by time *(26)*. Based on this earlier observation, one can make the hypothesis that an SSF process might be advantageous as the monosaccharides are released throughout the process, which might support a coconsumption of glucose and xylose, which based on earlier experience would allow faster xylose consumption. From the present study, it can be concluded that using SSF as process configuration is advantageous as higher final ethanol concentrations were reached on both substrates (Table 2). However, more residual xylose was found in SSF fermentations in comparison with SHF fermentations at the time the fermentation was terminated, indicating that the xylose consumption was not improved in SSF in comparison with SHF.

The most important difference between the two substrates used, vinasse and starch free fibers, was that vinasse contained significantly higher levels of lactate and glycerol than starch free fibers. Organic acids are known to be inhibitory to *S. cerevisiae* as discussed earlier. In the present study, two to threefold higher specific ethanol productivites in phase 1

were found on the starch free fibers substrate as compared with the vinasse both for the SSF and the SHF process, respectively, confirming that the starch free fibers substrate was less inhibitory to *S. cerevisiae* than vinasse was.

In conclusion, the three investigated strains could all ferment xylose from the two evaluated substrates, however, the ethanol productivity on xylose was slow in comparison with utilization of glucose both using SHF or SSF. There are recent reports, were xylose consumption rates have been improved in recombinant xylose fermenting *S. cerevisiae* strains, by using metabolic engineering and/or random mutagenesis *(27,28)*, these strains will probably lead to better performance under SSF conditions. In this work, the SSF strategy on starch free fibers appeared to be the best for obtaining maximum ethanol levels and highest ethanol productivity with the F12 strain. Higher specific ethanol productivities in phase 1 were reached using starch free fibers than vinasse, and the difference is most likely as a result of higher amounts of inhibitory compounds in the latter. To construct an efficient xylose fermenting strain, both the strain background and the metabolic engineering strategy have to be taken into account in relation to the substrate it will be fermenting, i.e., influence on metabolism by potential inhibitory components in the applied substrate.

Acknowledgment

We wish to thank Beatriz Palmarola-Adrados, Lund University, Sweden, for assistance in preparing the starch free fibers.

References

1. Lewis, S. M. (1996), Fermentation alcohol. In: *Industrial Enzymology.* 2nd ed., Godfrey, T., West, S., (eds.), MacMillan Press Ltd, London UK, pp. 11–48.
2. Bacic, A. and Stone, B. A. (1980), *Carbohydr. Res.* **82,** 372–377.
3. Izydorczyk, M. S. and Biliaderis, C. G. (1993), *Cereal Chem.* **70,** 641–646.
4. Smith, M. M. and Hartley, R. D. (1983), *Carbohydr. Res.* **118,** 65–80.
5. Ishii, T. (1997), *Plant Sci.* **127,** 111–127.
6. Saha, B. C. (2000), *Biotechnol. Adv.* **18,** 403–423.
7. Sheehan, J. and Himmel, M. E. (1999), *Biotechnol. Prog.* **15,** 817–827.
8. Wooley, R., Ruth, M., Glassner, D., and Sheehan, J. (1999), *Biotechnol. Prog.* **15,** 794–803.
9. de Vries, R. P., Kester, H. C. M., Poulsen, C. H., Benen, J. A. E., and Visser, J. (2000), *Carbohydr. Res.* **327,** 401–410.
10. Sϕrensen, H. R., Meyer, A. S., and Pedersen, S. (2003), *Biotech. Bioeng.* **81,** 726–731.
11. Sϕrensen, H. R., Pedersen, S., Viksϕ-Nielsen, A., and Meyer, A. S. (2005), *Enz. Microb. Technol.* **36,** 773–784.
12. Ingram, L. O., Gomez, P. F., Lai, X., et al. (1998), *Biotech. Bioeng.* **58,** 204–214.
13. Aristidou, A. and Penttilä, M. (2000), *Curr. Opin. Biotechnol.* **11,** 187–198.
14. Hahn-Hägerdal, B., Wahlbom, C. F., Gardonyi, M., van Zyl, W., Cordero-Otero, R., and Jönsson, L. F. (2001), *Adv. Biochem. Eng. Biotechnol.* **73,** 53–84.
15. Zaldivar, J., Nielsen, J., and Olsson, L. (2001), *Appl. Microbiol. Biotechnol.* **56,** 17–34.
16. Sonderegger, M., Jeppsson, M., Larsson, C., et al. (2004), *Biotech. Bioeng.* **87,** 90–98.
17. Roca, C., Nielsen, J., and Olsson, L. (2003), *Appl. Environ. Microbiol.* **69,** 4732–4736.

18. Bro, C., Regenberg, B., Forster, J., and Nielsen, J. In silico aided metabolic engineering of *Saccharomyces cerevisiae, Metab. Eng.*, in press.
19. Verduyn, C., Postma, E., Scheffers, W. A., and van Dijken, J. P. (1990), *J. Gen. Microbiol.* **136,** 395–403.
20. Stenberg, K., Galbe, M., and Zacchi, G. (2000), *Enzyme Microb. Technol.* **26,** 71–79.
21. Maiorella, B., Blanch, H. W., and Wilke, C. R. (1983), *Biotechnol. Bioeng.* **23,** 103–121.
22. Pampulha, M. E. and Loureiro-Dias, M. C. (1989), *Appl. Microbiol. Biotechnol.* **31,** 547–550.
23. Jeppsson, M., Johansson, B., Hahn-Hägerdal, B., and Gorwa-Grauslund, M. F. (2002), *Appl. Environ. Microbiol.* **68,** 1604–1609.
24. Jeppsson, M., Träff, K., Johansson, B., Hahn-Hägerdal, B., and Gorwa-Grauslund, M. F. (2003), *FEMS Yeast Res.* **3,** 167–175.
25. Verho, R., Londesborough, J., Penttilä, M., and Richard, P. (2003), *Appl. Environ. Microbiol.* **69,** 5892–5897.
26. Roca, C., Haack, M. B., and Olsson, L. (2004), *Appl. Microbiol. Biotechnol.* **63,** 578–583.
27. Wahlbom, C. F., van Zyl, W. H., Jönsson, L. J., Hahn-Hägerdal, B., and Cordero Otero, R. R. (2003), *FEMS Yeast Res.* **3,** 319–326.
28. Kuyper, M., Hartog, M. M. P., Toirkens, M. J., et al. (2005), *FEMS Yeast Res.* **5,** 399–409.