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Comparison of SHF and SSF processes for the bioconversion of steam-exploded wheat straw

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Two processes for ethanol production from wheat straw have been evaluated — separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). The study compares the ethanol yield for biomass subjected to varying steam explosion pretreatment conditions: temperature and time of pretreatment was 200°C or 217°C and at 3 or 10 min. A rinsing procedure with water and NaOH solutions was employed for removing lignin residues and the products of hemicellulose degradation from the biomass, resulting in a final structure that facilitated enzymatic hydrolysis. Biomass loading in the bioreactor ranged from 25 to 100 g l^{-1} (dry weight). The enzyme-to-biomass mass ratio was 0.06. Ethanol yields close to 81% of theoretical were achieved in the two-step process (SHF) at hydrolysis and fermentation temperatures of 45°C and 37°C, respectively. The broth required addition of nutrients. Sterilisation of the biomass hydrolysate in SHF and of reaction medium in SSF can be avoided as can the use of different buffers in the two stages. The optimum temperature for the single-step process (SSF) was found to be 37°C and ethanol yields close to 68% of theoretical were achieved. The SSF process required a much shorter overall process time (≈30 h) than the SHF process (96 h) and resulted in a large increase in ethanol productivity (0.837 g l^{-1} h^{-1} for SSF compared to 0.313 g l^{-1} h^{-1} for SHF). *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 184–192.

Keywords: SSF; SHF; steam explosion pretreatment; lignocellulosic biomass

Introduction

Wheat straw is an abundant source of biomass, readily available as agricultural residue. Because its cellulose content is high — roughly 34 wt.% dry weight — it is a good source of sugar in the bioprocess for ethanol for use as a fuel extender. Wheat straw hydrolysates are considered important to Europe's economy in contrast to that of the USA where corn stover and bagasse are mainly used. Direct combustion in ovens is another possible energetic utilisation of wheat straw but is less attractive from an economic point of view.

The bioconversion of lignocellulosic biomass to ethanol occurs in two steps. The cellulosic chain is first depolymerised to yield the monomeric unit, glucose, which is subsequently fermented to produce ethanol. The enzymatic complex cellulase that catalyses the hydrolysis usually operates with highest efficiency at temperatures of at least 45°C, and at a pH of 4.8 in 50 mM Na-acetate buffer. In contrast, the optimum temperature for the fermentation of glucose to ethanol with the commercial yeast, *Saccharomyces cerevisiae*, is reported to be 37°C or less, at a pH of 5.0 in 100 mM phosphate buffer [5]. Sterility precautions and the addition of salts and other components, such as yeast extract, could be required for the fermentation broth. Efforts over recent years have therefore been directed at the optimisation of the separate hydrolysis and fermentation (SHF) process performed under varying bioconversion conditions. However, the glucose produced during biomass saccharification strongly inhibits the cellulase activity, particularly the β-glucosidase component that catalyses the hydrolysis of cellobiose to glucose. Cellulases are also inhibited by cellobiose. These factors result in a large reduction in reaction rate [2,12].

In the simultaneous saccharification and fermentation (SSF) of lignocellulosic biomass, on the other hand, the glucose produced can be rapidly converted to ethanol by the yeast. This continuous removal of inhibitors from the reaction medium minimises the depression of enzyme activity [5,7,11], thereby offering the possibility of high production rates. A further advantage of SSF over the sequential process (SHF) is the cost reduction resulting from use of only one bioreactor.

The aim of this study was to compare SHF and SSF yields for wheat straw bioconversion. Because pretreatment is of great importance to the ethanol yield, the biomass was first processed in a steam explosion plant at different temperatures and for different times. This physical process, based on the combined effect of steam and pressure release, disrupts the lignin barrier and enhances accessibility of cellulose fibres to enzymatic attack. Steam explosion is one of the most attractive pretreatment processes due to its low use of chemicals, low energy consumption, and efficient biomass disruption characteristics [6,10,16]. Hemicellulose and lignin can be removed quite easily following steam explosion. The final structure of the biomass presents larger pores and higher surface area than it did before treatment. This facilitates the hydrolysis catalysed by enzymes. In the present study, the importance of both pretreatment conditions and process conditions (biomass loading, composition of fermentation broth, temperature, enzyme-to-biomass ratio) was investigated and the SSF and SHF processes were compared with regard to product formation.

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Materials and methods

Biomass pretreatment and composition

The wheat straw was pretreated in a batch plant located at the Research Centre of ENEA (Trisaia, Italy). A fixed amount of biomass, 0.5 kg, was processed in each cycle. The steam temperature was 200°C or 217°C. Pretreatment time was for 3 or 10 min. The "severity parameter" (R_0) was used to map the destruction, disaggregation, and depolymerization of wheat straw. R_0 was calculated using the relation reported in Refs. [1,14]: $R_0 = t \exp[(T-100)/14.75]$, where T=temperature (°C) and t=time (min). The pretreated biomass was then thoroughly washed with NaOH solutions and distilled water in order to remove accidental precipitates of non-constitutive lignin and hemicellulose as follows: the biomass (roughly 30 g l^{-1}) was first stirred for 30 min at 65°C in NaOH solution at 1.5 g l^{-1}; the suspension was subsequently filtered (using Whatman filter paper 541) and the solid was resuspended a second time in NaOH under the same conditions; this procedure was repeated three times at NaOH concentrations of 0.15, 0.015, and 0.00015 g l^{-1}, and then twice more in distilled water before filtering the mixtures.

The acid-soluble fraction of lignin in the biomass was determined by UV spectrophotometry at 205 nm following the procedure TAPPI Useful Methods 250. The residual lignin in the fibres and the carbohydrate content were determined using modified TAPPI T13 m-54. The carbohydrate content (the sum of cellobiose, glucose, xylose, and arabinose) was determined by HPLC. Glucose and cellobiose were assumed to be products of cellulose hydrolysis, and xylose and arabinose to be products of hemicellulose hydrolysis. Chromatographic procedures and conditions were the same as those reported below in the *Product determination* section.

The composition of native and steam-exploded biomass thoroughly washed with NaOH solutions and distilled water is reported in Table 1. The ash content (ASTM-1106, modified) and the extractive fraction of the native wheat straw were 7.6% and 6.7%, respectively. The biomass was stored at 120°C for 48–72 h in a convection oven before the dry weight was measured. All determinations were made in duplicate. The water content of the biomass, collected in the final stage of pretreatment, amounted to 75 wt.% on average.

Enzymatic hydrolysis

Two enzymatic preparations, available under the trade names X70L and C80L, by SAF-ISIS (France) were used. Protein contents (31 g l^{-1} in X70L and 279 g l^{-1} in C80L) were measured by the Lowry–Hartree method [9], using crystalline serum albumin as standard. β-Glucosidase was assayed using a final concentration of 10 mM cellobiose (Sigma, USA). Endoglucanase, exoglucanase, and xylanase activities were assayed using a final concentration of 0.25% w/v carboxymethylcellulose (Sigma), Avicel (Machery Nagel, Germany), and xylan (Sigma), respectively. The FPase activity was measured using filter paper Whatman no. 1 (1×6 cm^2 in 1 ml). Specific enzyme activities, enzymatic units per milligram of protein, are quoted in Table 2. Units are expressed as the amount of enzyme that catalyses the formation of 1 μmol of reducing sugar per minute at 45°C in 50 mM Na-acetate buffer pH 4.8.

A jacketed vessel of 350 ml working volume was used as the cellulose hydrolysis reactor. The hydrolysis temperature (45°C) was regulated by means of a water jacket attached to a circulating water bath. Stirring of the reaction medium (250 rpm) was provided by magnetic bars. Hydrolysis pH was kept to 4.8 with 50 mM Na-acetate buffer. These conditions have been shown to be optimal in previous experiments on the activity and storage stability of the two cellulase complexes [17]. Two runs were carried out under each set of experimental conditions.

Fermentation conditions

Control fermentations were carried out in duplicate using 300 ml Erlenmeyer flasks at 37°C in a New Brunswick G25-KC controlled environment incubator shaker (220 rpm) (New Brunswick Scientific, Edison, NJ, USA). The fermentation broth (100 ml) contained yeast extract (2.5 g l^{-1}) from Oxoid (England), $(NH_4)_2HPO_4$ (0.25 g l^{-1}) and $MgSO_4 \cdot 7H_2O$ (0.025 g l^{-1}) from Aldrich (England). The pH was initially adjusted to 5.0 with 100 mM phosphate buffer. The flasks were autoclaved for 15 min at 121°C. Sterile glucose (60 g l^{-1}) was then added aseptically. The fermenters were inoculated with dry *S. cerevisiae* (Baker's yeast, type I) from Sigma at 3 g l^{-1} (dry weight), added directly. The same conditions and procedures were adopted for the fermentation of the biomass hydrolysate, using 50 mM Na-acetate buffer pH 4.8 in place of the phosphate buffer. All experiments and controls were performed in duplicate. At determined time intervals, at least two samples were withdrawn aseptically and used for determining the chemical concentrations.

SSF experiments

SSF experiments were carried out in 300 ml flasks stirred at 220 rpm conforming to 100 ml medium for 50 h. The temperature was maintained at 37°C and the pH was initially adjusted to 4.8 with 50 mM Na-acetate buffer. The weight percentage of pretreated substrate was 10% based on oven-dried material. The base composition of the SSF medium was: 3 g l^{-1} dry yeast cell (*S. cerevisiae*, Baker's yeast, type I from Sigma), enzyme loadings were 30 and 60 mg g biomass^{-1}, 2.5 g l^{-1} yeast extract, 0.25 g l^{-1} $(NH_4)_2HPO_4$, 0.025 g l^{-1} $MgSO_4.7H_2O$. The SSF experiments were performed under non-sterile conditions. To determine rates of

Table 1 Weight percentage of lignocellulosic components in native and steam-exploded wheat straw thoroughly washed with NaOH solutions and distilled water

logR_0	Temperature (°C)	Time (min)	Cellulose (wt.%)	Hemicellulose (wt.%)	Lignin (wt.%)
(Native)			34.3	25.70	21.60
3.42	200	3	48.6	36.60	18.98
3.93	200	10	60.8	17.15	22.14
3.94	217	3	65.3	11.51	18.16
4.45	217	10	59.4	9.20	26.50

Effect of pretreatment conditions.

Table 2 Specific activities in the cellulase complexes

	β-Glucosidase	Endoglucanase	Exoglucanase	Xylanase	FPase
X70L	0.39	5.74	0.33	13.90	0.29
C80L	0.42	6.02	0.29	0.53	0.31

Enzymatic units per milligram of protein.

hydrolysis and fermentation, samples were withdrawn periodically for sugar and ethanol analyses.

Product determination

In hydrolysis studies, the total reducing sugars were measured by means of the Somogyi–Nelson colorimetric method [13] employing glucose as standard. Glucose was determined using GOD-Perid kit supplied by Boehringer Mannheim (Germany). In SSF and fermentation studies, glucose and ethanol were analysed by HPLC (Perkin Elmer, USA) with a refractive index detector (LC-25, Perkin Elmer) and a Supelcogel CA column (300 mm × 7.8 mm) from Supelco (USA). The column temperature was regulated at 70°C by means of a column heater (Bio-Rad, USA). Double-distilled and degassed water was used as the mobile phase at a flow rate of 0.4 ml min^{-1}. Samples (1 ml) were collected from the broth and centrifuged. An aliquot of supernatant (100 μl) was subsequently deproteinised with the addition (1 ml) of $HClO_4$ solution (2.0 wt.%). The mixture was centrifuged at 11,000×*g* for 10 min at 15°C and the supernatant was removed for the analyses.

Results and discussion

SHF

Effect of pretreatment conditions and enzyme composition: A series of experiments was first carried out to study the effect of enzyme activity balance and severity factor on the rate and extent of hydrolysis of wheat straw. In these experiments, the reaction conditions were the same as described previously. The bioreactor was loaded with 25 g l^{-1} (dry weight) of biomass. The yields of reducing sugars and glucose, based on both the biomass (g_b) and the mass of cellulose (g_c) in the biomass, were calculated for 24 and 48 h of incubation. Composites of the two enzymic preparations were prepared and added to the hydrolysis reactor (1.5 g l^{-1}). The weight percentages of X70L and C80L were varied in the range 0–100 wt.%. This procedure was found in previous studies [3,8,15] to result in a good enzyme activity balance. The amount of reducing sugars was measured on a molar basis because of the difficulty in performing a complete analysis of all the oligosaccharides. Besides, sugars other than glucose are less important as they cannot be fermented by the yeast used in this study. Their determination was nevertheless significant for the exact calculation of process selectivity by the molar mass balance. The results of a comparison of hydrolysis yields at different compositions of cellulase mixture and severity parameters are shown in Table 3. Several observations can be made. The composition of the enzymatic mixture did not significantly affect the saccharification yield, the differences being within the limits of experimental error. This is true both for 24 and 48 h of hydrolysis, and for both reducing sugar and glucose yield. The only exception was for the case of biomass hydrolysis following pretreatment at the lowest severity parameter, $\log R_0$=3.42. The results suggest that lower yields of reducing sugar and glucose may be obtained using the pure cellulases than with the enzyme composite. When the hydrolysis was prolonged from 24 to 48 h, the formation of both reducing sugars and glucose was slightly enhanced. It is possible that the pretreatment improved accessibility of the enzyme to the cellulose fibres, reducing the time needed for hydrolysis. Alternatively, the fact that the glucose yield, based on the cellulose content in the biomass, was less than unity may indicate the importance of endproduct inhibition and cellulase deactivation.

Table 3 Biosaccharification of steam-exploded wheat straw

$\log R_0$	C80L (wt.%)	24-h hydrolysis at 45°C				48-h hydrolysis at 45°C			
		Reducing sugars		Glucose		Reducing sugars		Glucose	
		mmol g_b^{-1}	mmol g_c^{-1}	g g_b^{-1}	g g_c^{-1}	mmol g_b^{-1}	mmol g_c^{-1}	g g_b^{-1}	g g_c^{-1}
3.42	0	2.036	4.395	0.207	0.426	2.090	4.300	0.227	0.467
	25	2.331	4.796	0.231	0.475	2.471	5.084	0.262	0.539
	50	2.240	4.069	0.226	0.465	2.416	4.971	0.262	0.539
	75	2.212	4.551	0.231	0.475	2.420	4.979	0.262	0.539
	100	1.877	3.862	0.191	0.393	2.053	4.224	0.225	0.463
3.93	0	2.363	3.886	0.302	0.497	2.448	4.026	0.311	0.511
	25	2.446	4.023	0.317	0.521	2.577	4.238	0.333	0.548
	50	2.391	3.933	0.302	0.497	2.459	4.044	0.318	0.523
	75	2.425	3.988	0.317	0.521	2.476	4.072	0.320	0.526
	100	2.361	3.833	0.311	0.512	2.465	4.054	0.320	0.526
3.94	0	2.675	4.096	0.346	0.530	2.734	4.186	0.355	0.544
	25	2.656	4.067	0.344	0.527	2.745	4.203	0.355	0.544
	50	2.728	4.177	0.354	0.542	2.844	4.355	0.369	0.565
	75	2.705	4.142	0.348	0.533	2.781	4.258	0.355	0.544
	100	2.639	4.041	0.341	0.522	2.743	4.200	0.352	0.539
4.45	0	2.280	3.839	0.357	0.601	2.370	3.990	0.372	0.626
	25	2.383	4.012	0.374	0.630	2.427	4.087	0.380	0.640
	50	2.305	3.881	0.369	0.621	2.416	4.068	0.378	0.636
	75	2.378	4.004	0.371	0.625	2.340	3.940	0.366	0.616
	100	2.283	3.844	0.354	0.596	2.416	4.068	0.372	0.626

Yields as function of the pretreatment severity parameter and the composition of the enzyme composite.

However, neither of these hypotheses is proven by the results presented here, and their significance awaits further experimentation. A comparison of the results obtained using biomass steam-exploded at different temperatures for different times shows that the severity factor is a good parameter for predicting the efficiency of enzymatic hydrolysis. The analysis is more complex, however, as the amount of glucose produced per gram of cellulose is a continuously increasing function of the severity parameter. In contrast, the amount of glucose formed per gram of biomass increases with severity parameter levels from 3.42 to 3.94 and remains almost unchanged at $\log R_0$=4.45. In the hydrolysis of biomass pretreated at almost the same severity parameter, $\log R_0$=3.93 and $\log R_0$=3.94, a higher pretreatment temperature (217°C rather than 200°C) resulted in higher yields than a longer pretreatment time (10 rather than 3 min). These observations can be quantified with reference to the results reported in Table 1. An increase in severity parameter determines, within uncertainty limits of ±5%, a progressive decrease in hemicellulose content. The percentage of cellulose increases from 48.6 at $\log R_0$=3.42 to 65 at $\log R_0$=3.94 owing to more effective removal of the other components. In contrast, at $\log R_0$=4.45, the cellulose content decreased to 59.4%. It is possible that steam explosion at a high severity factor may have caused an initial breakdown of the cellulose fibre and a consequent loss of glucose. The almost equal glucose yield obtained at $\log R_0$=3.94 and 4.45 would be a consequence. This suggests that wheat straw pretreatment should be performed at $\log R_0$=3.94 because of the lower energy demand in the steam explosion process.

Effect of biomass rinsing procedure on hydrolysis reaction: The experiments so far discussed were performed directly with the biomass, pretreated as described above. Under these conditions, however, enzyme inhibitors can be formed during the pretreatment process. Lignin residues can be present and can precipitate in water onto the cellulose fibre. Therefore, in order to promote effective enzymatic attack, the biomass samples were thoroughly washed in different conditions and their hydrolysis was tested. The product release was determined under standard hydrolysis conditions. Because the results presented in Table 3 indicate that a composite of the two cellulases containing 25 wt.% C80L gave the highest glucose yields, the reaction was carried out with this enzymic composition. The reducing sugars and glucose yields, after saccharification times of 24 and 48 h, are presented in Table 4. The results indicate that the glucose yield is related to the biomass rinsing, the rate of hydrolysis increasing when the wheat straw is thoroughly washed. Biomass rinsing with NaOH was superior to just water rinsing. Water rinsing may have reduced the concentration of enzyme inhibitors and rinsing with NaOH may have increased the accessibility of the cellulose fibres by avoiding precipitation of lignin residues. It is apparent that the larger availability of cellulose determines the need for a longer hydrolysis time. The glucose yield after a reaction time of 48 h was roughly 20% higher than after 24 h. A maximum glucose yield of 1.258 g g_c^{-1} was reached with the biomass pretreated at a severity parameter of 4.45 after rinsing with NaOH. This value is close to the stoichiometric one (1.11 g g_c^{-1}) within the limits of experimental error. However, owing to the cellulose content of the biomass, the highest glucose productivity (0.747 g g_b^{-1}) was achieved with wheat straw pretreated at $\log R_0$=3.94.

Effect of biomass loading on bioprocess productivity and glucose yield: The subsequent investigation concerned the dependence of saccharification rate on biomass loading. When the amount of biomass in the reactor was increased to 75 g l^{-1} or above, stirring became difficult as most of the water in the system are required to soak the biomass: the biomass remains partially segregated at the bottom of the reactor and the contact between the enzyme and the biomass is less effective. Hydrolysis carried out at low biomass loadings, on the other hand, resulted in both low glucose production per unit reactor volume and low glucose concentration. It is desirable to achieve high glucose concentrations, however, as the optimum value for the fermentation step is 150 g l^{-1}. Low values increase the energy demand for concentration of the hydrolysate stream prior to the fermentation step.

Pretreatment at severity parameter $\log R_0$=3.94 followed by rinsing with NaOH solution and water gave the highest glucose yield per gram of biomass. Therefore, the study was continued with wheat straw steam-exploded for 3 min at 217°C. The effect of

Table 4 Effect of rinsing procedure of steam-exploded biomass on hydrolysis yield

$\log R_0$	Rinsing	24-h hydrolysis at 45°C				48-h hydrolysis at 45°C			
		Reducing sugars		Glucose		Reducing sugars		Glucose	
		mmol g_b^{-1}	mmol g_c^{-1}	g g_b^{-1}	g g_c^{-1}	mmol g_b^{-1}	mmol g_c^{-1}	g g_b^{-1}	g g_c^{-1}
3.42	–	2.331	4.796	0.231	0.475	2.471	5.084	0.262	0.539
	Water*	2.083	4.286	0.223	0.459	2.404	4.946	0.280	0.576
	NaOH**	2.883	5.932	0.361	0.743	3.228	6.642	0.428	0.880
3.93	–	2.446	4.023	0.317	0.521	2.577	4.238	0.333	0.548
	Water*	3.237	5.324	0.491	0.808	3.254	5.352	0.564	0.928
	NaOH**	3.590	5.904	0.612	1.006	3.938	6.477	0.683	1.123
3.94	–	2.656	4.067	0.344	0.527	2.745	4.203	0.355	0.544
	Water*	3.222	4.933	0.482	0.738	3.399	5.204	0.527	0.807
	NaOH**	3.187	4.880	0.554	0.848	3.555	5.443	0.627	0.960
4.45	–	2.383	4.012	0.374	0.630	2.427	4.087	0.380	0.640
	Water*	2.817	4.743	0.458	0.771	2.992	5.038	0.527	0.887
	NaOH**	3.880	6.533	0.617	1.038	4.391	7.393	0.747	1.258

*Thoroughly washed with distilled water.
**Rinsed as reported in *Materials and methods* section.

biomass loading in the reactor was investigated. Four amounts of biomass were tested (25, 50, 75, and 100 g l^{-1}). Glucose formation was monitored in the presence of cellulase composites from X70L and C80L. The weight ratio of X70L to C80L was kept constant at 3:1. The glucose yield (g g_b^{-1}) and the volumetric glucose productivity (g h^{-1} l^{-1}) were determined as functions of the biomass loading from the experimental curves of glucose production *versus* reaction time. The results are reported in Figure 1. Saccharification of the biomass started at a relatively high rate and then slowed down: for short process times, the yield was low and the productivity was high, the opposite being the case for extended process times. Productivity was found to be a decreasing function of yield and attained its highest value at a biomass loading of 50 g l^{-1}. In contrast, the highest productivity was observed at 75 and 100 g_b l^{-1}, where the reactor yield reached the value of 0.6 g g_b^{-1}. This result is related to the rheology of the system: because of stirring difficulties, the rate of hydrolysis was initially low at high biomass loading; as the cellulose depolymerises, the rheology of the medium improves and hydrolysis proceeds at a faster rate. Results plotted in Figure 1 suggest two possible alternatives for selecting process conditions: either to work with 50 g l^{-1} of biomass in order to attain good productivity without depressing the yield too much or to operate with 100 g l^{-1} of biomass in order to achieve good productivity at the highest yield. This latter condition could be preferable as it coincides with the highest concentration of fermentable sugars in the reactor (60 g l^{-1}). The final glucose concentration in this case, which is roughly 85% of theoretical (based on the average cellulose content in the biomass), was reached after 72 h of hydrolysis. Longer times were not considered as these would result in a progressive decrease in volumetric productivity.

Optimisation of fermentation conditions: A previous work [4] identified the presence of inhibitors of yeast activity in the hydrolysis stream. In addition, stresses brought about by the action of the shake fermenter cause the biomass to damage the yeast cells. For this reason, the fermentation of commercial glucose was studied in the presence of biomass and in broth prepared with the liquid collected at the end of the rinsing procedure. Control experiments were performed with glucose only. The sugar concentration was maintained at 60 g l^{-1}, the highest value reached in the hydrolysis step. As previous experiments [17] showed that adjustment of pH from the optimum hydrolysis value of 4.8 to 5.0 (the usual value chosen for fermentation with *S. cerevisiae*) is unimportant, as is a change of buffer from acetate to phosphate, glucose formation was studied in 50 mM Na-acetate buffer at pH 4.8.

Glucose consumption and ethanol yield were determined (Figure 2). Neither the preparation of fermentation broth with the liquid collected from rinsing procedures nor the addition of the biomass modified the length of the lag phase or the ethanol yield, the latter remaining close to the stoichiometric level. The longer lag phase measured in this study, in comparison with that reported for ethanol fermentation with *S. cerevisiae*, is a result of the use of lyophilised yeast cells rather than preincubated yeast. The results indicate that the yeast cells were adequately robust to withstand the stress induced by the solid biomass; also that any inhibitor that could be present in the solid–liquid mixtures collected from the steam explosion plant is adequately removed during the biomass rinsing procedure.

A series of fermentations with sterile commercial glucose was also performed in the presence of the two enzymatic preparations,

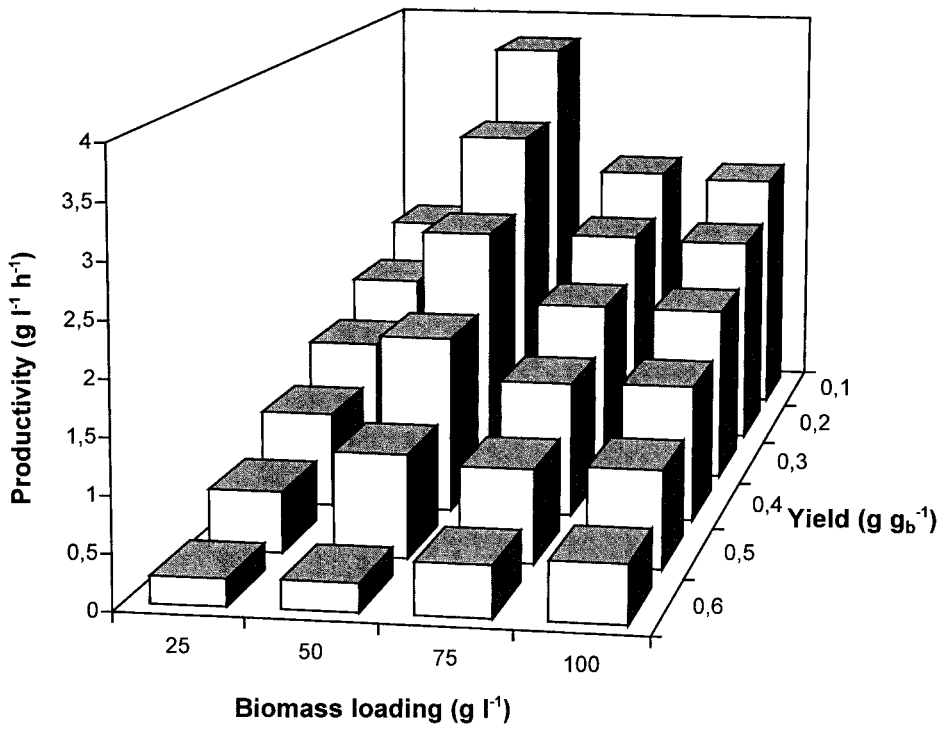

Figure 1 Glucose productivity (g l^{-1} h^{-1}) and yield (g g_b^{-1}) as function of biomass loading in the enzymatic hydrolysis step. Reaction conditions: 45°C, 50 mM Na-acetate buffer pH 4.8, stirring 250 rpm, and 25 wt.% of C80L as enzyme composite. Biomass: severity parameter: $\log R_0 = 3.94$; NaOH rinsing procedure.

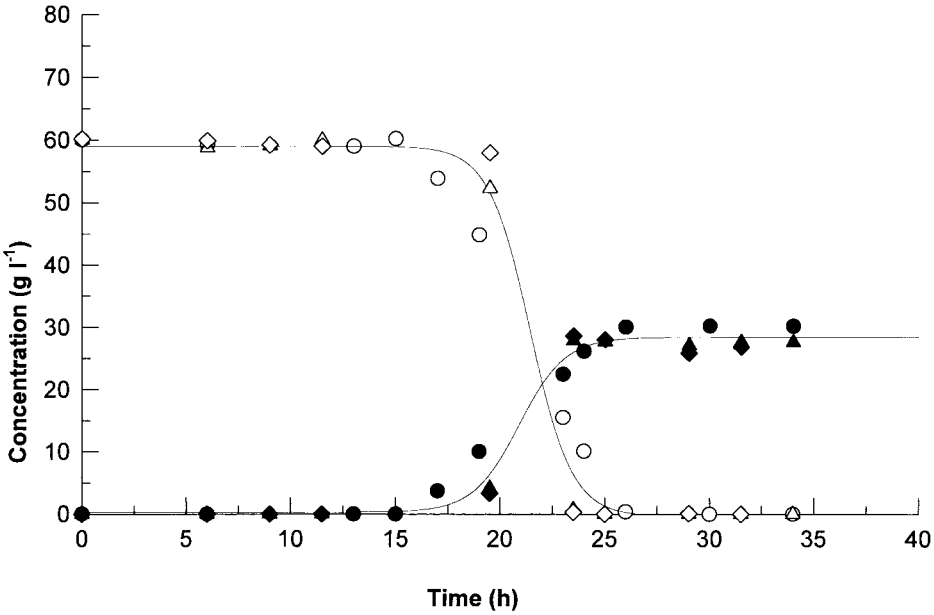

Figure 2 Time dependence of glucose consumption (open symbols) and ethanol formation (closed symbols) in shake flask fermentations. Fermentation conditions: 37°C, 150 rpm, 100 ml broth, 3 g l^{-1} *S. cerevisiae*, sterile commercial glucose 60 g l^{-1}. (○/●) Pure broth; (△/▲) addition of water from NaOH rinsing procedure; (◇/◆) addition of water from NaOH rinsing procedure and biomass ($\log R_0 = 3.94$) residual from hydrolysis (40 g l^{-1}).

C80L and X70L. The concentrations of X70L (4.5 g l^{-1}) and C80L (1.5 g l^{-1}) were the same as in the hydrolysis reaction. Fermentation was run for 55 h with freeze-dried *S. cerevisiae* cells (3 g l^{-1}). The results (Figure 3) show that in the presence of X70L, the lag phase was much longer: 30 h, compared with 13 h for the control case and for fermentation in the presence of C80L. Ethanol yield was reduced to 90% of the level attained in the presence of C80L.

A further series of fermentations was performed with the sugar streams obtained after 72 h of hydrolysis of wheat straw at 45°C. Wheat straw was pretreated at $\log R_0$ of 3.94 and thoroughly washed with NaOH and water. The ethanol yield from sugar

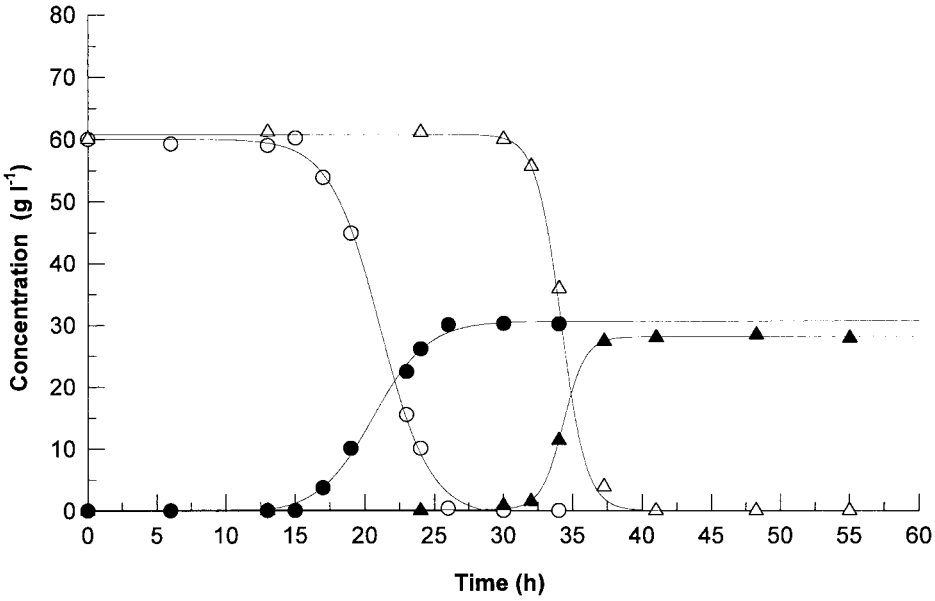

Figure 3 Time dependence of glucose consumption (open symbols) and ethanol formation (closed symbols) in shake flask fermentations in the presence of enzymatic preparations. Fermentation conditions: 37°C, 150 rpm, 100 ml broth, 3 g l^{-1} *S. cerevisiae*, 60 g l^{-1} sterile commercial glucose. (○/●) Addition of 1.5 g l^{-1} of C80L; (△/▲) addition of 4.5 g l^{-1} of X70L.

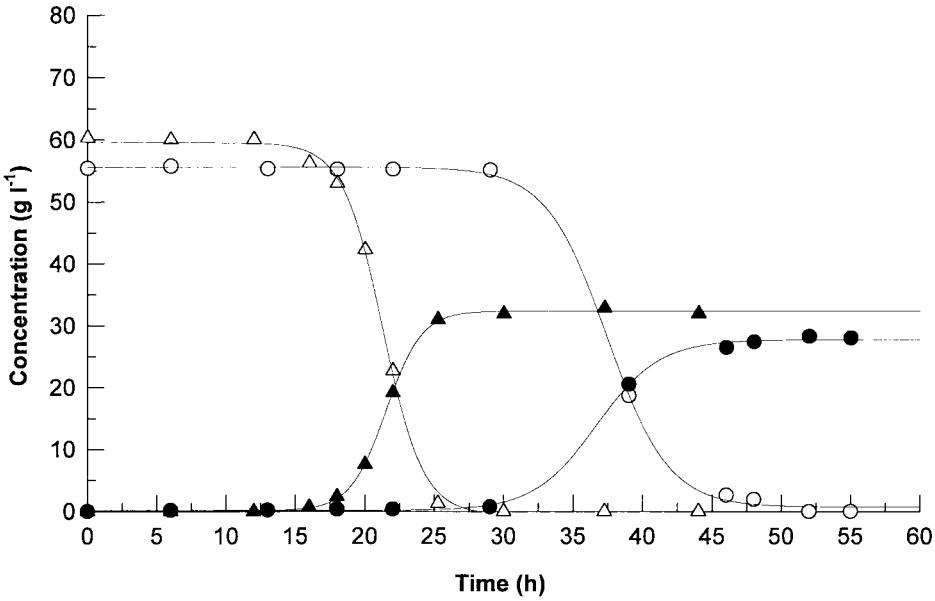

Figure 4 Shake flask fermentations of the sugar stream from hydrolysis of 100 g l^{-1} of biomass ($\log R_0 = 3.94$) for 72 h at 45°C. Fermentation conditions: 37°C, 150 rpm, 100 ml broth, 3 g l^{-1} *S. cerevisiae*. Glucose consumption (open symbols) and ethanol formation (closed symbols). (△/▲) Hydrolysis performed with only C80L and (○/●) hydrolysis performed with the C80L/X70L composite.

streams produced using the C80L-X70L composite was compared with that of the hydrolysate obtained using only the C80L. The spent biomass was present and the broth was prepared with the hydrolysis medium to which $(NH_4)_2HPO_4$ (0.25 g l^{-1}) and $MgSO_4 \cdot 7H_2O$ (0.025 g l^{-1}) had been added. All the other fermentation conditions of previous experiments were maintained. The effect of the presence of X70L (Figure 4) confirmed the results of fermentation. The lag phase and the fermentation time were longer when X70L was employed. An ethanol yield of 98% of the theoretical value was attained after 28 h fermentation of the hydrolysate of the wheat straw with C80L. The ethanol yield was 13% lower when the fermentation

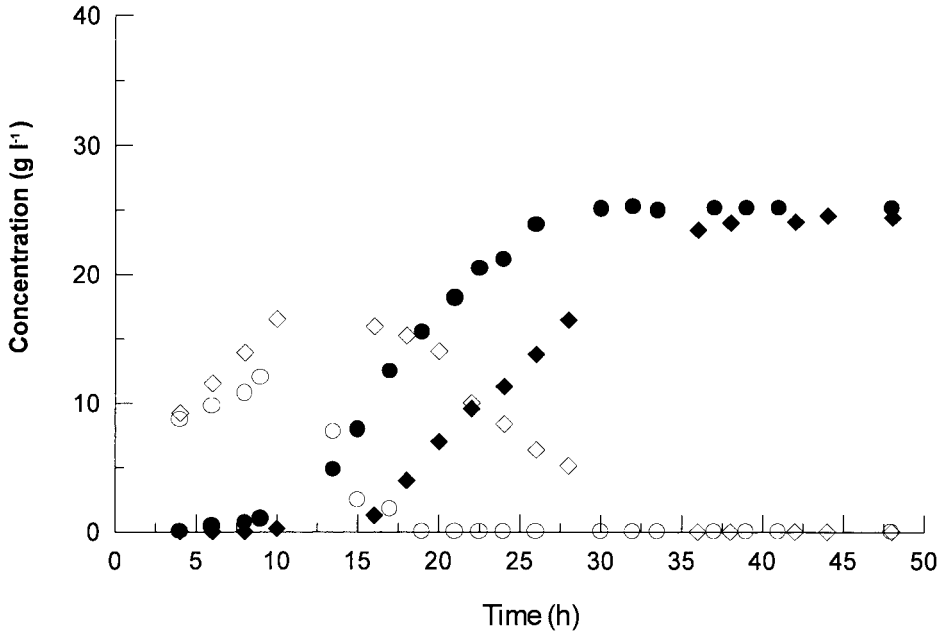

Figure 5 Effect of temperature on simultaneous saccharification and fermentation of wheat straw (100 g l^{-1}, $\log R_0 = 3.94$) in 100 ml broth, with 3 g l^{-1} *S. cerevisiae* and C80L and 150 rpm. Glucose concentration (open symbols) and ethanol concentration (closed symbols). (○/●) 37°C and (◇/◆) 40°C.

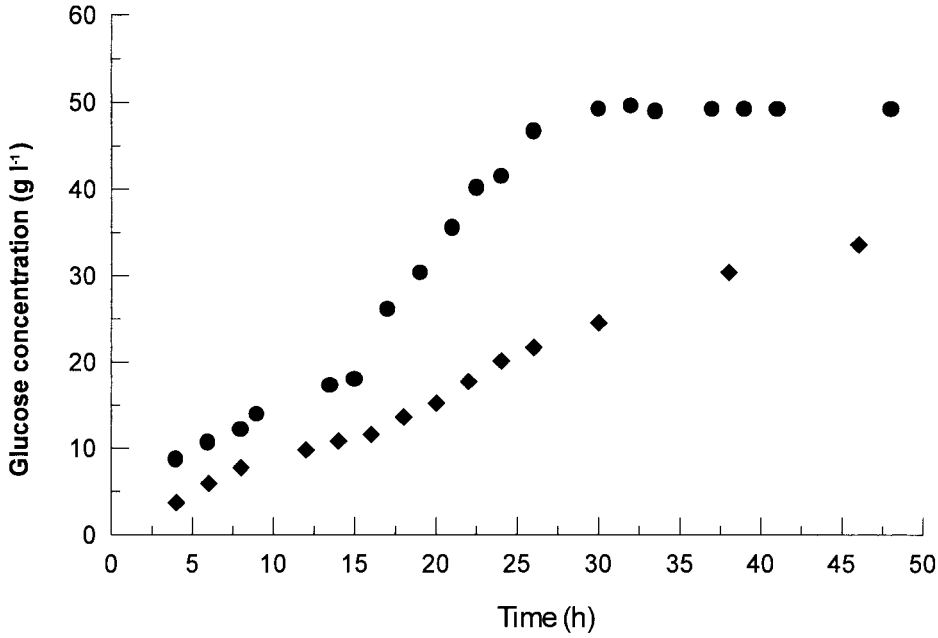

Figure 6 Effect of enzyme-to-biomass ratio on glucose production during simultaneous saccharification and fermentation of wheat straw (100 g l^{-1}, logR_0 = 3.94) in 100 ml broth, with *S. cerevisiae* 3 g l^{-1} and C80L at 37°C and 150 rpm. (◆) 0.03 g g^{-1}; (●) 0.06 g g^{-1}.

was performed with sugar streams from biosaccharification carried out with the enzymatic composites containing also the X70L.

SSF

Ethanol yield in the fermentation of commercial glucose and wheat straw hydrolysate, carried out with and without X70L, indicate the likely presence of inhibitors of *S. cerevisiae*. The SSF of wheat straw was therefore performed using solely the C80L complex. Biomass at 100 g l^{-1} loading was pretreated at a severity parameter of 3.94. In an effort to establish whether or not ethanol production is dependent on process temperature and enzyme-to-biomass weight ratio, SSF runs were carried out at 37°C and 40°C and at enzyme-to-biomass ratios of 0.03 and 0.06. All other parameters were kept constant. The ethanol and residual glucose concentrations in the system were plotted as functions of process time (Figure 5). An increase in temperature from 37°C to 40°C was detrimental since it induces an extension of the lag phase and of the time needed for completing the bioconversion. The ethanol yield at 37°C was as high as 68% after only 30 h. The glucose yield was evaluated, taking into account cellulose content in the biomass and fermentation stoichiometry. The S-shaped curve of glucose production is characteristic of a series mechanism for cellulose conversion into ethanol in which glucose is the intermediate product. The highest glucose concentration was attained after approximately 12 h of bioconversion.

Ethanol production in the SSF was substantially faster than in the SHF, suggesting that there may be a lower enzyme inhibition by glucose. Because of the higher enzyme activity, the possibility of using a smaller quantity of enzyme was also explored. The experiments were performed at 37°C with wheat straw at 100 g l^{-1} pretreated at logR_0 of 3.94. Two quantities of enzymes were tested, corresponding to 0.03 and 0.06 g of enzyme per gram of biomass. The concentration of glucose was calculated from the instantaneous value determined by analysis and from ethanol production assuming the stoichiometric ratio. The results (Figure 6) show that under the conditions tested, enzyme concentration was a significant factor, the lower concentration resulting in considerably longer bioconversion time and a much lower glucose yield.

Conclusions

A comparison of SHF with SSF indicated that the bioprocess was completed in approximately 30 h with SSF as opposed to 96 h with SHF (72 h for hydrolysis and 24 h for fermentation). It was possible to attain 68% of the theoretical ethanol yield (per unit weight of biomass) with SSF. In contrast, 81% of the theoretical ethanol yield was attained in the SHF. Because the overall process time was much longer for the SHF, the ethanol productivity (gram per unit volume and time) was much higher in the SSF. A further advantage of using a single bioreactor (SSF) is the reduction of investment and operation costs. The levels of glucose and ethanol production in SSF indicated that the conversion of glucose into ethanol was close to the theoretical value. In contrast, the conversion of cellulose into glucose was lower in the SSF (70% of the stoichiometric value) than that in SHF (83% of the stoichiometric value). This may be the result of a much shorter process time.

The optimum temperature for SSF was 37°C with *S. cerevisiae* as the fermenting organism. The optimum enzyme-to-biomass ratio was 0.06 for both SSF and SHF.

A number of other conclusions can be made from this study. Steam explosion is an effective pretreatment for wheat straw. Both time and temperature pretreatment affect the overall yield of saccharification and fermentation. The use of enzymatic composites of two cellulase complexes is advantageous in the hydrolysis step as overall enzymatic activity can be balanced. However, crude enzyme preparations can contain inhibitors that depress *S.*

cerevisiae activity in both the fermentation step of SHF as well as in the SSF.

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