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Thermostable Enzymes in Lignocellulose Hydrolysis

Liisa Viikari[1] (✉) · Marika Alapuranen[2] · Terhi Puranen[2] · Jari Vehmaanperä[2] · Matti Siika-aho[3]

[1]University of Helsinki, P.O. Box 27, 00014 Helsinki, Finland
liisa.viikari@helsinki.fi

[2]ROAL, Valta-akseli, 05200 Rajamäki, Finland

[3]VTT Technical Research Centre of Finland, P.O. Box 1000, 02044 Espoo, Finland

Abstract Thermostable enzymes offer potential benefits in the hydrolysis of lignocellulosic substrates; higher specific activity decreasing the amount of enzymes, enhanced stability allowing improved hydrolysis performance and increased flexibility with respect to process configurations, all leading to improvement of the overall economy of the process. New thermostable cellulase mixtures were composed of cloned fungal enzymes for hydrolysis experiments. Three thermostable cellulases, identified as the most promising enzymes in their categories (cellobiohydrolase, endoglucanase and β-glucosidase), were cloned and produced in *Trichoderma reesei* and mixed to compose a novel mixture of thermostable cellulases. Thermostable xylanase was added to enzyme preparations used on substrates containing residual hemicellulose. The new optimised thermostable enzyme mixtures were evaluated in high temperature hydrolysis experiments on technical steam pretreated raw materials: spruce and corn stover. The hydrolysis temperature could be increased by about 10–15 °C, as compared with present commercial *Trichoderma* enzymes. The same degree of hydrolysis, about 90% of theoretical, measured as individual sugars, could be obtained with the thermostable enzymes at 60 °C as with the commercial enzymes at 45 °C. Clearly more efficient hydrolysis per assayed FPU unit or per amount of cellobiohydrolase I protein used was

obtained. The maximum FPU activity of the novel enzyme mixture was about 25% higher at the optimum temperature at 65 °C, as compared with the highest activity of the commercial reference enzyme at 60 °C. The results provide a promising basis to produce and formulate improved enzyme products. These products can have high temperature stability in process conditions in the range of 55–60 °C (with present industrial products at 45–50 °C) and clearly improved specific activity, essentially decreasing the protein dosage required for an efficient hydrolysis of lignocellulosic substrates. New types of process configurations based on thermostable enzymes are discussed.

Keywords Thermostable · Cellulases · Cellobiohydrolase · Endoglucanase · β-Glucosidase · Lignocellulose · Hydrolysis

1 Introduction

The present challenge is to substantially increase the production and use of biofuels for the transport sector. In order to reach the future goals of substituting fossil based fuels, it will be necessary to promote the transition towards second generation biofuels. These can be produced from a wider range of feedstock, including lignocellulosic raw materials. Biomass resources can be broadly categorised as agricultural or forestry-based, including secondary sources derived from agro- and wood industries, waste sources and municipal solid wastes. Fuels from lignocellulosic biomass have a higher potential to reduce greenhouse gas emissions, and hence are an important means to fulfil the CO_2 emissions targets, as compared with first generation biofuels. Lignocellulosic raw materials comprise an abundant source of carbohydrates (cellulose and hemicellulose) for a variety of biofuels, including bioethanol. The conversion technologies of lignocellulosic raw materials are, however, more complex and need novel enzyme systems and advanced fermentation technologies. The rate-limiting step in the conversion of cellulose to fuels is hydrolysis, especially the initial attack on the highly ordered, insoluble structure of crystalline cellulose. In spite of recent achievements, further developments are still needed to improve the overall economy of the lignocellulose-to-ethanol process. These novel conversion techniques would also be applicable for the production of other sugar platform-based chemicals.

2 Enzymatic Hydrolysis of Cellulose

Plant cellulose exists in a highly crystalline form. In addition, it is associated with hemicellulose and surrounded by lignin, which may also be covalently bound to hemicellulose. Pretreatments aim at increasing the surface area of cellulose by either removing lignin or solubilising hemicellulose, disrupting

the crystallinity and/or by increasing the pore volume. Hydrolysis of cellulose requires the co-operation of three classes of cellulolytic enzymes, namely cellobiohydrolases (CBH, EC 3.2.1.91), endo-β-1,4-glucanases (EG, EC 3.2.1.4) and β-glucosidases (BG, EC 3.2.1.21). The CAZY (carbohydrate active enzymes) [16] classification system collates glycosyl hydrolase (GH) enzymes into families according to sequence similarity, which have been shown to reflect shared structural features. Most of the initial cellulase work was concentrated on the biochemistry, genetics and process development of the mesophilic fungus *Trichoderma reesei*. This fungus is one of the most powerful secretors of extracellular proteins. It is industrially used for the production of various homologous and heterologous proteins. Also several thermostable enzymes have been expressed in this host, as reviewed by Bergquist et al. [8].

Trichoderma reesei produces several cellulases which act synergistically in the degradation of cellulose. Eight major cellulase genes have so far been identified from the *T. reesei* genome; two cellobiohydrolases (CBH I and II, i.e. Cel7A and Cel6A), and six endoglucanases (EG I–VI, i.e. Cel7B, Cel5a, Cel12A, Cel45A, Cel61A, Cel74A) [24]. All known *T. reesei* cellulases, with one exception (Cel12A), have a two-modular structure. They consist of a catalytic module and a carbohydrate binding module (CBM) connected with a linker region. Cel7A (CBHI) is the major cellulose produced by *T. reesei*; it has been reported to hydrolyse solid cellulose and constitutes about 60% of the cellulases expressed [51, 73]. It has been shown that Cel7A hydrolyses the cellulose chain from the reducing end and it is believed that the chain is hydrolysed processively [3, 19, 55]. Cel6A, on the other hand, preferably hydrolyses the cellulose chain from the non-reducing end [55, 73]. It constitutes about 10–15% of total cellulase proteins [51]. The Cel7B is the major endoglucanase, forming about 6–10% of total *T. reesei* cellulase [51, 73]. It has activity against solid and soluble substrates, such as CMC, as well as against xylan, PASC and glucomannans [71]. Also the endoglucanase Cel5A is reported to have activity against solid (Avicel, BMCC) and soluble (CMC, mannan) substrates [31, 38, 44], but not on xylans. This enzyme comprises about 1–10% of the total cellulases in *T. reesei* [51, 73]. The minor endoglucanases Cel12A, Cel61A and Cel45A are reported to hydrolyse solid (Avicel, filter paper) and soluble (CMC, glucomannan) substrates with diverse specific activities. Two β-glucosidase encoding genes from *T. reesei* have been cloned [2, 74]. Cel74A [24] is a xyloglucanase having also endoglucanase activity against β-glucan and CMC [28].

3 Thermostable Cellulases

Thermostable enzymes are gaining wide industrial and biotechnical interest due to the fact that they are more stable and thus generally better suited

for harsh process conditions. The concept of thermostability is, however, not very clear, and the thermostability is a relative term. The enzymatic activity is known to increase with increasing temperature up to the temperature where inactivation starts to occur [25]. Thermostability is usually defined as the retention of activity after heating at a chosen temperature for a prolonged period. The drawback is that it only measures how well an enzyme tolerates high temperature and does not take into consideration the number of variables affecting this measurement. The most appropriate way to express thermostability is to measure the half-life of enzyme activity at elevated temperatures. Thermostable enzymes are produced both by thermophilic and mesophilic organisms. Although thermophilic microorganisms are a potential source for thermostable enzymes, the majority of industrial thermostable enzymes originate from mesophilic organisms. Thermophilic bacteria have, however, received considerable attention as sources of highly active and thermostable enzymes.

Thermostable enzymes in the hydrolysis of lignocellulosic materials have several potential advantages: higher specific activity (decreasing the amount of enzyme needed), higher stability (allowing elongated hydrolysis times) and increased flexibility for the process configurations. The two first characteristics would expectedly improve the overall performance of the enzymatic hydrolysis even at the range of conventional enzymes active at around 50 °C. Thus, carrying out the hydrolysis at higher temperature would ultimately lead to improved performance, i.e. decreased enzyme dosage and reduced hydrolysis time and, thus, potentially decreased hydrolysis costs. Thermostable enzymes would expectedly also allow hydrolysis at higher consistency due to lower viscosity at elevated temperatures and allow more flexibility in the process configurations. The characteristics of thermostable cellulases are reviewed in Table 1. The enzymes are categorised as endo- or exoglucanases, based on the information available.

Several hyperthermostable cellulolytic enzymes have been isolated from various thermophilic bacteria including the anaerobic *Thermotoga* [11, 14, 21], *Anaerocellum thermophilum* [82] and *Rhodothermus* strains [34]. Significant research efforts have been invested in the thermophilic bacterial cellulosome systems of Clostridia (reviewed by [17]). Concepts for the direct conversion of lignocellulose into ethanol using clostridial co-culture process have been studied [33]. In addition, thermostable ascomycete cellulases have been characterised [30, 37, 57]. Several mesophilic or moderately thermophilic fungal strains are also known to produce thermostable enzymes. These enzymes are stable and active at temperatures that are essentially higher that the optimum temperatures for the growth of the microorganism [65]. Some filamentous fungi produce cellulases that retain relatively high cellulose-degrading activity at elevated temperatures, particularly those from the species *Talaromyces emersonii* [27, 50, 78], *Thermoascus aurantiacus* [26, 59, 70], *Chaetomium thermophilum* [48], *Myceliophthora ther-*

Table 1 Thermostable cellulases

Organisms	Enzymes	Characteristics of enzymes				Refs.
		MW (SDS PAGE) (kDa)	pH optimum	T optimum (°C)	Stability	
Acidothermus cellulolyticus	Endoglucanase I	57.420–74.580	5.0	83	Inactivated at 110 °C	[18, 32, 67]
Anaerocellum thermophilum	Endoglucanase	230	5–6	95–100	Half-life 40 min at 100 °C	[82]
Bacillus sp. KSM-S237	Endoglucanase	86	8.6–9.0	45	30% of activity remained after 10 min at 100 °C	[29]
Caldocellum saccharolyticum	Endoglucanase	na			na	[76]
Caldocellulosiruptor saccharolyticus	Endoglucanases Exoglucanases	na	7.0	68–70	na	[7, 76]
Chaetomium thermophilum	Endoglucanase	68	4.0	60	Stable at 60 °C > 60 min, half-life 7 min at 90 °C	[42]
Cladosporium sp.	Endoglucanase Exoglucanase	na	4–6	60	Stable at 60 °C for 24 h	[1]
Clostridium stercorarium	Endoglucanase	100	6.0–6.5	90	Stable for several days	[13]
Clostridium stercorarium	Exoglucanase	87	5–6	75	Stable at 70 °C for 3 days	[12]
Clostridium thermocellum	Endoglucanase	83	6.6	70	33% of activity remained after 50 h at 60 °C	[22]
Clostridium thermocellum	Endoglucanase	76	7.0	70	50% of activity remained after 48 h at 60 °C	[61]
Melanocarpus albomyces	Endoglucanase	20	6–7	70	70% of activity remained after 60 min at 80 °C	[47]
Rhodothermus marinus	Endoglucanase	49	7.0	95	50% of activity remained after 3.5 h at 100 °C, 80% after 16 h at 90 °C	[34]

na: not available

Table 1 (continued)

Organisms	Enzymes	Characteristics of enzymes				Refs.
		MW (SDS PAGE) (kDa)	pH optimum	T optimum (°C)	Stability	
Rhizomucor pusillus	Endoglucanase	na	5.5	60	Stable at 60 °C for 84 h	[68]
Sporotrichum sp.	Endoglucanase	33	4.5–5	70	Stable at 70 °C for 30 min	[35]
Streptomyces sp.	Exoglucanase	44	4	60	30% of activity remained after 10 min at 100 °C	[57]
Streptomyces sp.	Endoglucanase	26	4	60	20% of activity remained after 10 min at 100 °C	[57]
Talaromyces emersonii	Exoglucanase (CBH IA)	66	3.6	78	Half-life 34 min at 80 °C	[78]
Thermoascus aurantiacus	Endoglucanase		4.5	75	Half-life 98 h at 70 °C and 4.1 h at 75 °C	[26]
Thermomonospora curvata	Endoglucanase	100	6.0–6.5	70–73	Stable for 48 h at 60 °C, half-life 30 min at 80 °C	[41]
Thermotoga sp.	Exoglucanase	36	6.8–7.8	100–105	Half-life 70 min at 108 °C, 2 min at 117 °C	[64]
Thermotoga maritima	Endoglucanase	27	6.0–7.5	95	Half-life 2 h at 95 °C	[14]
Thermotoga neapolitana	Endoglucanase (CelA)	29	6.0	95	Half-life > 240 min at 100 °C	[11]
	Endoglucanase (CelB)	30	6.0–6.6	106		

na: not available

mophila [63], *Thielavia terrestris* and *Corynascus thermophilus* [45]. Thermophilic β-glucosidases have been obtained from e.g. *Aureobasidium* sp. [66], *Chaetomium thermophila* [79], *Talaromyces emersonii* [15], *Thermoascus aurantiacus* [23, 26, 59, 70] and *Thermomyces lanuginosa* [40]. The literature data shows that a number of enzymes are stable at temperatures around 70 °C for elongated periods, but the data does not allow comparison of the properties under similar conditions.

4 Process Concepts

The enzymatic hydrolysis of the pretreated raw material and the fermentation of the hydrolysed sugars can be performed separately or simultaneously, commonly referred to as SHF (separate hydrolysis and fermentation) or as SSF (simultaneous saccharification and fermentation). The SSF process configuration has been generally considered more favourable for reducing the ethanol production costs [72, 81]. The hydrolysis rate in the separate hydrolysis is strongly inhibited by the accumulation of the end products, cellobiose and glucose [60]. In the simultaneous hydrolysis and fermentation, the end product inhibition is alleviated by the continuous removal of glucose by the fermenting organism. In the separate hydrolysis and fermentation the most severe end product inhibition caused by cellobiose has been overcome by adding an adequately high amount of β-glucosidase. For the same reason, the enzyme dosage needed is obviously lower in the SSF. Other claimed advantages of the SSF are the lower risk of contamination and reduction of investment costs by combined reactors. The low concentration of free glucose and the presence of ethanol make it more difficult for contaminating microorganisms to take over the fermentation and decrease the ethanol yield. The drawback of the SSF is that the conditions, i.e. the pH and temperature of the hydrolysis and fermentation, are suboptimal in a combined process. The optimal temperature for the enzymatic hydrolysis is clearly higher than that of the presently used fermenting organisms. Another drawback of the SSF is the difficulty in optimising the fermentation of techniques, i.e. by running continuous fermentation or recirculating and reusing the yeast due to the presence of the solid residues from the hydrolysis.

To improve the overall process economics and to achieve a faster hydrolysis rate by using thermostable enzymes, various modifications of the present process configurations can be considered (Fig. 1). After the pretreatment, the temperature of the substrate is high, and is reduced to achieve the operating temperature in the following process stages. In the traditional SSF, the temperature is about 35 °C. In a separate hydrolysis and fermentation process, the first total hydrolysis stage is carried out at about 45–50 °C with the present commercial enzymes, or above 60 °C with novel thermostable

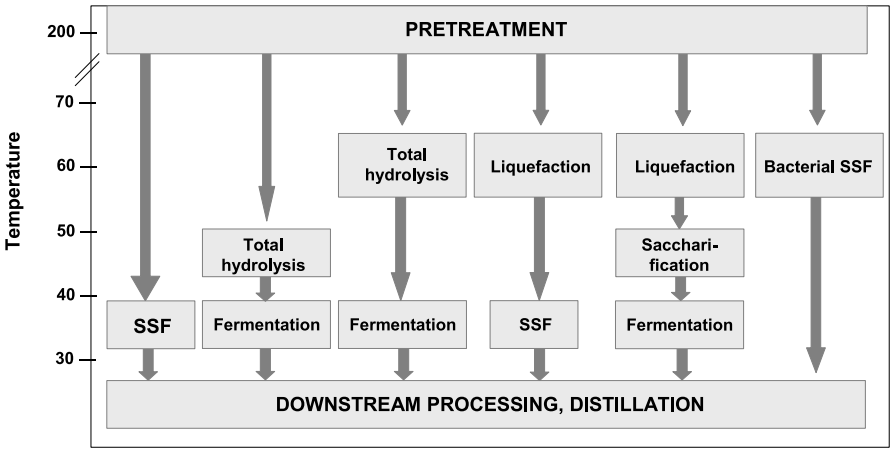

Fig. 1 Configurations of hydrolysis and fermentation processes at different temperatures using thermostable enzymes

enzymes. Other options include a partial prehydrolysis at higher temperatures, denoted as liquefaction, where the viscosity of the substrate is decreased using a chosen composition of thermostable cellulases based on one or several enzymes. The liquefaction stage, i.e. an enzymatic treatment improving the rheological properties (improved flowability, reduced viscosity) of the slurry, can significantly improve the mixing properties of the substrate slurry [83]. This partial hydrolysis can be carried out even with the limited number of thermostable cellulolytic and hemicellulolytic enzymes available. Using a set of thermoactive enzymes in the prehydrolysis, it was possible to reduce the viscosity and increase the sugar formation [83]. The high viscosity is a consequence of a high initial substrate consistency, needed to achieve a high final sugar and ethanol concentration and to decrease the distillation costs [69]. With a theoretical ethanol yield of 25–30% of the raw material, the raw material consistency should be at least 15% (d.w.) to reach an ethanol concentration of 4–5%. Some of the technical obstacles related to high consistency can thus be overcome by a rapid decrease of viscosity. After a liquefying partial hydrolysis, the saccharification stage using a complete or complementary set of hydrolytic enzymes, either simultaneously or separately from the fermentation (SSF or SHF), can be carried out. A separate hydrolysis stage (SHF) can be carried out at elevated temperatures with the complete set of hydrolytic thermostable enzymes needed for a chosen substrate. Finally, thermostable enzymes could be supplemented to bacterial fermentations using anaerobic, ethanol producing strains, such as Clostridia, to improve their conversion rate of cellulosic substrates into sugars (SSF or consolidated bioprocessing). Thus, new thermostable enzymes would allow the design of more flexible process con-

figurations, based on the availability of novel thermostable lignocellulolytic enzymes.

The performance of chosen thermostable cellulolytic enzymes with present commercial fungal enzymes was compared in this paper. The reference enzyme preparations contain the whole set of cellulolytic enzymes, i.e. cellobiohydrolases and endoglucanases, as well as several hemicellulolytic activities and β-glucosidases. These enzymes work at temperatures up to about 45 °C in long-term hydrolysis conditions and up to 50 °C in short-term conditions. New enzyme compositions were designed and tested in the hydrolysis of various steam pretreated raw materials.

5 Evaluation of Novel Thermophilic Enzymes; Materials and Methods

Enzymes

The thermostable enzyme preparations were kindly provided by ROAL, Finland. The genes encoding three thermostable enzymes: cellobiohydrolase (CBH/Cel7A) from *Thermoascus aurantiacus*, fused with the *T. reesei* CBHI cellulose binding domain (CBM), endoglucanase (EG/Cel45A) from *Acremonium thermophilum* and a xylanase and β-glucosidase (BG/Cel3A) from *T. aurantiacus* were inserted under the control of a strong *T. reesei cbh1* promoter and transformed into a host strain where all the major cellulase genes were deleted (phenotype CBHI/Cel7A – CBHII – Cel6A – EGI/Cel7B – EGII/Cel5A). Fermenter supernatants produced at pilot scale were used to produce various mixtures of the thermostable enzymes. The background activities in the deletion strains were measured. The composition of the mixture of the three thermostable enzymes was optimised based on the average cellulase composition of *T. reesei*. The enzyme components were mixed in different ratios and the total cellulase activity of the mixtures was measured at 50 °C (as FPU mL^{-1}) and used as the basis of enzyme dosing. In addition, a family 10 thermostable xylanase from *T. aurantiacus*, cloned and expressed in the *T. reesei* deletion strain, was added to some preparations to ensure complete hydrolysis.

Celluclast 1.5 L FG (Novozymes, Denmark) and Econase CE (ABEnzymes, Finland), eventually supplemented with BG from Novozym 188 (Novozymes, Denmark) were used as reference enzymes. The standard enzyme dosage was 10 FPU g^{-1} cellulose for Celluclast 1.5 L FG, supplemented with additional BG (100–500 nkat g^{-1} cellulose). Assuming an average 50% cellulose content of the lignocellulose substrates, the enzyme dosage was thus 5 FPU g^{-1} substrate. For the hydrolysis experiments at elevated temperatures, higher dosage of Celluclast (22 FPU g^{-1} cellulose) was used.

The total cellulolytic activity used as a basis for dosing of the enzyme mixtures was evaluated using the FPU activity, measured against Whatman no. 1 filter paper [36]. The EG activity was assayed using hydroxyl ethyl cel-

lulose as substrate [36]. The CBH activity was determined by using 4-methyl umbelliferyl-β-D-lactoside as substrate, estimating the effect of EGs by carrying out the assay with or without 20 mM cellobiose in the reaction [6]. The xylanase activity was assayed using birchwood glucuronoxylan as substrate [4] and that of BG using *p*-nitrophenyl-β-D-glucopyranosidase as substrate [5]. Protein was assayed according Lowry et al. [43]. All the enzyme activity assays were carried out at pH 5.

Substrates

The substrates used were steam pretreated, washed spruce solid fraction kindly provided by Guido Zacchi at the Lund University, Sweden, and steam pretreated corn stover kindly provided by Francesco Zimbardi at ENEA, Italy. The solid fraction of spruce substrate after the pretreatment was separated from the liquid fraction by filtration, washed and used in the hydrolysis experiments. The composition of the fibre fractions of the substrates is presented in Table 2. In addition to the insoluble fibre fraction, the corn stover substrate contained significant amounts, about 17% (d.w.) of solubilised mono- and oligosaccharides, solubilised mainly from hemicelluloses. Based on secondary analytical enzymatic hydrolysis and HPLC analysis, the carbohydrates in the soluble fraction consisted of xylose (74%), arabinose (15%), galactose (5%) and glucose (6%). Comparative hydrolysis experiments were carried out using crystalline cellulose (Avicel, Sigma).

Table 2 Composition of substrates used in the hydrolysis experiments

	Spruce	Corn stover
Pretreatment conditions	215 °C, 4 min	195 °C, 5 min
Catalysts	SO_2 impregnation	No
Composition of solid fraction(%)		
• Glucan	50.8	56.0
• Xylan	0.11	9.3
• Mannan	0.16	bdl
• Lignin and others	49	35

bdl below detection limit

Hydrolytic Properties of *T. reesei* Enzymes at High Temperatures

The hydrolysis experiments were carried out at a substrate consistency of 10 g L^{-1} in 50 mM sodium acetate, pH 5, in a volume of 100 mL, and incubated in shake flasks with shaking (200 rpm) at different temperatures from 55 °C to 70 °C. Duplicate shake flasks were sampled (5 mL sample) at 2 h, 4 h, 6 h, 24 h, 48 h and 72 h from the start of the hydrolysis. Possible evaporation was checked by weighing and corrected when necessary by adding a correspond-

ing amount of water. The release of hydrolysis products was followed during the hydrolysis.

Hydrolytic Properties of Thermostable Enzyme Mixtures

The performance of the thermostable enzyme mixtures was studied in hydrolysis experiment in test tubes (5 mL). Enzyme mixtures were dosed on the basis of FPU activity to the substrates (10 g L^{-1} dry matter) suspended in 50 mM sodium acetate, pH 5. The standard enzyme dosage was 10 FPU g^{-1} cellulose. Triplicate samples were incubated with mixing at 35 °C, 45 °C, 55 °C or 60 °C for 24 h, 48 h or 72 h. Reference samples with inactivated enzymes and corresponding substrates were also prepared.

Chemical Analysis

The release of hydrolysis products was measured as reducing sugars assayed by the DNS method using glucose as standard [10]. The results were corrected by taking into account the blank samples containing corresponding amounts of inactivated enzymes and substrate. The mono- and oligosaccharides formed were also analysed by high-performance anion-exchange chromatography on a Dionex 4500i series chromatograph with pulsed amperometric detection (HPAEC-PAD), as described earlier [75].

6
Composition of the Thermophilic Enzyme Mixtures

The tested thermostable fungal enzymes, classified as cellobiohydrolases (CBHs) or endoglucanases (EGs) based on the activity determinations, were chosen by preliminary screening and characterisation. Several thermostable CBHs from various thermophilic organisms were purified and characterised (Voutilainen et al, manuscript in preparation). The gene of the most potential CBH isolated from *Thermoascus aurantiacus* was fused with the *T. reesei* CBHI cellulose binding domain (CBM). In addition, an EG from *Acremonium thermophilum*, a β-glucosidase and a xylanase from *T. aurantiacus* were used to compose the thermostable mixtures. Fermenter supernatants produced in pilot scale were used to obtain the thermostable cellulase mixtures. The optimal ratio of EG to CBH amount (measured as protein of the enzyme mixtures) was determined on the basis of FPU activity of the preparations. The highest FPU activity was obtained by an EG to CBH protein ratio of 3 : 10, which corresponded well to the respective ratio of the native *T. reesei* enzymes. This ratio also gave the highest sugar yields in the hydrolysis of the steam pretreated corn stover substrate (results not shown) and was used as the standard basis for various mixtures. Three different mixtures were used in this work, differing with respect to the xylanase activity (Table 3). The xylanase-free preparation (TM 1) was first used for the spruce substrate

Table 3 Activity ratios of the thermostable enzyme mixtures (TM) used in the hydrolysis experiments

Enzyme mixture	EG : CBH (nkat : nkat)	BG : CBH (nkat : nkat)	XYL : CBH (nkat : nkat)
TM 1	0.53	3.5	0
TM 2	0.53	3.5	17.3
TM 3	0.53	3.5	8.8

The enzymes were composed of a thermostable cellobiohydrolase (CBH), endoglucanase (EG) and β-glucosidase (BG) (mixture TM 1) supplemented either with high (TM 2) or low (TM 3) amounts of xylanase (XYL). The activities of the enzyme mixtures are expressed as the ratio of the added key activity (EG, BG or XYL) to the CBH activity of the enzyme mixture

and the xylanase-containing preparations (TM 2 and TM 3) for the corn stover substrate. As it has frequently been observed that xylanases enhance the hydrolysis of lignocellulosic substrates containing even low amounts of residual xylan [9], preparations with xylanase activity were later used for both substrates.

7 Performance of Commercial Fungal Preparations at Elevated Temperatures

The activities of commercial reference preparations were first measured at higher temperatures in order to evaluate their general performance and to estimate the role of the background activities originating from the production strain. The hydrolysis of the pretreated spruce substrate by the commercial preparations (with and without added β-glucosidase, BG) at various temperatures from 50 to 70 °C was estimated during the first 24 h of the hydrolysis. The native *Trichoderma* cellulases and the *Aspergillus* BG were rapidly inactivated during the first 2 h of hydrolysis of the pretreated spruce substrate at temperatures above 60 °C (Fig. 2). The hydrolysis ceased after 24 h at 60 °C and after 48 h at 55 °C (results not shown). As expected, the effect of the added BG on the sugar yield was significant. The relative inactivation of BG was more pronounced even at 60 °C (Fig. 2b). The hydrolytic effect of the rather high loading (about 20 FPU g^{-1} cellulose) of *T. reesei* and *Aspergillus* enzymes was obviously due to the initial stage of hydrolysis during which the enzymes remained active. The hydrolysis yield of sugars from spruce during the first 2 h was 15% of the theoretical maximum at 70 °C, 22% at 65 °C and 33% at 60 °C. There were indications that the temperature optimum of the commercial *T. reesei* enzymes in the hydrolysis of the pretreated spruce substrate was about 5 °C lower than on pure cellulose (results not shown).

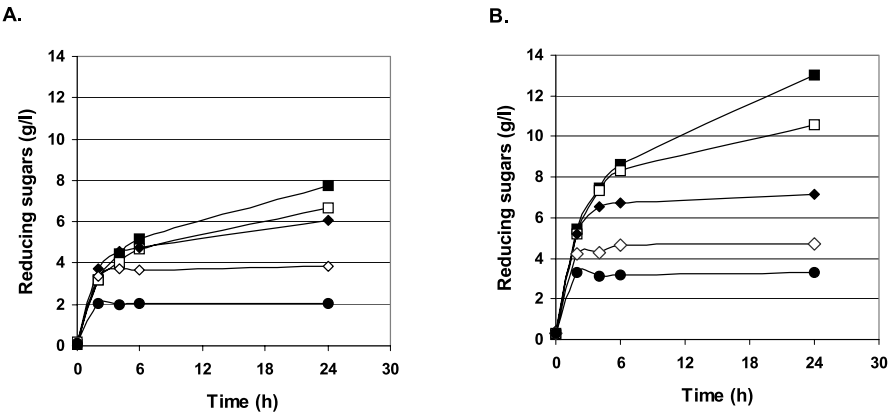

Fig. 2 Hydrolysis of washed, steam pretreated spruce substrate (cellulose content 18.3 g L^{-1}) with Celluclast 1.5 L FG alone (**A**) or supplemented with Novozym 188 (**B**) at various temperatures at pH 5. The dosage of Celluclast was 22 FPU g^{-1} cellulose and the Novozym 188 β-glucosidase 550 nkat g^{-1} cellulose. ■50 °C, □55 °C, ♦60 °C, ◇65 °C and • 70 °C

8
Evaluation of New Thermostable Enzyme Mixtures

Mixtures of selected thermostable enzymes (Table 2) were first evaluated for their hydrolytic efficiency by measuring the FPU activities at different temperatures (Fig. 3). The temperature optima of the new thermostable mixtures in the FPU activity assay were 5–10 °C higher than those of the commercial enzyme mixtures when a relatively short reaction time (60 min) in this assay was used. The relative FPU activity was set at the value of 100 at the reference point at 50 °C. The maximum FPU activity of the novel enzyme mixture was about 25% higher at the optimum temperature at 65 °C as compared with the highest activity of the commercial reference enzyme at 60 °C. As could be expected, at lower temperature (35 °C), corresponding to the fermentation temperature of traditional yeasts in a simultaneous saccharification and fermentation process (SSF), the FPU activities of the thermostable preparations were slightly lower than those of the commercial *T. reesei* enzymes.

The thermostable enzyme mixture without added xylanase activity (TM 1) was evaluated on pure cellulose (Avicel) and compared with the commercial enzyme preparations (Celluclast supplemented with β-glucosidase) at 45 °C, 55 °C and 60 °C in a 48 h hydrolysis (Fig. 4). On pure cellulose, the mixture of thermostable enzymes gave nearly similar hydrolysis results at 60 °C as the *T. reesei* enzymes at 45 °C, i.e. thus enabling an increase of temperature of about 15 °C. At 60 °C, the hydrolysis yield of Avicel was about three- to four-fold better with the thermostable enzymes than with the commercial fungal enzymes. The highest hydrolysis yield was about 90% of the theoretical.

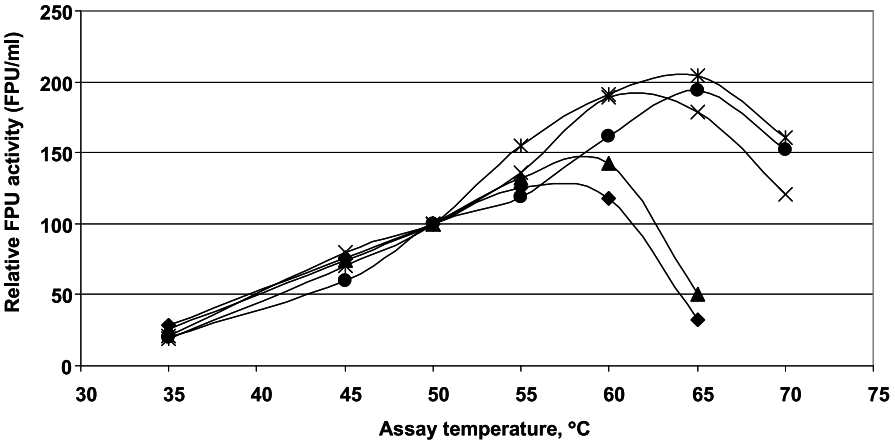

Fig. 3 Temperature optimae of cellulase activity (FPU) of the thermostable enzyme mixtures and of the commercial enzyme preparations (Econase or Celluclast supplemented with Novozym 188). ♦ Econase + Novozym 188, ▲ Celluclast + Novozym 188, ∗ TM 1, × TM 2, ● TM 3

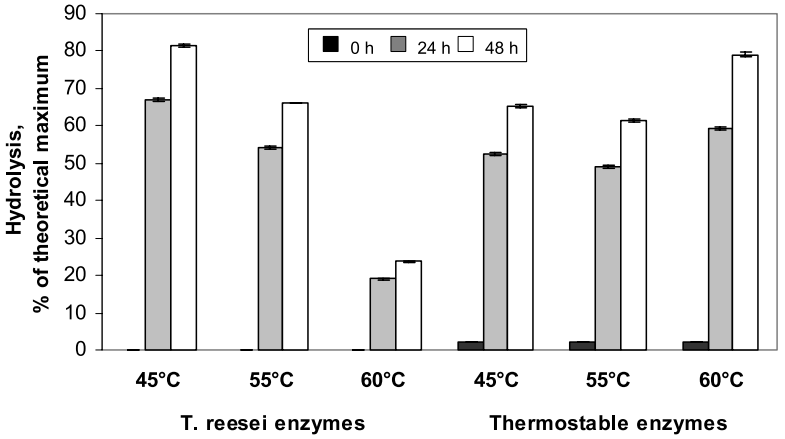

Fig. 4 Hydrolysis of cellulose (Avicel, 10 mg mL^{-1}, *left*) with Celluclast and the thermostable enzyme mixture (TM 1) at 45, 55 and 60 °C. Hydrolysis yield was measured as reducing sugars. Enzyme dosages: Celluclast 10 FPU g^{-1} cellulose, supplemented with 100 nkat Novozym 188 g^{-1} substrate (total activity 12 FPU g^{-1}); thermostable enzyme 10 FPU g^{-1} cellulose. Hydrolysis time 72 h at pH 5, triplicates with mixing. ■0 h, ▩24 h, □48 h

On the spruce substrate, the thermostable enzyme mixture resulted in an even more significant improvement in the performance at higher hydrolysis temperature as compared with the commercial enzymes. Thus, the hydrolysis yield was about threefold better at 55 °C and about fivefold better at 60 °C

using the thermostable enzyme mixture (Fig. 5). The hydrolysis was, however, also decreased with the thermoenzyme mixture at 60 °C. When comparing the hydrolytic performance of the commercial enzymes by increasing the temperature from 45 °C to 60 °C on Avicel and on spruce, it can be observed that the increased hydrolysis temperature decreased the performance on the natural lignocellulose substrate significantly more: from 70–10% on spruce, as compared with 90–30% on Avicel within 48 h. Obviously, the spruce substrate, even washed, contained compounds that, with increasing temperature, inhibited or inactivated not only the *T. reesei* enzymes, but also the thermostable enzymes.

High temperature enzyme mixtures suitable for hemicellulose-containing raw materials were evaluated in the hydrolysis of steam pretreated corn stover substrate (Fig. 6). With this raw material, the hydrolysis by the thermostable enzyme mixture at 45 °C was better than with the commercial preparation. The hydrolysis was still efficient at 55 °C and only slightly decreased at 60 °C with the thermostable enzyme mixture. The relative decrease of the hydrolytic performance of both enzyme preparations was less pronounced on the corn stover substrate than with the spruce substrate at elevated temperatures. Based on HPLC analysis (Table 4) of the corn stover hydrolysates, the yield of glucose was around 90–95% of the theoretical after 72 h. The corresponding yield of xylose was about 80–90% at temperatures up to 60 °C. The hydrolysis yields of the minor monosaccharide sugars, arabinose and galactose, were not significantly improved by the thermophilic enzyme mixtures, indicating the absence of corresponding thermostable enzymes, i.e. arabinosidases and galactanases in the mixtures. In the hydrolysis of the

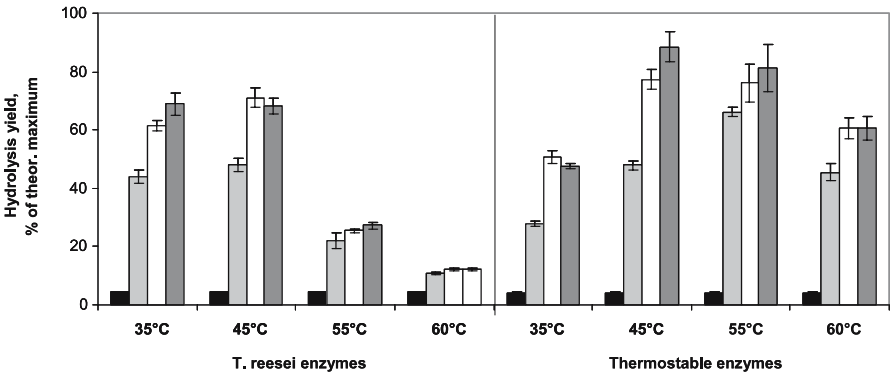

Fig. 5 Hydrolysis of pretreated washed spruce (10 mg mL^{-1}) with Celluclast and the thermostable enzyme mixture (TM 3) at temperatures from 35 to 60 °C. Hydrolysis yield was measured as reducing sugars. Enzyme dosages: Celluclast 5 FPU g^{-1} substrate, supplemented with 100 nkat Novozym 188 g^{-1} substrate; thermostable enzyme 5 FPU g^{-1} substrate. Hydrolysis time 72 h at pH 5, triplicates with mixing. ■0 h, ▩24 h, □48 h and ▨72 h

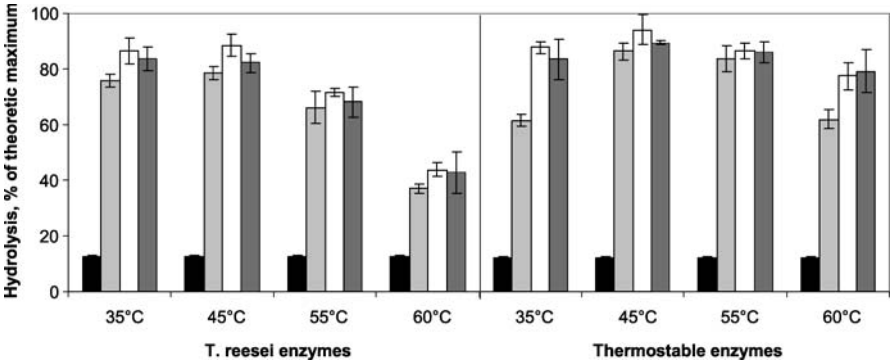

Fig. 6 Hydrolysis of pretreated corn stover (10 mg mL^{-1}) with Celluclast and the thermostable enzyme mixture (TM 3) at temperatures from 35 to 60 °C. Hydrolysis yield was measured as reducing sugars. Enzyme dosages: Celluclast 5 FPU g^{-1} substrate, supplemented with 100 nkat Novozym 188 g^{-1} substrate; thermostable enzyme 5 FPU g^{-1} substrate. Hydrolysis time 72 h at pH 5, triplicates with mixing. ■0 h, ▩24 h, □48 h and ▨72 h

Table 4 Sugars released from steam pretreated spruce and corn stover (% of the initial sugar component in the substrate), analysed by HPLC

Enzymes	Hydrolysis temp. (°C)	Sugars released from spruce % of theoretical Glucose	Sugars released from corn stover % of theoretical Glucose	Xylose	Arabinose	Galactose
Commercial enzymes (Celluclast + Novozym 188)	35	76	76	80	25	9
	45	75	83	81	25	13
	55	26	67	74	20	11
	60	9	28	50	6	4
Thermostable mixture (TM 3)	35	51	95	84	31	12
	45	95	90	84	36	15
	55	82	96	97	31	8
	60	56	81	85	22	2

Enzyme dosage was for reference enzymes: Celluclast 5 FPU g^{-1} substrate supplemented with 100 nkat g^{-1} Novozym 188; and for thermostable enzyme (TM 3) 5 FPU g^{-1} substrate. Hydrolysis time 72 h at pH 5, triplicates with mixing. Release of xylose, mannose and arabinose from spruce substrate was below the reliable detection limit (less than 0.1% of the substrate)

spruce substrate (Fig. 5, Table 4), only glucose was released. The individual sugar analyses corresponded well with the measured values of the reducing sugars.

9
Performance of the Thermostable Enzymes at Lower Temperatures

The performance of the thermostable enzymes at a lower temperature, the 35 °C commonly used in SSF, was compared. The *T. reesei* deletion strains produced only low amounts of background cellulase activities, mainly due to the presence of native EGIII (Cel12A) and EGV (Cel45A). However, the deletion strains used for the production of thermoenzymes produced some hemicellulases. For practical use, any mesophilic background activity enhancing the hydrolysis can be considered useful, but in order to evaluate the performance of the thermophilic enzymes, the level of remaining background activities was evaluated. The FPU activity in the background was negligible and the endoglucanase activity was very low as compared to the commercial preparations. Most of the endoglucanase activity, 85–90%, was inactivated during the thermal treatment at 60 °C, pH 6.5 for 2 h. Obviously, the EGV activity was the most stable remaining activity. Thus, the background activities originating from the *T. reesei* deletion strains had only a minor contribution to the total hydrolysis above 65 °C.

The actual hydrolysis performance of the new thermostable enzyme mixtures on various pretreated lignocellulose substrates (spruce and corn stover) at 35 °C showed some variations as compared with the *T. reesei* enzymes: on

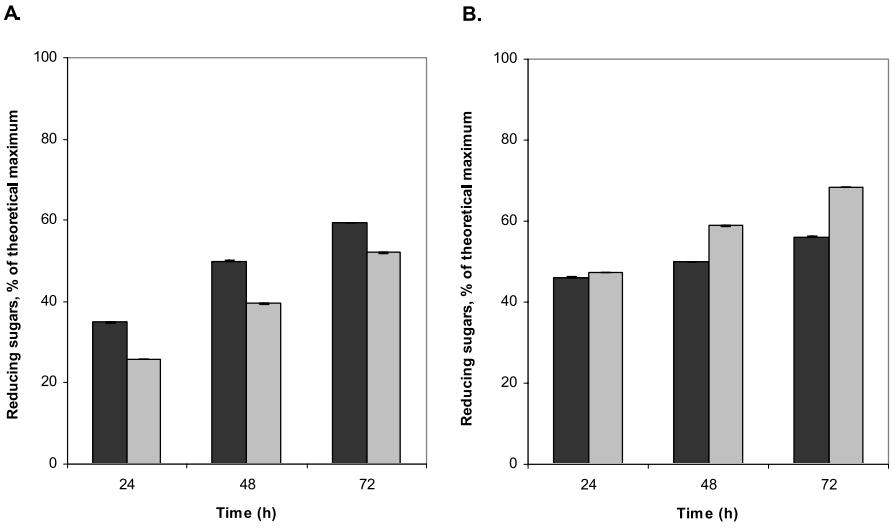

Fig. 7 Hydrolysis of steam pretreated washed spruce (**a**) and unwashed corn stover (**b**) by Celluclast (■) and (▩) the thermostable enzymes (TM 1 for spruce and TM 2 for corn stover) at 35 °C. Enzyme dosages: Celluclast 5 FPU g^{-1} substrate, supplemented with 100 nkat Novozym 188 g^{-1} substrate; thermostable enzymes 5 FPU g^{-1} substrate, substrate concentration 10 g L^{-1}, hydrolysis time 72 h at pH 5, triplicates with mixing

spruce, the sugar yield obtained by the thermophilic enzymes was generally lower and on corn stover higher than with the commercial *T. reesei* enzymes (Fig. 7). The result was the same, irrespective of the presence of the thermoxylanase in the preparation (TM 1 in Fig. 7 or TM 3 in Fig. 5). Thus, with this substrate the relatively lower cellulase activity at 35 °C is obviously the reason for the poorer hydrolysis at the lower temperature. In contrast, on the xylan-containing substrate, corn stover, the additional xylanase activity in the thermostable enzyme mixture had a more profound effect. The total xylanase activity was somewhat higher in the thermostable preparation, emphasising the importance of hemicellulases in the hydrolysis of substrates containing residual xylans. Further research would be needed to study in detail the structural differences of both cellulose and hemicellulose in the two substrates and their impact on the performance of the enzyme patterns used.

10 Discussion

When designing novel thermostable enzyme systems, the structural features of the substrates determine the number of enzymes needed for total hydrolysis. The crystallinity of cellulose, the available surface area and the distribution of lignin and hemicellulose are the major substrate-related factors limiting the hydrolysis rate of cellulose. An efficient pretreatment is the most straightforward solution for improving the hydrolysis rate and decreasing the amount of enzymes needed. Using various pretreatment techniques, either most of the hemicellulose or lignin is removed. It has been observed that the removal of hemicellulose has a direct correlation with the efficiency of the hydrolysis [56]. Even low amounts of residual xylan can limit the extent and rate of the hydrolysis. This can be overcome by addition of suitable hemicellulases, especially xylanases, to substrates with high original xylan content. Usually, the xylanase activity in commercial *T. reesei* preparations has been adequately high to overcome this limitation on xylan-containing substrates. Lignin content and distribution has also been proposed to be a substrate-related factor that affects the efficiency of enzymatic hydrolysis [49]. The close association of lignin and cellulose may prevent swelling of the fibrous substrate and result in limited accessibility of enzymes. The role of lignin or lignin-derived compounds in destabilising or deactivating enzymes is obviously also crucial. High temperature and pressure during the pretreatment result in a variety of soluble inhibitors for the enzymes and the yeast. In this work, it was observed that the inhibition of cellulases on lignin-containing substrates was increased at higher temperatures (Figs. 4 and 5).

The optimal cellulase composition varies depending on the substrate used but usually, the major cellulases comprise two cellobiohydrolases (about 60–70% of total protein), and two major and several minor endoglucanases

(about 25% of the total protein). Various models and mechanisms for the synergistic action of cellulases have been proposed. These studies have focused on the *T. reesei* exo–exo synergism [31, 53, 77, 80] or on the endo–exo synergism [39, 46, 52, 80]. The key role of β-glucosidase in a separate hydrolysis process has been clearly demonstrated, and is due to the end product inhibition of especially cellobiohydrolases caused by cellobiose [20, 54]. In *T. reesei* this activity is partly mycelium-bound and obviously limits the enzyme performance in commercial *T. reesei* preparations. Therefore, β-glucosidase is usually supplemented, generally originating from *Aspergillus niger*. Interestingly, in this work it was shown that just three major thermostable cellulases, i.e. one cellobiohydrolase and one endoglucanase supplemented by β-glucosidase, used in a preliminarily optimised ratio were able to produce a hydrolysis yield comparable with that obtained with the whole set of cellulolytic and accessory enzymes present in the commercial *T. reesei* preparations. Further research would be necessary to clarify the detailed mechanisms of these enzymes. Although the endo–exo synergism was obviously efficient enough to result in a high sugar yield, it could be further improved by optimising the thermostable cellulase components. The optimal ratio of the major enzymes was shown to be close to that of *T. reesei*. In this work, the individual thermostable cellulases were preliminarily screened based on their activity profiles and not based on their synergistic action. Therefore, the hydrolysis result can be considered extremely promising. Previously, thermostable enzymes from different organisms have not been combined to form new efficient mixtures. Expectedly, further optimisation, as well as supplementation of other synergistically acting enzymes would further improve the hydrolytic efficiency. In the present work, only thermostable xylanase was added to the mixture of the three cellulases (endoglucanase, cellobiohydrolase and β-glucosidase).

Previously, thermostable enzymes have only been studied as individually added proteins to improve the performance of the cellulases from *T. reesei* [62]. The *T. reesei* cellulase system is rapidly inactivated at temperatures above 45 °C, and the optimal temperature is generally considered to be below 45 °C on substrates requiring longer hydrolysis times, e.g. due to higher substrate consistency. Crude culture filtrates from various moderately thermophilic fungi (*C. thermophilum, T. terrestris, T. aurantiacus, C. thermophilus, M. thermophila*) were added on the protein basis to a commercial *T. reesei* preparation. Obviously, due to the relatively high proportion of *T. reesei* enzymes in the mixture, and the consequent inactivation of these enzymes at elevated temperatures, no improvement of the hydrolysis at higher temperatures could be observed. The main advantage was expected to be due to more active endoglucanases or due to a improved improved ratio of endoglucanase and cellobiohydrolase in the crude fermentation broth. In addition, unidentified enzyme activities in the preparations may also have caused some effects.

In this work, the individual cloned thermostable enzymes were produced with a *T. reesei* strain where the four genes encoding the major cellulases, i.e. Cel7A, Cel6A, Cel7B and Cel5A, had been deleted. Thus, only the minor endoglucanases Cel12A, Cel61A and Cel45A, as well as xylanases and other accessory enzymes, were present in the *T. reesei* background. In addition, most of these activities were inactivated in a thermal treatment. Only the Cel45A was somewhat more resistant to thermal inactivation and retained most activity at higher temperatures. Thus, the hydrolysis results were non-disputably obtained due the cloned thermostable enzymes, and the background activities were negligible. This was also clear from the hydrolysis experiments with the commercial *T. reesei* enzymes, showing clearly a decreased performance at temperatures of 50 °C or above.

In addition to improved performance in the hydrolysis of lignocellulosic substrates, thermophilic enzymes allow the design of more flexible process configurations. Traditionally, *T. reesei* enzymes are used either in a separate hydrolysis and fermentation process (SHF) or in a simultaneous saccharification and fermentation (SSF) process. It is commonly stated that the major advantage of the SHF is that both process steps (hydrolysis and fermentation) can be run under optimal conditions. Typically, hydrolysis of the SHF is carried out at around 45–50 °C at pH 5, and the fermentation at 35 °C at a lower pH. The SSF, on the other hand is usually carried out at 35 °C at pH 4.5–5. A more efficient hydrolysis is expected to take place at higher temperatures. In this work, using thermostable enzymes, it was indeed possible to obtain about 10 °C higher operation temperature than with the present commercial *T. reesei* enzyme preparations. The applicable hydrolysis temperature could be 60–65 °C for the hydrolysis of corn stover substrate and about 55 °C for spruce substrate. The hydrolysis rates at 55 °C were higher than those of the commercial enzymes at 45 °C. The enzymatic hydrolysis at higher temperatures would potentially reduce the reaction time and the enzyme loading.

It can be concluded that the cloned thermostable enzymes in preliminarily optimised preparations clearly demonstrate that the hydrolysis of lignocellulosic raw materials can be further improved, leading to potential savings in the hydrolysis costs. Previously, it has been shown that the costs of cellulases can be radically decreased, e.g. by improving the specific activity, by omitting the downstream processing of enzyme production or by improving the production process by other means. A mixture of only four thermostable enzymes was shown to be superior to the present commercial *T. reesei* preparations, which are comprised of at least ten enzymes acting synergistically on cellulose and on other components of the lignocellulosic substrates under optimal conditions. Further supplementation of other cellulases or accessory enzymes would expectedly further improve the hydrolysis result and the overall economy of the process.

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