The Use of Cellulases from a β-Glucosidase-Hyperproducing Mutant of *Trichoderma reesei* in Simultaneous Saccharification and Fermentation of Wheat Straw

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Conidia of the cellulolytic strain *Trichoderma reesei* F-522 were mutagenized with UV irradiation and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). A visual agar plate detection system was developed, using esculin and ferric ions, to identify mutants of *T. reesei* with increased β-glucosidase activity. Selected mutants were tested for production of extracellular cellulases in shake flasks on autohydrolyzed wheat straw as carbon source. The most active mutant V-7 showed about 6-times higher activity of β-glucosidase than the parent strain F-522, whereas the filter paper degrading and endo-1,4-β-D-glucanase activities increased by 45% and by almost 31%, respectively. Cellulase preparations obtained from the parent and mutant strains were then used along with *Kluyveromyces fragilis* cells for ethanol production from ethanol-alkali pulped straw in the simultaneous saccharification and fermentation (SSF) process. From 10% (w/v) of straw pulp (dry matter), 2.5% (w/v) ethanol was obtained at 43°C after 48 h using cellulase derived from the parent strain of *T. reesei*. When the β-glucosidase-hyperproducing mutant V-7 was employed, the ethanol yield in the SSF process increased to 3.4% (w/v), the reaction time was shortened to 24 h and no cellobiose was detected in straw hydrolyzates.

INTRODUCTION

Production of ethanol from lignocellulosics is a subject of intensive research because ethanol can be either blended with gasoline as a fuel extender and octane enhancing agent or used as a neat fuel in internal combustion engines.[1] The utilization of low-cost and renewable plant biomass requires a pretreatment of this material to enhance its susceptibility to microbial or enzymatic attack, mainly by removing lignin which is a nonfermentable component that makes cellulose unavailable for biodegradation.[2]

In recent years, an ethanol production process has been developed which utilizes *Trichoderma reesei* cellulase and yeast cells in the simultaneous saccharification and fermentation (SSF) of cellulose to ethanol.[3] The direct production of alcohol from cellulose in an SSF process alleviates the problem of end product inhibition by glucose during saccharification as well as eliminates the need for separate reactors for saccharification and fermentation.[4]

Trichoderma reesei is known to be one of the best producers of cellulases but, although this organism excretes high levels of cellulose solubilizing activities, the extramycelial yields of β-D-glucosidase produced in the culture filtrates are low.[5,6] The use of such preparations in the enzymatic saccharification of cellulose lead to a significant accumulation of cellobiose in cellulose hydrolyzates. It is well known that this intermediate decreases the rate of saccharification because it is a stronger inhibitor of cellulase enzymes than glucose.[7] On the other hand, the inability of industrial glucose-fermenting yeasts to ferment cellobiose[8] results in incomplete conversion of cellulose hydrolyzate to ethanol. These drawbacks may be overcome by selection of *T. reesei* mutants with higher β-glucosidase activity,[9] supplementation of the cellulase complex with a β-glucosidase from another source (often an *Aspergillus* species)[10] or selection of yeast mutants that are able to ferment glucose and cellobiose simultaneously.[11]

Recently,[12] the authors have selected the thermotolerant yeast strain *Kluyveromyces fragilis* FT23 capable of producing a high ethanol yield in cellulolysis conditions. The present article presents the screening of a hyperproductive β-glucosidase mutant of *T. reesei* and the utility of its cellulase complex together with yeast cells in simultaneous saccharification and fermentation of pretreated wheat straw to ethanol.

MATERIALS AND METHODS

Microorganism

Trichoderma reesei strain F-522 (QM9414) from the Czechoslovak Collection of Microorganisms in Brno was used as a starting strain for mutation. Conidia for mutagene-

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sis and inoculations were produced on potato dextrose agar slants for seven days at 28°C.

Mutagenesis and Isolation of β-Glucosidase Mutants

A water suspension of *T. reesei* conidia (10^7/mL) was exposed to UV irradiation at a dose of 2.5×10^3 erg/mm^2 and treated with 0.05% solution of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) for 15 min. To stop NTG activation, the conidial suspension was centrifuged (5 min at 10,000 g) and the washed conidia were resuspended in sterile distilled water. The survival rate after the combined mutagenic treatment was 0.1–0.3%. The mutagenized conidia were then plated on Vogel's agar medium[13] supported by 0.1% peptone, 1% cellobiose and 0.02% sodium deoxycholate as colony restrictor. Cultures were incubated at 28°C. Following colony formation, they were overlayered with agar containing esculin and ferric ammonium citrate.[14] Potential high yielding β-glucosidase mutants were visualized as those colonies which were surrounded by a brown-black zone which is produced by ferric ions and esculin in the presence of β-glucosidase. The diameter of the zone and intensity of the color were taken as the indexes of the extracellular β-glucosidase.

Pretreatment of Wheat Straw

Wheat straw was milled in a knife mill and a medium fraction (0.2 to 0.5 mm) was either exposed for 10 min to autohydrolysis at 200°C,[15] or chemical pulping by a mixture of equal volumes of 96% ethanol and 8% NaOH (30 min at 170°C).[16] After the pretreatment the samples were thoroughly washed with water, pressed out to obtain straw of about 26 wt % dry matter, and stored at −18°C until use. The composition of native straw was 20.6% lignin, 43.2% hexosans, and 26% pentosans. The autohydrolysis-treated straw contained 30.6% lignin, 61.9% hexosans, and 1.7% pentosans. Ethanol-alkali pulping gave a straw material which contained 5.4% lignin, 68.2% hexosans, and 17.3% pentosans.

Flask Cultures

The most active mutants of *T. reesei* along with the parent strain were assayed for their cellulolytic activity in shake flask cultures. The enzyme production medium (pH 5.5) developed by Mandels and Weber[17] in 500-mL conical flasks, 100 mL each, supported by 0.1% proteose peptone, 0.02% Tween 80, and 1.5% autohydrolyzed wheat straw (dry basis) was autoclaved for 30 min at 117°C and seeded with 10% (v/v) of the 48 h mycelium, previously incubated with shaking in the same medium but with 2% glucose. The submerged culture was run for 6 days at 28°C on a rotary shaker at 220 rpm. The biomass formed (mycelium plus residual straw) was separated from the medium by centrifuging (20 min at 4000 g), and enzyme activity and extracellular protein were analyzed in the supernatant fraction.

Cellulase Source

Crude cellulase preparations for SSF-experiments were obtained from the post-culture fluid of *T. reesei* by ultrafiltration (UM10 membrane, Amicon) in an Amicon ultrafiltration cell (model 202). The concentrated enzyme solution (about twentyfold) was sterile filtrated (Schleicher and Schüll, disposable membrane filters) prior to use.

Yeasts

The yeast strain *Kluyveromyces fragilis* FT23 selected earlier[12] was employed in SSF-experiments. The basal medium for yeast maintenance was (g/L): yeast extract (Oxoid), 10.0; NaCl, 1.0; $CaCl_2 \cdot 2H_2O$, 0.2; KH_2PO_4, 2.0; $FeCl_3 \cdot 6H_2O$, 0.01; $MgSO_4 \cdot 7H_2O$, 1.7; NH_4Cl, 2.0; and distilled water to give 1000 mL. The precultures (100 mL in 500-mL conical flasks) were inoculated from malt agar slants and incubated with shaking for 24 h at 28°C in the basal medium with 20 g/L glucose until sugar had been exhausted.

Simultaneous Saccharification and Fermentation

SSF-reaction-mixtures consisted of 10% ethanol-alkali modified wheat straw (dry basis), which had previously been autoclaved for 15 min at 121°C, 40 FP cellulase units/g substrate, 14–64 U of β-glucosidase/g substrate, 10% yeast inoculum, and basal medium to give 100%. The SSF-mixture had a pH of about 5.1. All tests were carried out in 200-mL conical flasks with 100-mL working volume. Ethanol evaporation was prevented by rubber stoppers with fermenting tubes filled with 50% H_2SO_4. Temperature was 43°C. The reaction mixtures were shaken for 48 h at 150 rpm. Samples were harvested aseptically and assayed for ethanol, glucose, reducing sugars, and final pH.

Saccharification and Subsequent Fermentation

Chemically modified straw (10% dry basis) was saccharified for 48 h at 43°C with 40 FPU/g and 14–64 U/g of β-glucosidase from *T. reesei* in 100 mL of basal medium (pH 5.1). Saccharification was run in 200-mL conical flasks on a water bath shaker at 150 rpm. After sterile centrifugation (10 min at 10,000 g) the hydrolyzate liquid was assayed for glucose and reducing sugars. It was then subjected to fermentation in a 200-mL flask by adding 10% of yeast inoculum and reincubated by 48 h at 43°C under anaerobic conditions (see SSF process). The production of yeast biomass was determined in the incubation mixture, and after centrifugation the supernatant was analyzed for ethanol yield and sugar uptake.

Analytical Procedures

Cellulase (FPase) activity was assayed by the method of Mandels et al.,[18] using Whatman no. 1 filter paper as a substrate and was expressed as filter paper activity in terms of filter paper unit (FPU). β-1,4-Glucosidase (βGDase) activity was measured with cellobiose as a substrate[19] and the free glucose release was monitored by the glucose oxidase method.[20] Endoglucanase (CMCase) activity was estimated with carboxymethylcellulose as a substrate.[18] One unit of enzyme activity (*U*) is defined as the amount of the enzyme which liberates 1 μmol of reducing sugars (calculated as glucose) per minute under the assay conditions. In the case of βGDase, one unit represents 1 μmol/min of substrate converted and 2 μmol/min of glucose formed, respectively. Reducing sugars were measured by the Somogyi-Nelson method.[21,22] The content of sugars in straw hydrolyzates was determined by gas-liquid chromatography as previously described.[23] Glucose was analyzed using the glucose oxidase method.[20] Ethanol was determined according to Kellermann.[24] Lignin analysis was performed using a gravimetrical method[25] and corrected for ash. The content of hexosans and pentosans was determined with o-toluidine reagent.[26] Soluble protein was assayed by the method of Schacterle and Pollack.[27] The growth of yeast cells (biomass) was monitored by correlating cell dry weight with optical density at 650 nm.[28]

Calculations

The percent of saccharification was calculated using the equation:

$$\text{Saccharification (\%)} = \frac{\text{reducing sugars} \times 0.9}{\text{carbohydrates in straw}} \times 100$$

RESULTS AND DISCUSSION

Mutation Studies

The conidia of the parent strain *Trichoderma reesei* F-522 were subjected to mutagenesis by using a combined UV-NTG technique. Out of 4000 colonies growing on agar plates containing a β-glucosidase detection system, 50 strains that showed the greatest diameter of the enzymatic zone were isolated and further tested for cellulase and protein production in shake flask cultures on autohydrolyzed wheat straw as inducing carbon source. The results for the best six mutants along with the parental strain are presented in Table I. The activity of β-glucosidase of the investigated mutants in comparison with a starting nonmutagenized strain *T. reesei* F-522 was generally higher (from two times in the case of *T. reesei* IX-2 to nearly six times in *T. reesei* V-7). These results are parallel to those achieved for the enhancement of β-glucosidase production in other cellulolytic fungi, namely, *Trichoderma harzianum*,[29] *Aspergillus terreus*,[30] *Fusarium graminearum*[31] and *Sclerotium rolfsii*.[32] The increase in β-glucosidase activity has been found to be usually associated with enhanced synthesis of other cellulase components (except the mutants II-58 and VII-323) as well as with higher extracellular protein production. The most active mutant V-7 showed β-glucosidase activity about 6-times higher, filter paper-degrading activity 45% higher, and endoglucanase activity increased by 31% in comparison with the parent strain. The increased yield of cellulases production by the selected mutant was stable in subsequent subcultures.

Table I. Cellulase and extracellular protein production by parent and mutant strains of *Trichoderma reesei* after 6 days submerged growth on Mandels and Weber medium with 1.5% autohydrolyzed wheat straw.

T. reesei strain	Cellulase complex activity[a] (U/mL)			Extracellular protein (mg/mL)
	FPase	CMCase	βGDase	
Parent				
F-522	0.51	23.1	0.20	2.8
Mutant				
I-123	0.62	28.3	0.52	3.5
II-58	0.40	25.7	0.81	2.9
V-7	0.74	30.2	1.15	3.4
VI-274	0.78	27.4	0.60	3.0
VII-323	0.60	20.9	0.93	3.2
IX-2	0.71	32.3	0.43	3.3

[a] Abbreviations: FPase = cellulase, CMCase = endo-1,4-β-D-glucanase, βGDase = 1,4-β-D-glucosidase.

The post-culture fluids of the best mutant and parent strain obtained after 6-day growth on waste cellulose were concentrated by ultrafiltration and used as cellulase source in SSF-experiments. Cellulase preparations were applied for straw saccharification at a constant concentration of 40 FPU/g substrate which, as previously described,[33] guaranteed a high degree of straw conversion to sugar syrup. At this FPase dose the activity of β-glucosidase for the parent and mutant strain was 14 and 64 U/g of straw, respectively.

Simultaneous Saccharification and Fermentation

Conversion of ethanol-alkali pulped straw to ethanol by combined hydrolysis and fermentation was carried out using *T. reesei* cellulase and the thermotolerant yeast strain *Kluyveromyces fragilis* FT23 selected earlier.[12]

To determine the basic factors of hydrolysis and fermentation (i.e., the degree of saccharification, sugars content in straw hydrolyzates and yeast biomass), which cannot be defined in SSF process, a two-step system was carried out simultaneously (saccharification and subsequent fermentation) in which hydrolysis and fermentation were separated. The results of SSF experiments are summarized in Tables II and III. When 10% wheat straw was fermented at 43°C by a mixture of *T. reesei* cellulase derived from the parent strain and *Kluyveromyces* cells, 2.5% (w/v) of ethanol was obtained after 48 h. Glucose was completely metabolized in this process (Table II). At the same time

Table II. Simultaneous saccharification and fermentation[a] of chemically modified straw by *Trichoderma reesei* cellulase preparations derived from the parent and βGDase mutant strain and *Kluyveromyces fragilis* cells.

Cellulase source	Activity[b] (U/g straw) FPase	βGDase	Time (h)	Ethanol percent (w/v)	Glucose in hydrolyzate (mg)	Final pH
T. reesei F-522 (parent)	40	14.1	24	1.9	—	5.0
			48	2.5	0	4.9
T. reesei F-522-V-7 (mutant)	40	64.2	24	3.4	—	4.7
			48	3.4	0	4.7

[a] Substrate, 10%; yeasts (simultaneously added), 10%; 43°C; initial pH 5.1.
[b] Abbreviations as in Table I.

Table III. Saccharification and subsequent fermentation[a] of chemically modified straw by *Trichoderma reesei* cellulase preparations derived from the patent and βGDase mutant strain and *Kluyveromyces fragilis* cells.

Cellulase source	Activity[b] (U/g straw) FPase	βGDase	Time (h)	Hydrolysis: Saccharification (%)	Hydrolysis: Glucose content (%)	Fermentation: Ethanol percent (w/v)	Fermentation: Ethanol yield[c] % theoretical	Fermentation: Glucose in hydrolyzate (mg)	Fermentation: Yeast dry weight (mg/100 mL)	pH
T. reesei F-522 (parent)	40	14.1	0	—	—	—	—	—	190	5.1
			24	77.8	45.4	1.8	77.6	—	580	4.9
			48	86.1	54.3	2.4	86.6	0	695	4.8
T. reesei F-522-V-7 (mutant)	40	64.2	0	—	—	—	—	—	195	5.1
			24	87.9	73.5	3.3	88.0	—	720	4.7
			48	90.1	74.8	3.4	89.0	0	735	4.6

[a] The hydrolysis and fermentation conditions are the same as in Table II. The yeasts were added after 48 h hydrolysis.
[b] Abbreviations as in Table I.
[c] Calculated from the glucose content.

the degree of straw saccharification and glucose content in hydrolyzate were 86 and 54%, respectively. Basing on the glucose formed, the ethanol yield was about 87% of the theoretical value. Moreover, an over threefold increase in yeast biomass with a simultaneous small decrease of the medium pH was observed (Table III). Unfortunately, the chromatographic analysis of the products of straw hydrolysis, using the cellulase preparation from the parent strain of *T. reesei,* still showed 20% of cellobiose in straw hydrolyzates (Table IV). The presence of considerable amounts of cellobiose in the reaction mixture resulted from a low yield of β-glucosidase in the enzymatic complex of the parent strain of *T. reesei.*

The use of the cellulase preparation from the mutant V-7 with 4.5-times higher β-glucosidase activity increased the ethanol yield in the SSF process to 3.4% (w/v) and shortened the reaction time to 24 h (Table II). Furthermore, this procedure also involved a distinct increase in

Table IV. Content of sugars in hydrolyzates of chemically modified straw after 48 h hydrolysis[a] using *Trichoderma reesei* cellulase preparations derived from the parent and βGDase mutant strain.

Cellulase source	Activity[b] (U/g straw) FPase	βGDase	Hydrolysis products (% dry wt) D-Glucose	D-Cellobiose	D-Xylose	L-Arabinose	Others
T. reesei F-522 (parent)	40	14.1	53.4	20.1	9.8	1.5	15.2
T. reesei F-522-V-7 (mutant)	40	64.2	74.0	trace	10.3	1.7	13.9

[a] Hydrolysis conditions: wheat straw, 10%; temperature, 43°C; pH 5.1.
[b] Abbreviations as in Table I.

the content of glucose (from 20 to 28%) during the hydrolysis stage as compared with that reached by using cellulase preparation derived from the parent strain (Table III). As can be seen from the data in Table IV the application of the enzyme complex from a β-glucosidase-hyperproducing mutant completely removed cellobiose from straw hydrolyzates by hydrolysing it to glucose, the content of which increased to 74%.

The presented results showed that the relationship between β-glucosidase and cellulase in the enzymatic complex of *T. reesei,* besides the yeasts used, is an important factor determining effective conversion of cellulose to ethanol. The high activity of β-glucosidase in the enzymatic complex of cellulases increased the rate of saccharification of straw with glucose as the main hydrolysis product. On the other hand, in our SSF experiments the use of the thermotolerant yeast strain fermenting glucose at higher temperatures enabled the obtaining of a high production efficiency of ethanol from wheat straw in a short time.

The obtained results indicate that further studies are required to examine the use of the complex of cellulases of the *T. reesei* mutant for hydrolysis of wheat straw in the SSF process by using laboratory fermenters.

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