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Recycle of cellulases and the use of lignocellulosic residue for enzyme production after hydrolysis of steam-pretreated aspenwood

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SUMMARY

Various modes of substrate and enzyme addition were used to hydrolyze a 10% concentration (w/v) of steam-exploded, water-and-alkali extracted aspenwood with *Trichoderma harzianum* E58 cellulases. Although cellulose conversion was high (94–100%), enzyme recovery was low in all cases. Low enzyme recovery was due to a combination of thermal inactivation and adsorption of the cellulases onto the lignocellulosic residue. Enzyme recycle was not feasible as the activity of the recovered cellulases towards crystalline cellulose was low. However, the residual material from enzyme hydrolysis was a suitable carbon source for cellulase enzyme production by *T. harzianum* based on enzyme yield and hydrolytic potential. These residues could only be used up to a 1% substrate concentration, since at higher substrate loadings cellulase production was reduced, likely because of lignin inhibitors.

INTRODUCTION

The commercial production of ethanol from lignocellulosic feedstock requires technical improvements to achieve economical production [12]. Recycle of the cellulase enzymes after hydrolysis of the substrate could improve the economics of the process. A major problem in cellulase recycle is the adsorption of the enzyme onto the substrate [8,15]. The proportion of enzyme remaining adsorbed to the residual cellulosic substrate after hydrolysis is dependent on enzyme/substrate ratio, percent hydrolysis, and lignin content of the substrate. Vallander and Eriksson [15] found that recycle of the enzyme is feasible when the enzyme-residue complex is also recycled. However, if applied to a continuous process, this procedure will result in increasing substrate levels due to recycle of inert material (i.e. lignin). As the process continues, a decreasing proportion of the solids in the reactor will be cellulose, and mixing problems will increase.

Steam-pretreated aspenwood, following water-and-alkali extraction, is routinely used for both hydrolysis and enzyme production. Glucose yield obtained per tonne of wood is reduced in proportion to the amount needed for enzyme production. The amount of substrate diverted to enzyme production could be decreased by recycling the residue remaining after hydrolysis to the enzyme production reactor.

In an earlier paper, we discussed the relationship between quantitative enzyme recovery and substrate conversion [8]. To obtain quantitative enzyme recovery during hydrolysis, near complete conversion of the cellulose to sugars was necessary. The optimum level of enzyme loading for hydrolysis, using the enzymes derived from *T. harzianum* E58, was 50 IU filter paper activity (FPU)/g substrate [7]. For a commercial process, the substrate loading must be greater than 10% to keep process costs to a minimum. The present paper investigates enzyme recycle under process conditions of 10% substrate and 50 FPU/g enzyme loading.

MATERIALS AND METHODS

Cellulase preparation. The fungus *Trichoderma harzianum* E58 was obtained from the Forintek Culture Collection. A spore inoculum was used to initiate growth in shake flasks containing Vogel's [11] medium and 1% glucose. After 3 days at 28 °C the mycelial suspension was used to initiate growth in either 500-ml Erlenmeyer flasks containing 150 ml Vogel's medium or in a 30-l New Brunswick fermentor containing 20 l Vogel's medium.

The inoculated shake flasks, containing various lignocellulosic substrates, were incubated for 4 days at 28 °C

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and 110 rpm. The culture filtrates were harvested by filtration through Whatman glass microfibre filters and assayed for enzymatic activity.

The fermentor-produced cellulase preparation, used for the hydrolysis of 10% substrate, was grown on 1% Solka Floc BW300. The culture filtrate was concentrated by ultrafiltration using a Pellicon Cassette System (Millipore), fitted with a polysulfone membrane with a molecular cut-off of 10000 Da, then freeze dried.

The purified exoglucanase, derived from *T. harzianum* E58, was kindly donated by Dr. L.U.L. Tan.

Assays. Total reducing sugars were determined colorimetrically using dinitrosalicylic acid reagent [10].

Enzymatic activity was determined by the method of Mandels et al. [6]. One ml of a suitably diluted culture filtrate was added to 1 ml 0.05 M citrate buffer at pH 4.8. The substrates for the filter paper, endoglucanase, and β-glucosidase assays were a 1 × 6-cm strip of Whatman No. 1 filter paper, 10 mg carboxymethyl cellulose (CMC medium viscosity, D.S. 0.7, Sigma) and 10 mg salicin (Sigma), respectively. All three assays were performed at 50 °C. The filter paper assay was incubated for 1 h, and the endoglucanase and β-glucosidase assays were incubated for 30 min. All three assays were terminated by adding 3 ml dinitrosalicylic acid reagent to each tube and boiling the tubes for 5 min. The tubes were cooled to room temperature and the absorbance read at 575 nm. One unit of enzyme activity was defined as 1 μmol glucose equivalents released per minute.

Sugar analysis. The sugars produced during enzymatic hydrolysis were analyzed on a Varian HPLC system (Model 5000) interfaced with a Varian CDS 401 data system. An HPX-87H column (Bio-Rad) was operated with a guard column (Cation H; Bio-Rad) using 0.01 N sulphuric acid as the eluent. The flow rate was 0.6 ml/min, and the column temperature was 70 °C. A Varian RI-3 refractive index detector was used to monitor the column effluent.

All experiments and assays were performed in duplicate, and the values averaged. The variability between duplicates was within 5%.

Substrate preparation. Aspenwood chips were saturated with steam at 240 °C for 80 s, then steam-exploded. A 5% slurry of the material was extracted at room temperature for 2 × 1 h with water to produce the substrate, SEA-WI (steam-exploded aspenwood-water insolubles). A portion of this material was subsequently extracted with 0.4% NaOH (2 × 1 h), then washed thoroughly with water until the washings were neutral, to produce SEA-WAI (steam-exploded aspenwood-water and alkali insolubles). The chemical composition of SEA-WI was 62.5% cellulose and 30.7% total apparent lignin, while SEA-WAI was 84.4% cellulose and 9.0% total apparent lignin.

The hydrolysis residue, SEA-WAI, HR, was obtained by enzymatic hydrolysis of SEA-WAI. The unhydrolyzed residue was collected by centrifugation at 10000 rpm for 30 min. Soluble sugars were removed by resuspension of the pellet in distilled water and recentrifugation.

RESULTS

Cellulase recycle

To parallel process conditions, the steam-treated, water-and-alkali-washed aspenwood was hydrolyzed at a 10% concentration (w/v). Agitation of a 10% concentration of steam-treated wood is difficult because of its low bulk density (90–120 g/l). Complete conversion at this substrate level requires long residence times. Enzyme inactivation, however, increases with the residence time. To overcome these problems, the substrate and enzyme were added to the hydrolysis reactor by a fed-batch procedure. Fig. 1 shows the combinations of substrate and enzyme addition used in this study for the hydrolysis of 10% SEA-WAI. High conversion levels were achieved for all the systems (Table 1), with the highest hydrolysis level (100%) obtained when a double fed-batch method (i.e. two-step addition of enzymes and substrate) was used.

The culture filtrates from the various hydrolysis reactions were filtered using an ultrafiltration unit fitted with a 10 kDa molecular weight cut-off membrane. The cellulase enzymes and any other high molecular weight compounds were retained by the membrane; the sugars and low molecular weight compounds produced during hydrolysis were removed. Recovery of endoglucanase and filter paper activity was low, but recovery of β-glucosidase activity was much higher (Table 2). The low recovery of cellulase enzymes following extensive hydrolysis could not be solely due to adsorption onto cellulose, as there was only a low level of glucan remaining in the residue. The original substrate contained 84.4% cellulose and 9.0% lignin. After extensive hydrolysis, a lignin-rich

TABLE 1

Hydrolysis of 10% SEA-WAI using protocols outlined in Fig. 1

Experiment no.	Hydrolysis products[a]		Percent[b] hydrolysis
	glucose, g/l	cellobiose, g/l	
1	83.1	7.4	96.9
2	81.6	7.6	95.6
3	80.2	7.8	94.3
4	83.1	8.2	97.8
5	85.1	8.5	100.3

[a] Sugar determinations were performed by HPLC.
[b] Glucan content of SEA-WAI was 84.4%.

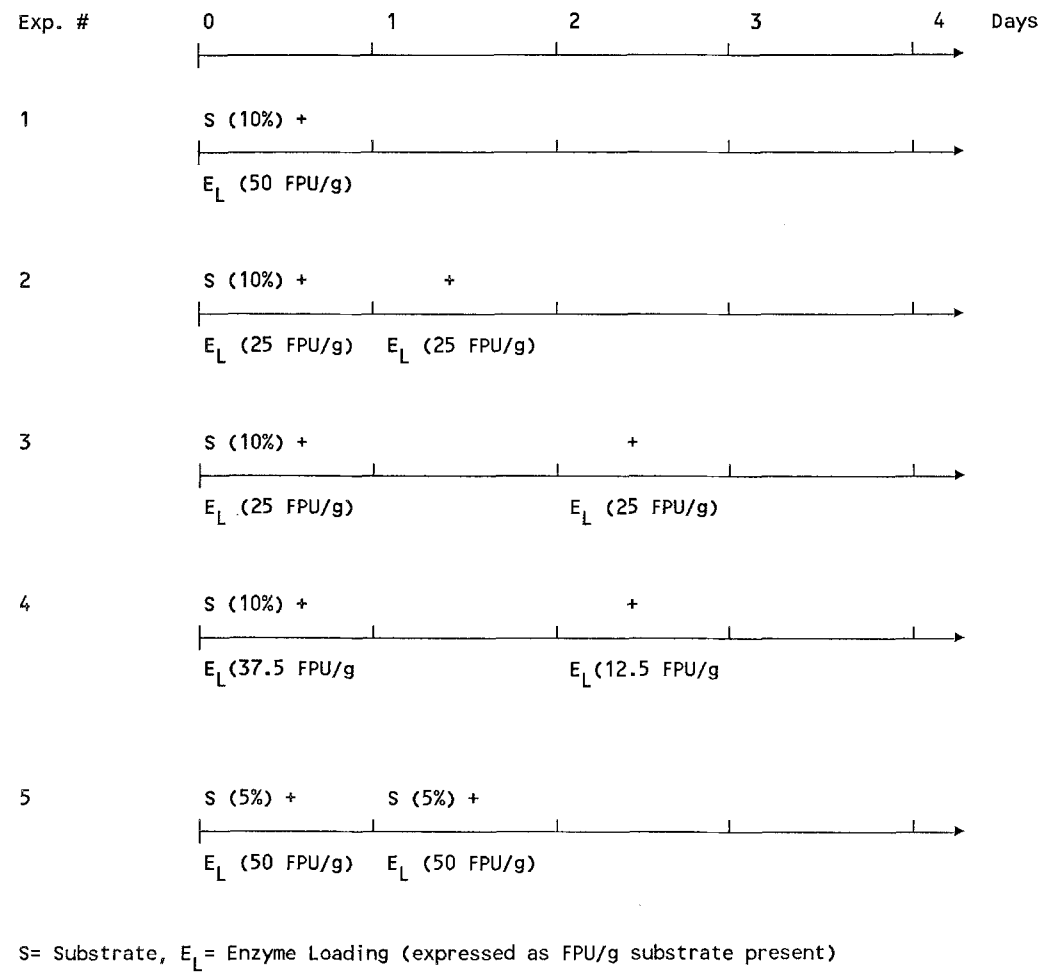

Fig. 1. Comparison of protocols for enzymatic hydrolysis of 10% SEA-WAI at an enzyme loading of 50 FPU/g substrate. Various combinations of enzyme and substrate were added during the 4 days of incubation.

TABLE 2

Recovery of cellulase enzymes following hydrolysis of 10% SEA-WAI

Experiment no.	Percent hydrolysis[a]	Enzyme activities (% of original)[b]		
		endoglucanase	β-glucosidase	filter paper activity
3	94.3	8.6	39.9	10.8
2	95.6	11.5	41.6	16.1
1	96.9	15.2	43.0	22.9
4	97.8	12.0	46.0	16.9
5	100.3	12.4	40.6	17.5

[a] Hydrolysis was performed as described in Fig. 1.

[b] Original enzyme activity was endoglucanase, 86.6 IU/ml; Filter paper activity, 5.0 FPU/ml; and β-glucosidase, 5.2 IU/ml.

residue remains. Other researchers [2,14] have demonstrated that the cellulase enzymes have a strong affinity for the lignin-carbohydrate complex. The low recovery values can be partially explained by the strong adsorptive properties of the cellulases.

A major problem in adsorption studies is that the activity of the free enzymes is measured, and adsorption of the remaining enzyme is inferred. After 4 days hydrolysis, a portion of the enzyme system may have been denatured, but our test would have reported these as adsorbed enzymes. The thermal stability of the enzymes may be such that little activity remains after 96 h at 45 °C, or alternatively, degradative products may form during hydrolysis which denature the enzymes. These concerns were partially addressed by examining the extent of thermal inactivation during hydrolysis. Enzyme stability over a 4-day incubation period was studied. The enzyme system was buffered and agitated to duplicate the hydrolysis conditions, but in the absence of substrate. The endoglucanase component appeared to be the most thermolabile, losing 20% of its activity over a 4 day period (Fig. 2). The preparation lost 10% of the filter paper activity and 15% of the β-glucosidase activity after 4 days. To evaluate the effect of thermal inactivation on the hydrolytic efficiency of the cellulases, the ability of these cellulases to hydrolyze cellulose was tested. Enzyme samples were recovered at various times during incubation at 45 °C, then incubated with fresh crystalline cellulose for 24 h at 45 °C

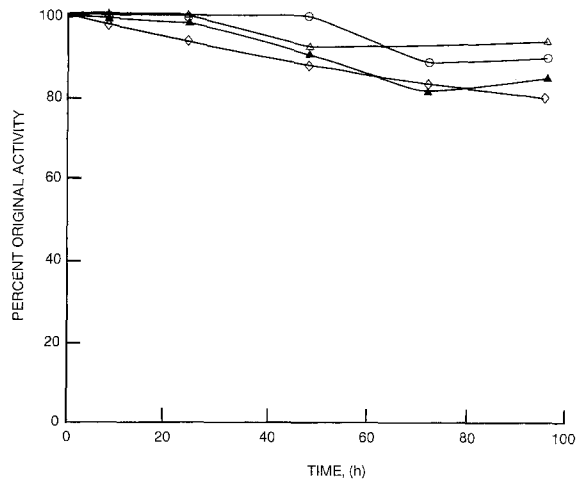

Fig. 2. Thermal stability of the endoglucanase (◊), β-glucosidase (▲), and filter paper (○) activities of *T. harzianum* E58 cellulases and purified exoglucanase (△) activity after incubation at 45 °C, pH 4.8. Enzyme activity (IU/mg protein) profile for the cellulase preparation was: endoglucanase, 13.30; β-glucosidase, 0.45; filter paper activity, 0.67. Protein concentrations of the incubated cellulases and purified exoglucanases were 1.12 and 1.04 mg/ml.

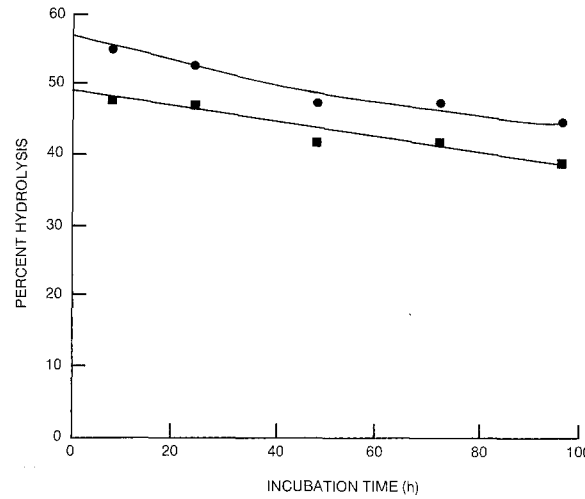

Fig. 3. Effect of thermal inactivation on the hydrolytic efficiency of the preincubated cellulases to degrade a 2% (w/v) concentration of Solka Floc BW300 to reducing sugars (●) and glucose (■). Enzyme fractions were collected at various times during incubation at 45 °C (Fig. 2). The enzyme solutions were filter-sterilized and added, without dilution, to the hydrolysis systems. After 24 h incubation at 45 °C the amount of sugars released by the various systems were compared. Initial enzyme loading, at $t = 0$ h, was 30 FPU/g substrate.

(Fig. 3). Enzymes that had been preincubated for 4 days at 45 °C lost 20% of their ability to degrade Solka Floc BW300 to glucose, indicating that enzyme inactivation is a contributing factor to enzyme loss during hydrolysis.

The glucose-to-cellobiose ratio was approximately 10:1 in all the hydrolysis modes examined (Table 1), suggesting there was insufficient β-glucosidase in the enzyme preparation. Conversion to glucose may be enhanced by supplementation of the systems with β-glucosidase. Because the β-glucosidase recovery was fairly high (40–46%), it was worthwhile examining the recycled enzymes as a source of supplemental β-glucosidase.

The recovered enzymes were combined with fresh enzyme and used to hydrolyze 10% SEA-WAI. The activities of the different systems were assayed and in all cases the supplemented systems had greater enzyme activities than the control (Table 3). After hydrolysis, the systems supplemented with the recovered enzymes had less cellobiose built-up than the control. This was due to the higher β-glucosidase activity in the recycled systems. There was not, however, an increase in the extent of hydrolysis as was expected with the higher cellulase loading. In some cases, the percentage hydrolysis decreased with the addition of recovered enzymes (Table 3).

We surmised from the data that substances present in the recovered cellulase fraction may be inhibitory to enzyme action. It is known that oxidized phenolic

TABLE 3

Hydrolysis[a] of 10% SEA-WAI using fresh cellulase enzymes supplemented with various recovered cellulase enzymes

Source of recovered enzymes[b] expt. no.	Enzyme loading, IU/g			Hydrolysis products		Percentage hydrolysis
	endoglucanase	β-glucosidase	filter paper activity	glucose g/l	cellobiose g/l	
3	419.8	38.1	23.1	59.0	6.4	70.1
2	435.7	38.7	24.8	61.2	6.5	72.6
1	456.6	39.1	27.0	63.3	6.5	74.8
4	438.7	40.2	25.1	63.2	6.8	75.0
5	438.8	38.0	25.1	64.2	6.8	76.1
Fresh-only Control	371.9	24.8	19.6	61.9	9.1	76.2

[a] Hydrolyses were performed at 45 °C for 4 days.

[b] Recovered enzymes were ultrafiltered and then added to fresh cellulase enzymes to obtain the above enzyme loadings.

compounds can attach to proteins and react covalently to inactivate many enzymes [13]. Phenolic compounds, derived from the lignin fraction, may bind to the cellulases and be retained with the enzyme fraction during ultrafiltration. The source of the inhibition was investigated by examining the high molecular weight fraction after 4 days hydrolysis of 10% SEA-WAI (Table 4). After ultrafiltration through a 10 kDa membrane, the high molecular weight fraction was boiled to denature the cellulases. Half of this mixture was filtered to remove the precipitated proteins, while the other half was sonicated to obtain a suspension of the inactivated proteins. The two systems were combined with fresh cellulases and applied to hydrolysis of a 10% concentration of Solka Floc BW300. In comparison to the control, the high molecular weight fractions had no significant effect on the long term hydrolysis (data not shown). Apparently, there are no inhibitors present in the high molecular weight fraction that interfere with enzyme activity.

TABLE 4

Batch hydrolysis[a] of 10% SEA-WAI at an enzyme loading of 50 FPU/g substrate

Incubation time, h	Hydrolysis products		% Residual cellulose[b]
	glucose g/l	cellobiose g/l	
4	20.4	4.4	61.9
8	28.6	4.5	54.4
24	53.4	4.9	31.7
48	68.7	4.3	18.5
96	79.4	3.8	9.3
168	87.6	3.9	1.9

[a] Hydrolysis was performed at 45 °C with cellulases derived from *T. harzianum* E58.

[b] Original cellulose content was 84.4%.

Klyosov has demonstrated a correlation between the tightness of cellulase adsorption and the ability of the bound cellulases to solubilize crystalline cellulose [4]. Similarily, Beldman et al. [1] have shown that the degree of synergy between the exoglucanase and endoglucanases is dependent on their individual adsorption characteristics. Weakly adsorbed endoglucanases, in combination with exoglucanase, exhibited lower percent solubilization of crystalline cellulose than the strongly adsorbed endoglucanases. In this experiment, the enzymes most capable of attacking crystalline cellulose may remain bound to the residue, while the weakly adsorbed enzymes remain in solution and are available for recycling. The addition of these poorer quality enzymes to fresh substrate may have little or no impact on the efficiency of hydrolysis, and may interfere in hydrolysis by competing for adsorption sites with the crystalline-degrading cellulases.

Residue recycle

The first stage in enzyme production is preparation of the seed inoculum, a step which requires a readily-consumed carbon source. For the wood-to-glucose process to be commercially feasible, the glucose produced in-house must be cheaper than purchased glucose. The glucose produced in-house would be an ideal carbon source for inoculum production. However, inhibitors present in the hydrolysis stream may interfere with fungal growth. Growth of *T. harzianum* E58 on the sugar syrups produced during batch hydrolysis of 10% SEA-WAI was investigated. The hydrolysates were collected at various times during hydrolysis and diluted to obtain a 1% sugar

TABLE 5

Use of cellulose hydrolysates for production of *T. harzianum*[a] E58 biomass

Time, h	% Hydrolysis	C/G ratio[b]	Biomass yield g/g sugars
4	38.1	0.22	0.53
8	45.6	0.16	0.53
24	68.3	0.09	0.52
48	81.5	0.06	0.54
96	90.7	0.05	0.58
168	98.1	0.04	0.56
Control		0.00	0.58

[a] *T. harzianum* E58 was grown on 1% sugar for 4 days, 28 °C.
[b] Hydrolysis was performed on 10% SEA-WAI at an enzyme loading of 50 FPU/g substrate, 45 °C (Table 4). The ratio of cellobiose (C) to glucose (G) in the hydrolysates is given above.

concentration in the media. After 3 days incubation, all the available sugars had been consumed for all the systems (Table 5). Biomass yields were comparable for all the systems, indicating that the presence of inhibitors was not a problem for fungal growth at this sugar concentration.

The production of the enzymes in a wood-to-glucose process is a costly component. The best substrate for high enzyme production in *T. harzianum* E58 appears to be a cellulosic material [9] and the most feasible candidate would be the incoming feedstock. This approach, however, decreases the potential glucose from the feedstock because some of the feedstock must be diverted to enzyme production. The long hydrolysis time and high enzyme loading required for complete hydrolysis make it practical to look at a hydrolysis scheme where the target would be 80–90% hydrolysis of the cellulose component. In such a scheme, the accumulation of lignocellulosic residue from the hydrolysis reactor would be high and a waste treatment plant would be required to dispose of the material. An alternative plan is to use this residue as the cellulose source for enzyme production. Hydrolysis of cellulose generally results in the formation of a highly recalcitrant form of cellulose [5]. The production of cellulase enzymes is partially controlled by the accessibility of the cellulose to the cellulase enzyme [9], with the more recalcitrant celluloses inducing greater enzyme production. Early work demonstrated the feasibility of this approach with Solka Floc, a pure cellulose substrate [8]. Other researchers have obtained promising results using the residue remaining after hydrolysis of wheat straw [3]. A major concern is that the high amount of lignin present in the residue may have a negative impact on the use of this cellulosic substrate. Vohra and co-workers [16] noted lignin and its components had a negative effect on enzyme production in *T. reesei*.

Our first attempts at using the residue from the hydrolysis of steamed aspenwood for cellulase production were unsuccessful. Prior to using the residue for growth, the residue was dried, which may have had a detrimental effect on the structural properties of the substrate. When the residue was kept wet, and combined with fresh steam-treated wood, high levels of cellulase enzymes were produced (Table 6). As hydrolysis proceeded, the cellulose

TABLE 6

Cellulase enzyme production by *T. harzianum* E58[a] grown on SEA-WAI and SEA-WAI, HR[b]

Growth substrate	Ratio	Enzyme activities, IU/ml		
		endoglucanase	β-glucosidase	Filter paper activity
SEA-WAI	100	10.6	0.53	0.61
SEA-WAI:	75 : 25	10.4	0.56	0.55
SEA-WAI, HR^{24h}	50 : 50	10.6	0.58	0.72
SEA-WAI:	75 : 25	10.4	0.58	0.56
SEA-WAI, HR^{48h}	50 : 50	11.7	0.65	0.85
SEA-WAI:	75 : 25	10.9	0.61	0.64
SEA-WAI, HR^{96h}	50 : 50	12.4	0.70	0.85
SEA-WAI:	75 : 25	9.8	0.60	0.67
SEA-WAI, HR^{168h}	50 : 50	11.5	0.74	0.74

[a] *T. harzianum* E58 was grown on 1% substrate for 4 days at 28 °C.
[b] SEA-WAI, $HR^{t\,h}$ is the hydrolysis residue remaining after hydrolysis of 10% SEA-WAI for various lengths of time, as described in Table 4.

TABLE 7

Cellulase enzyme yield from growth of *T. harzianum* E58[a] grown on SEA-WAI and SEA-WAI, HR[b]

Growth substrate	Ratio	Cellulose content %	Enzyme yield, IU/g cellulose		
			endoglucanase	β-glucosidase	Filter paper activity
SEA-WAI	100	84	1262	63.1	72.6
SEA-WAI:	75:25	71	1465	78.9	77.5
SEA-WAI, HR^{24h}	50:50	58	1828	100.0	124.1
SEA-WAI:	75:25	68	1529	85.3	82.4
SEA-WAI, HR^{48h}	50:50	51	2294	127.5	166.7
SEA-WAI:	75:25	66	1652	92.4	97.0
SEA-WAI, HR^{96h}	50:50	47	2638	148.9	180.8
SEA-WAI:	75:25	64	1531	93.8	104.7
SEA-WAI, HR^{168h}	50:50	43	2674	172.1	172.1

[a] *T. harzianum* E58 was grown on 1% substrate for 4 days at 28 °C.

[b] SEA-WAI, $HR^{t\,h}$ is the hydrolysis residue remaining after hydrolysis of 10% SEA-WAI for various lengths of time, as described in Table 4.

content of the substrate decreased, while the lignin concentration increased. The cellulase production per gram of substrate, however, was found to be inversely proportional to the cellulose content (Table 7). The hydrolytic activity of the produced filtrates against 2% SEA-WI was tested (Table 8). Glucose yields were high, while cellobiose build-up was low (<2% of the cellulose content), indicating that inhibition of either the cellulases or β-glucosidase was not a problem.

At higher substrate concentrations, these residues may prove inhibitory to enzyme production. A 1 and 2% substrate concentration of the lignin residues, obtained after 72.2 and 87.6% hydrolysis of the substrate, was used for enzyme production. The 2% residue concentration was found to have a detrimental effect on enzyme production (Table 9). This effect was more pronounced with the more hydrolyzed fraction. In comparison, the fresh substrate, SEA-WAI, demonstrated only a slight decrease in enzyme production at a 2% substrate concentration.

TABLE 8

Hydrolytic potential of cellulase enzymes produced by *T. harzianum* E58[a] grown on SEA-WAI and SEA-WAI, HR[b]

Growth substrate	Ratio	Enzyme loading IU/g	Percentage hydrolysis of 2% SEA-WI[c] to glucose		
			day 1	day 2	day 3
SEA-WAI	100	15.3	49.7	64.8	84.2
SEA-WAI:	75:25	13.8	51.8	67.7	85.7
SEA-WAI, HR^{24h}	50:50	18.0	54.7	70.6	87.8
SEA-WAI:	75:25	14.0	46.1	56.9	75.6
SEA-WAI, HR^{48h}	50:50	21.3	60.5	74.9	91.4
SEA-WAI:	75:25	16.0	54.7	69.1	87.1
SEA-WAI, HR^{96h}	50:50	21.3	63.4	77.8	92.9
SEA-WAI:	75:25	16.8	51.1	64.1	84.2
SEA-WAI, HR^{168h}	50:50	18.5	59.8	73.4	90.0

[a] *T. harzianum* was grown on 1% substrate for 4 days.

[b] SEA-WAI, $HR^{t\,h}$ is the hydrolysis residue remaining after hydrolysis of 10% SEA-WAI for various lengths of time, as described in Table 4.

[c] Glucan content of SEA-WI was 62.5%.

TABLE 9

Cellulase enzyme production by *T. harzianum* E58 grown on SEA-WAI or SEA-WAI, HR

Growth substrate	Cellulose content %	Enzyme activities, IU/ml			Percentage hydrolysis of 2% SEA-WAI to glucose		
		endoglucanase	β-glucosidase	filter paper activity	day 1	day 2	day 3
1% SEA-WAI	84	7.8	0.63	0.59	49	62	80
2% SEA-WAI		7.6	0.64	0.49	47	61	80
1% SEA-WAI, HR^{96h}	28	10.5	0.79	0.75	56	66	85
2% SEA-WAI, HR^{96h}		7.2	0.74	0.60	50	63	82
1% SEA-WAI, HR^{168h}	13	6.2	0.70	0.64	52	65	84
2% SEA-WAI, HR^{168h}		4.9	0.63	0.44	43	54	73
0.5% SEA-WAI + 0.5% SEA-WAI, HR^{96h}	56	9.4	0.74	0.70	55	69	87
0.5% SEA-WAI + 0.5% SEA-WAI, HR^{168h}	48	11.2	0.75	0.75	59	70	87

DISCUSSION

The low recovery of cellulase enzymes from the hydrolysis of 10% SEA-WI could be largely attributed to adsorption of the enzymes onto the residue, although thermal inactivation and inactivation due to presence of low molecular weight inhibitors also contributed to the low cellulase recoveries. A 20% loss of enzymes can be attributed to thermal instability. The relative importance of adsorption and inhibition in affecting enzyme recovery is difficult to ascertain. Previous data [8] indicate that enzymatically digested steamed aspenwood with a cellulose content of 3–10% can adsorb 33% endoglucanase, 43.6% filter paper activity and 15% β-glucosidase. Enzyme recovery after hydrolysis was 13% endoglucanase, 16% filter paper and 42% β-glucosidase. In this hydrolysis, 54% of the endoglucanase, 40% filter paper activity and 34% β-glucosidase loss must be attributed to other factors such as thermal instability (20%) and inactivation by inhibitory substances, such as lignin [2]. As the concentration of the steam treated material increases, the loss of enzyme activity due to inhibitory substances may increase.

The presence of lignin-derived inhibitors and the adsorptive properties of the lignin/cellulose residue posed serious limitations to the technical feasibility of enzyme recycle following hydrolysis of steamed wood. The lignin-rich residue strongly adsorbed the cellulase enzymes, making them unavailable for recycle. More extensive delignification of the substrate may be necessary for successful enzyme recycle.

The hydrolysate syrups are suitable for fungal biomass production. These syrups had not been processed, indicating the inhibitors present in the hydrolysate did not affect fungal growth.

The residual material from enzyme hydrolysis is a good carbon source for enzyme production. This result supports earlier work, where it was shown that the more recalcitrant substrates promote high cellulase production [9]. Studies have shown that this process modification can increase the potential glucose from the incoming feedstock by 20% [7]. The hydrolysis residue has only been used at low substrate concentration and problems with inhibitory materials may surface when higher substrate concentrations are used. The concentration at which the residue could be used for cellulase production appears to be dependent on the cellulose composition of the substrate.

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