0273-2289/90/2301—0025$03.00

Application of a Magnetic Immobilized β-Glucosidase in the Enzymatic Saccharification of Steam-Exploded Lignocellulosic Residues

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Received February 27, 1989; Accepted May 4, 1989

ABSTRACT

A β-glucosidase preparation derived from *Aspergillus niger* was immobilized onto a magnetic support and used in the enzymatic saccharification of a lignocellulosic material. The enzyme was immobilized onto polyethyleneimine-glutaraldehyde activated magnetite (PAM) and also onto titanium (IV) oxide (TiO_2)-coated magnetite (TAM). Although >80% of the protein applied was immobilized, only 15–27% of the enzyme activity was recovered after immobilization. The β-glucosidase immobilized onto TiO_2-coated magnetite suffered from enzyme being removed from the matrix under hydrolysis-use conditions, whereas the PAM enzyme remained attached to the matrix. The physicochemical properties of the immobilized β-glucosidase preparations are described. Both immobilized β-glucosidase preparations were capable of completely hydrolyzing cellobiose. Recycling of the immobilized enzymes (IME) resulted in reduced rates of hydrolysis with each recycling of the enzyme, although cellobiose was still capable of being completely hydrolyzed. The reduced hydrolysis performance was attributable to physical losses of IME during recovery and, in the case of TAM, enzyme loss from the matrix. Supplementing cellulase digests of steam-explosion pretreated *Eucalyptus regnans* pulps with immobilized β-glucosidase resulted in enhanced hydrolysis. Cellulose-to-glucose yields of 80% of theoretical predictions resulted within 24 h.

The magnetically immobilized β-glucosidase could easily be recovered from the lignocellulose solids suspension in a stirred batch reactor by applying a magnetic field. The recycled immobilized enzyme continued to convert cellobiose into glucose in 80% yields over a 24-h period. This is the first report of a magnetically immobilized β-glucosidase preparation used in the enzymatic saccharification of a lignocellulosic material.

Index Entries: Magnetically immobilized β-glucosidase; cellobiose hydrolysis; lignocellulose, enzymatic saccharification of; polyethyleneimine–glutaraldehyde activated magnetite.

INTRODUCTION

Cellobiase (or 1,4-β-D-glucosidase, EC 3.2.1.21) is an important enzyme of the cellulase complex and is responsible for the hydrolysis of cellobiose, and other cello-oligosaccharides arising from cellulase hydrolysis of cellulose, into glucose (*1,2*). The complete hydrolysis of cellulose into glucose, therefore, is dependent upon the presence and amount of "active cellobiase" in the cellulase preparation used in enzymatic hydrolysis. The highly cellulolytic fungus, *Trichoderma reesei*, and many of its mutant strains are generally regarded as the best producers of cellulases (*3*). Consequently, these organisms have been used in studies at both the laboratory (*4–8*), and pilot plant (*8–10*), scale for the enzymatic saccharification of lignocellulosic materials. The main drawback, however, in using *T. reesei* cellulase preparations is that most are deficient in cellobiase activity and that glucose itself strongly inhibits the *T. reesei* β-glucosidase (*11*). In many of the studies reported on the enzymatic saccharification of cellulose, it has been the practice (*12,13*) to supplement the cellulase preparation with an exogenous β-glucosidase usually derived from another organism (e.g., *Aspergillus niger* (*13*), *A. phoenicus* (*14*)). This has resulted in increased conversions of cellobiose hydrolysis in reduced hydrolysis times, but would also increase the cost of the process since an extra enzyme is required. The cost could be reduced, however, if the enzyme could be recovered and reused. This can be accomplished by using an immobilized β-glucosidase preparation (*15–17*). If the β-glucosidase were to be immobilized on a conventional carrier support material (e.g., glass, silica, alumina, polyacrylamide, cellulose, chitin, and agarose), then the immobilized enzyme would have to be used downstream from saccharification because of difficulties associated with recovering this type of immobilized enzyme preparation from a hydrolyzed lignocellulose digest containing solids. An alternative approach would be to use a β-glucosidase immobilized onto a magnetic matrix. This would allow the immobilized enzyme preparation to be recovered from a solids suspension using its acquired magnetic properties, i.e., by application of a magnetic field. A magnetic immobilized preparation could be used concurrently with cellulase diges-

tion of lignocellulose, i.e., the reaction could be carried out in the same bioreactor vessel.

The preparation of a magnetic matrix for enzyme immobilization has been previously described (*18*). Magnetite (Fe_3O_4) was functionalized by treatment with polyethyleneimine and cross-linked with glutaraldehyde. The enzyme was covalently linked to the polymeric matrix containing entrapped magnetite via the aldehyde groups. This paper reports on the preparation of a magnetic immobilized β-glucosidase using the above approach and one in which the enzyme was immobilized onto titanium (IV) oxide-coated magnetite. This report also describes the immobilized enzyme's physicochemical properties and their employment in the enzymatic hydrolysis of steam explosion pretreated hardwood (*Eucalyptus regnans*) sawdust.

MATERIALS AND METHODS

Materials

Novozym 188 (a β-D-glucosidase preparation derived from *Aspergillus niger*), was obtained from Novo Industrie A/S, 2880 Bagsvaerd, Denmark. The enzyme preparation was dialyzed against 50 m*M* acetate buffer (pH 5.0) and concentrated by ultrafiltration using an Amicon Diaflo YM-10 membrane (mol wt cutoff of 10,000 dalton). Magnetite was obtained from Steetley Chemicals, Melbourne, Australia, and was pretreated with acid and alkali prior to use. Titanium (IV) chloride ($TiCl_4$) solution (15% $TiCl_4$ w/v in 15% HCl w/v), was obtained from BDH Chemicals Ltd., Melbourne, Australia. Cellobiose was purchased from Merck Chemicals, E. Merck, Darmstadt, West Germany. Polyethyleneimine (50% solution), glutaraldehyde, glucose oxidase (Type V, *Aspergillus niger*), peroxidase (Type II, horse radish) and *ortho*-dianisidine dihydrochloride were purchased from Sigma Chemical Co., St. Louis, MO.

Analytical Procedures

Protein was determined by the Lowry method (*19*), as modified by Hartree (*20*). Bovine serum albumin (Sigma, fraction V) was used as the standard. Glucose, the hydrolysis product of cellobiose, was determined by a modified glucose oxidase method, as outlined elsewhere (*13*).

Enzyme Assays

β-Glucosidase: β-Glucosidase activity was assayed by measuring the amount of *para*-nitrophenol (PNP) liberated from *para*-nitrophenyl-β-D-glucoside (PNPG), as previously described (*13*).

The activity of the immobilized enzyme was measured in a similar manner except that the free enzyme was replaced by 20–50 mg of immo-

bilized β-glucosidase and that the digests were shaken (150 rpm) during the 10 min incubation period. The unit of β-glucosidase activity is defined as the amount of enzyme required to liberate 1 μmol PNP/min under the conditions of assay.

Cellobiase. Cellobiase activity was determined with cellobiose as substrate and measuring the amount of glucose produced by a modified glucose oxidase method (*13*). A typical assay for the immobilized enzyme consisted of incubating cellobiose (1.0 mL of a 10% (w/v) solution in 50 m*M* acetate, pH 5.0), the immobilized enzyme (20–50 mg), and 50 m*M* acetate buffer (pH 5.0) in a final vol of 1 mL at 50°C for 10 min and stirred at 150 rpm. An aliquot of the digest (0.1 mL) was next removed, suitably diluted with water, and a portion of this (0.1 mL) taken for assay of glucose. The unit of cellobiase activity is defined as the amount of enzyme that produces 1 μmol glucose from cellobiose/min under the assay conditions.

Enzyme Immobilization

β-Glucosidase was immobilized onto polyethyleneimine–glutaraldehyde activated magnetite (PAM) by the procedure described by Dekker (*18*). This immobilized enzyme preparation is referred to as PAM-β-glucosidase. β-Glucosidase was also immobilized onto hydrous titanium (IV) oxide (TiO_2)-coated magnetite by the method outlined by Kennedy and Cabral (*21*); Hydrous TiO_2-coated magnetite was produced by the precipitation method and enzyme immobilized through chelation. This enzyme preparation is referred to as TAM-β-glucosidase.

Enzymatic Hydrolysis of Cellobiose

Enzymatic digests of cellobiose contained cellobiose (10%, w/v), buffer (50 m*M* acetate, pH 5.0), and immobilized β-glucosidase and were incubated at 50°C while shaking. At various times, aliquots were withdrawn, suitably diluted, and assayed for glucose.

Enzymatic Hydrolysis of Steam-Explosion Pretreated *Eucalyptus regnans* Pulp

E. regnans sawdust was pretreated by autohydrolysis-steam explosion in a "Siropulper," as described by Dekker et al. (*5*). Enzymatic digests contained pretreated *E. regnans* pulps (1 g dry weight), *Trichoderma reesei* C-30 cellulase (20 filter paper cellulase units (FPU) of activity), and 50 mM acetate buffer (pH 5.0) in a final vol of 10 mL and were incubated on a rotary shaker (100 rpm) at 50°C. Aliquots were withdrawn at various time intervals, centrifuged (3000 rpm/10 min) to remove insoluble material (lignin and undigested cellulose), and the supernatant recovered and assayed for glucose.

Digests supplemented with exogenous β-glucosidase, contained in addition, the free enzyme (Novozym 188, 20 U) or immobilized β-gluco-

sidase (20 U) and were run under identical conditions as a control (no added exogenous β-glucosidase). The FPU: β-glucosidase ratio in these digests was ca. 1:1, whereas that of the control was 1:0.29. Enzyme removal from digests containing IME during sampling was prevented by applying a magnetic field.

The immobilized enzyme (IME) was recovered after hydrolysis by washing the suspended-solids/IME-β-glucosidase digests thoroughly with water and buffer. A magnetic field was applied to recover the IME, and the solids portion removed by decanting. Several washes were required to remove >99% of the lignocellulosic solids following hydrolysis. For reuse, the IME was washed with buffer and drained. To the IME preparation was then added a further portion of lignocellulosic pulp, *T. reesei* cellulase solution and buffer and the digest incubated a further 24 h at 50°C. This procedure (a recycle) was repeated several times.

RESULTS

Enzyme Immobilization

The conditions for immobilizing enzyme onto activated magnetite functionalized by treatment with PEI and glutaraldehyde were established using lactase and reported elsewhere (*18*). β-Glucosidase could likewise be covalently coupled to the magnetic matrix prepared by this procedure. The amount of protein coupled was >80% of that applied for immobilization. However, the β-glucosidase activity retained on immobilization was rather low and represented an enzyme coupling efficiency of 18–27% (assayed toward cellobiose as substrate). The relationship between protein load of the enzyme per gram activated-magnetic matrix and immobilized β-glucosidase activity is shown in Fig. 1. The maximum amount of protein that could be loaded onto the PAM to obtain maximum immobilized β-glucosidase activity was about 50 mg/g matrix. This value does not represent all of the protein coupled since the Novozym 188 preparation was a crude enzyme preparation containing other enzymes (e.g., xylanase and β-xylosidase (*13*)), which were also coupled during the immobilization step (unpublished data). The amount of protein coupled to the matrix was, furthermore, found to be directly proportional to the amount of protein presented for coupling per constant amount of matrix used in the immobilization. A similar relationship also existed for the amount of "enzyme coupled" although a high proportion of the β-glucosidase activity (ca. 80%) was inactivated upon immobilization (Fig. 1). The reason for the inactivation of β-glucosidase upon immobilization may be attributable to the enzyme binding to the amine (or sulfhydryl) groups at, or in close proximity to, the active site of the enzyme. Changes in conformation owing to the binding of enzyme to matrix may also have affected the immobilized β-glucosidase activity.

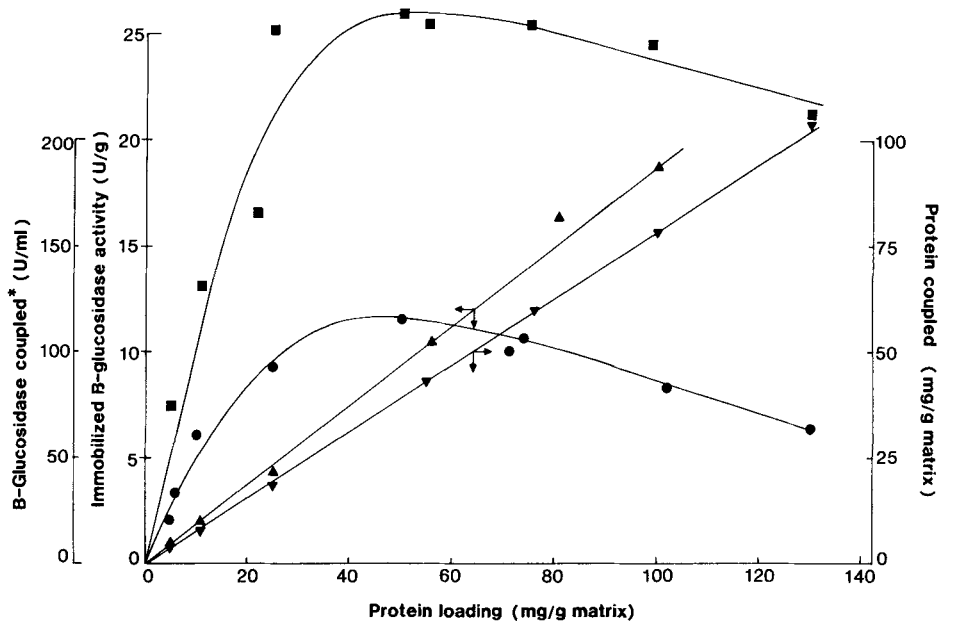

Fig. 1. The effect of protein loading on the amount of β-glucosidase immobilized and protein coupled on the magnetic matrix. Enzyme assayed toward: ■, cellobiose; and ●, PNPG. ▲ represents the amount of enzyme that disappeared during immobilization and represents the amount of enzyme (i.e., *E applied–E recovered) that is presumed to have coupled to the activated matrix; and ▼, protein coupled.

A titanated-magnetite (hydrous titanium (IV) oxide) preparation (*21*) was also used to prepare a magnetically immobilized β-glucosidase. Titanium analysis by atomic absorption spectroscopy (using a titanium element cathode lamp) revealed a titanium content of 2.4% (w/w) for the titanated-magnetite preparation. The amount of protein coupled was 98.5% of that applied, whereas the amount of β-glucosidase presumed coupled (assayed toward PNPG) was ca. 90% of that applied for immobilization (estimated by the activity remaining in the unbound fractions). However, the activity of the β-glucosidase retained on the immobilized matrix, when assayed toward both PNPG and cellobiose, was ca. 15–25% of that applied. Once again around 80% of the enzyme applied for immobilization was inactivated upon immobilization.

Both immobilized enzyme preparations were examined to determine whether enzyme was released from the immobilized matrix. The protocol for preparing IME included a thorough washing step with buffer and buffer containing 1*M* NaCl to displace any material that may have physically adsorbed onto the matrix during the immobilization step. The final IME preparation, therefore, was devoid of any physically adsorbed β-glucosidase. Both IME preparations were incubated in buffer (50 mM acetate, pH 5.0) and shaken at 50°C for up to 24 h. Assay for β-glucosidase activity in

Table 1
Comparison of the Physicochemical Properties
of Free (Novozym 188) and Immobilized β-Glucosidase Preparations

Property	Free β-glucosidase[a]	Immobilized β-glucosidase
pH optimum	4.5	4.0
Temperature optimum	60°C	65°C
Stability half-life at 50°C	<90 h	>96 h
Activation energy (kJ/mol/°K)	52.5	55.1[b]
		53.5[c]
Apparent K_m (m*M* cellobiose)	5.6	13.8[b]
		14.7[c]
V_m	33.7[d]	6.1[b,e]
		13.7[c,e]

[a]Taken from ref. *13*.
[b]Refers to PAM-β-glucosidase.
[c]Refers to TAM-β-glucosidase.
[d]μmol/min/mg protein.
[e]μmol/min/mg protein coupled/g matrix.

solution could not be detected for the PAM-β-glucosidase preparation, demonstrating that no leaching of enzyme from the matrix occurred and confirming that the enzyme was covalently attached to the matrix. However, the TAM-β-glucosidase preparation showed evidence of enzyme loss and the amount of enzyme released into solution increased with time. During these experiments, it was noted that the solution in which TAM-β-glucosidase was suspended became increasingly cloudy with time. This observation suggested that the TiO_2 layer (which contained the enzyme) was being removed from the magnetite. The cloudy solution after removal of the IME could be clarified by centrifugation, resulting in a gray-colored pellet that contained strong β-glucosidase activity. The amount of enzyme lost owing to leaching over a 24-h period was about 17% of the total immobilized TAM-β-glucosidase activity used in the experiment.

Physicochemical Properties

Some of the physicochemical properties of the immobilized β-glucosidase preparations are summarized in Table 1 and compared with the free enzyme. Enzyme immobilization lowered the pH optimum, but raised the optimal temperature of activity of the immobilized β-glucosidases compared to the free enzyme. The immobilized PAM-β-glucosidase preparation was remarkably stable when kept at 50°C for 96 h and retained ca. 86% of its original activity over this period. The free enzyme, by contrast, lost more than 50% of its original activity over the same period. The activation energy (E_A) for both the free and immobilized β-glucosidase prepara-

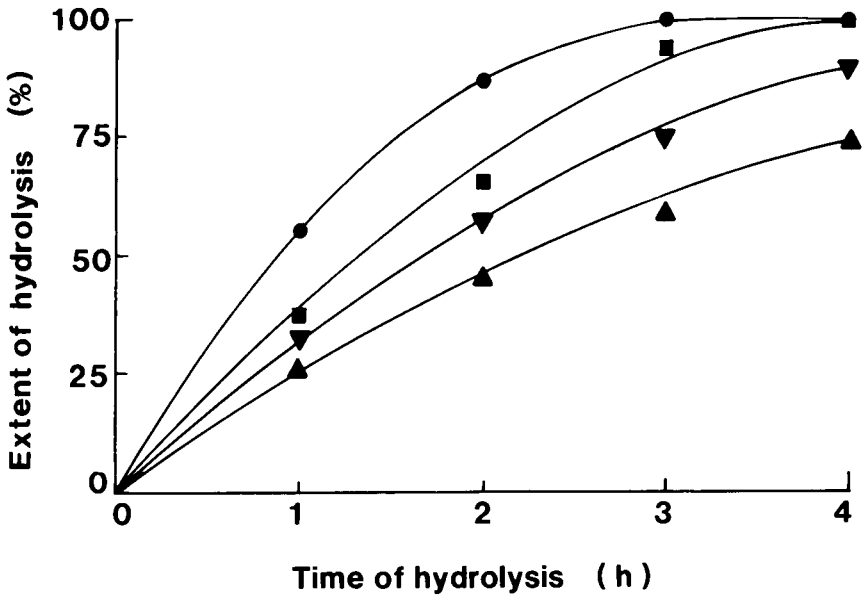

Fig. 2. Time course of hydrolysis of cellobiose by magnetically-immobilized β-glucosidase: ●, TAM-β-glucosidase (10 U); ■, TAM-β-glucosidase (5 U); ▼, PAM-β-glucosidase (10 U); and ▲, PAM-β-glucosidase (5 U).

tions ranged from 52.5–55.1 kJ/mol/°K, indicating that the enzyme may not have undergone any major conformational change upon immobilization.

The apparent K_m of both IME β-glucosidase preparations was higher than the free enzyme when assayed using both PNPG and cellobiose as substrates. However, the V_m was, in each case, lower for the immobilized enzymes. These findings indicated that immobilization sterically hindered enzyme access to substrate.

Enzymatic Hydrolysis of Cellobiose

The time course of hydrolysis of cellobiose by the immobilized β-glucosidase preparations is shown in Fig. 2. The hydrolysis performance (i.e., the rate and extent of hydrolysis) at equivalent β-glucosidase activities was found to be better for the TAM-β-glucosidase than the PAM-β-glucosidase preparation. The reason for this is owing to the higher V_m of the TAM-β-glucosidase preparation (*see* Table 1). Hydrolysis performance was shown to be dependent upon the amount of IME used in the digest. Hydrolysis was performed in a stirred batch reactor at 50°C. Total hydrolysis was achieved within 8 h by all of the preparations examined. The initial rate of hydrolysis decreased with each recycle of the immobilized enzyme (Table 2). This was more pronounced with the TAM-β-glucosidase preparation since the matrix partly disintegrated during recycling with subsequent enzyme loss. Recycling of both enzyme preparations did not,

Table 2
Hydrolysis Performance of the PAM-, and TAM-, β-Glucosidase Preparations upon Recycling and Reuse[a]

Time of hydrolysis, h	Rate of hydrolysis, mg glucose/min				Extent of conversion of cellobiose into glucose, (%)			
	Recycle Number,				Recycle Number,			
	1	2	3	4	1	2	3	4
PAM-β-glucosidase[b]								
1	0.46	0.35	0.26	0.20	27	18	14	11
2	0.43	0.30	0.25	0.20	59	36	30	23
4.5	0.28	0.27	0.23	0.19	73	69	59	48
24					100	100	100	100
TAM-β-glucosidase[c]								
1	0.61	0.32	0.26		35	18	14	
2	0.62	0.29	0.24		72	34	28	
4	0.43	0.25	0.23		98	59	50	
24					100	100	100	

[a]Digests contained 100g/L cellobiose, immobilized β-glucosidase, and acetate buffer (pH 5.0) and were incubated in a stirred reactor at 50°C.
[b]8.5 units.
[c]15.9 units.

however, preclude total hydrolysis of cellobiose, which was confirmed when the degree of hydrolysis was measured at 24 h. The hydrolysis performance (judged by the rate and extent of cellobiose hydrolysis) of the recycled immobilized β-glucosidase preparations is presented in Table 2. Since β-glucosidase was lost from the immobilized TAM-β-glucosidase preparation during the course of hydrolysis of cellobiose, it was difficult to distinguish whether hydrolysis was owing to the action of the free enzyme released into solution, or the enzyme retained in immobilized form. An experiment was designed in which TAM-β-glucosidase was thoroughly washed with buffer prior to hydrolysis and the washed immobilized enzyme used in hydrolysis. The results are shown in Fig. 3 and demonstrate that although the rate of hydrolysis was lower than the unwashed IME, the washed IME preparation was still, nevertheless, capable of totally hydrolyzing cellobiose, albeit at a slower rate.

The decrease in the hydrolysis performance of the PAM-β-glucosidase preparation was probably owing to physical losses of very fine particles of magnetite during recovery and recycling of IME. These particles presumably containing immobilized β-glucosidase, tended to float on the surface of the buffer solutions during the recovery and washing steps, and were not attracted by the bar magnet used in these experiments. Such losses of IME would obviously affect the rate of hydrolysis. The fine magnetic particles appeared to be produced as a consequence of repeated demagnetization/magnetization, washing, and shaking.

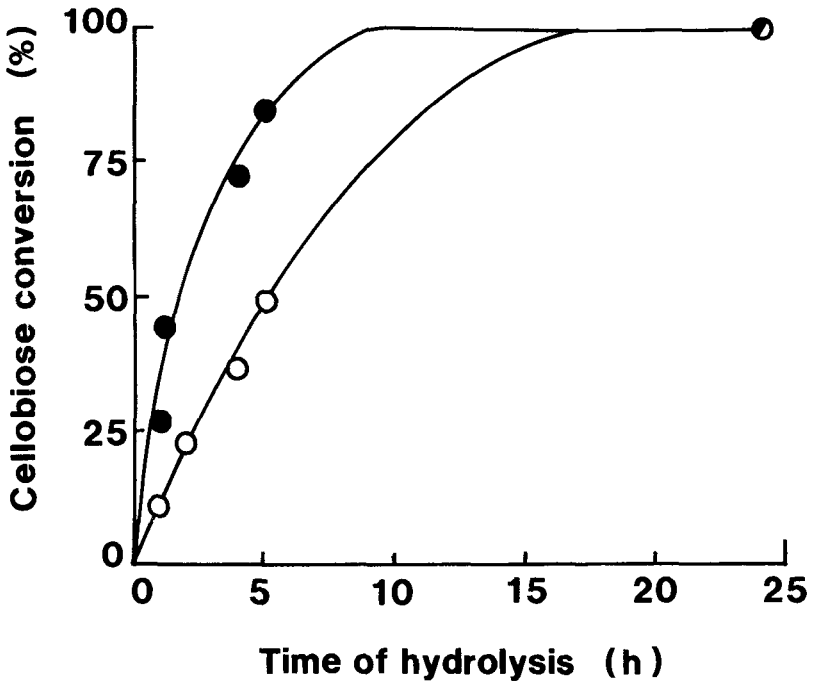

Fig. 3. The effect of washing of the immobilized TAM-β-glucosidase preparation on the hydrolysis activity toward cellobiose. ●, unwashed and ○, washed thoroughly with buffer prior to use.

Enzymatic Hydrolysis of Pretreated *Eucalyptus regnans* Sawdust

The enzymatic hydrolysis of *E. regnans* (pretreated by autohydrolysis-steam explosion) by *Trichoderma reesei* C-30 cellulases, supplemented with exogenous β-glucosidase, was performed in a stirred batch reactor at 50°C. The extent of conversion of cellulose into glucose by each of the cellulase preparations, supplemented with a different form of β-glucosidase at equivalent β-glucosidase activities, is shown in Table 3. Unsupplemented digests of *E. regnans* (i.e., a control) resulted in ca. 28% hydrolysis of cellulose in the pulps within 24 h, and 48% after 49 h. Digests supplemented with exogenous β-glucosidase resulted in conversion yields of 71.2, 63.3, and 74.9% of theoretical predictions at 24 h for the free, PAM- and TAM-β-glucosidase immobilized preparations, respectively, and almost total conversion within 49 h.

The effect of recycling and reuse of the IME preparations on the enzymatic hydrolysis of *E. regnans* cellulose is presented in Table 4. Recycling of the PAM-β-glucosidase preparation did not affect cellulose conversion into glucose over a recycling of four hydrolysis experiments. Each recovered IME batch after the first hydrolysis period managed to convert about 81% of the cellulose into glucose during a batch hydrolysis of 24 h.

Table 3
Enzymatic Saccharification of Autohydrolysis-Explosion Pretreated *Eucalyptus Regnans* Pulp in the Presence of Exogenous Added β-Glucosidase[a]

	Glucose, mg/mL		Cellulose conversion into glucose, %	
Enzyme	24 h	49 h	24 h	49 h
Control (no added β-glucosidase)[b]	18.4	31.5	28.3	48.2
Novozym 188 added[c]	46.5	62.3	71.2	95.5
PAM-β-glucosidase[c]	41.4	63.5	63.3	97.2
TAM-β-glucosidase[c]	48.9	66.1	74.9	100

[a]Twenty filter paper cellulase units/g pretreated *E. regnans* pulp.
[b]FPU to β-glucosidase ratio 1:0.29.
[c]FPU to β-glucosidase ratio 1:1.

Table 4
Effect of Multiple Recycles of Immobilized β-Glucosidase on the Enzymic Hydrolysis of Cellulose in *Eucalyptus Regnans* Pulp

Immobilized β-glucosidase preparation	Recycle number	Glucose, mg/mL[a]	Cellulose conversion into glucose, %[a]
TAM[b]	1	48.9	74.9
	2	45.1	69.0
	3	44.2	67.7
	4	41.1	62.9
PAM[c]	1	39.8	61.0
	2	53.5	81.9
	3	53.0	81.2
	4	53.4	81.9

[a]Measured at 24 h.
[b]TAM: TiO_2-activated magnetite.
[c]PAM: polyethyleneimine–glutaraldehyde activated magnetite.

Repeated washing of the IME, including a washing step with 1*M* NaCl solution to displace any physically-adsorbed enzymes, and prolonged exposure at 50°C, did not interfere with the extent of hydrolysis within 24 h for the immobilized PAM-β-glucosidase preparation although the actual immobilized β-glucosidase activity retained on the matrix was affected (*see* Table 5). Physical losses of IME did occur during recovery of the immobilized β-glucosidase preparations and ranged from 25 to 75% for the PAM- and TAM-β-glucosidase preparations, respectively, over four recyclings of IME. The reasons for the losses were as described above. The retention of activity was greater for the immobilized PAM-β-glucosidase preparation, whereas that for the TAM-β-glucosidase was lower and was

Table 5
Immobilized β-Glucosidase Recovery Yields After Multiple Recycling of the IME Used in the Enzymic Saccharification of *Eucalyptus Regnans* Pulps

Immobilized β-glucosidase preparation	Number of recycles	Recovery of IME, %		Retention of activity, %[a]
		By weight	By activity	
PAM	1	90	80 (31)[b]	88
	4	75	56 (22)	74
TAM	1	50	29 (28.6)	47
	4	25	9 (8.7)	29

[a]Calculated from the expression

$$E_R/E_A \times W_A/W_R \times 100\%$$

where E_A is the IME activity used for hydrolysis; E_R, recovered IME activity after hydrolysis; W_A is the weight of IME used for hydrolysis; and W_R, the weight of IME recovered after hydrolysis.

[b]Figures in parentheses represent the enzyme activity (U/g matrix in the recovered IME). The initial activity of the PAM- and TAM-immobilized β-glucosidase preparations were 39.2 and 97.4 U/g matrix, respectively.

mostly attributable to enzyme leaching and its loss during the recovery and washing stages. This would explain the lower cellulose to glucose conversions obtained when TAM-β-glucosidase was recycled (Table 4) and used in the enzymic saccharification of *E. regnans* pulp.

DISCUSSION

An attractive and important feature of using an immobilized β-glucosidase preparation in the hydrolysis of lignocellulosic materials is that the enzyme can be easily recovered for recycling. Use of conventional matrices for enzyme immobilization presents difficulties in recovering the immobilized enzyme preparations from solid suspensions such as those that occur in the enzymatic hydrolysis of lignocellulose. The problem of enzyme recovery can be overcome by using magnetic matrices for immobilization, which allow them to be recovered from a suspended-solids mixture by the application of a magnetic field. Employment of a magnetically-immobilized β-glucosidase preparation, as described herein, has demonstrated that it can be successfully applied in the enzymatic saccharification of lignocellulosic materials and that it can be easily recovered for reuse from a hydrolyzed lignocellulose suspension. Such separations would be difficult using a β-glucosidase immobilized onto a conventional support carrier since there are difficulties in separating the immobilized enzyme from the lignin (present in the digest in an insoluble form) and from partly- and undigested cellulose (both being insoluble). Thus, in using a β-glucosidase

immobilized onto a conventional support carrier (e.g., chitosan (*15*), chitin (*16*), and ion exchange resins (*17*)), requires that the hydrolysis sugars (cellobiose and glucose) be physically separated from the cellulose digests and then hydrolyzed separately downstream.

To the author's knowledge, there appears to be only one other report concerning a β-glucosidase immobilized onto a magnetic support. In this case, almond β-glucosidase was covalently coupled to aminobenzoic acid–formaldehyde polymers containing nickel and iron, thereby producing a ferromagnetic immobilized enzyme preparation (*23*). There was no report of this immobilized enzyme preparation being used to hydrolyze cellobiose or aid enzymatic saccharification of cellulose by cellulases. The work described herein, thus, is the first time that a magnetically-immobilized β-glucosidase has been used in the enzymatic hydrolysis of lignocellulosic materials.

A common feature of enzyme immobilization appears to be a loss of enzyme activity upon covalent coupling to the support. For example, up to 10% of the β-glucosidase coupled onto chitosan (using glutaraldehyde) retained activity (*15*). Similarly, 50% of the original enzyme activity was lost upon a β-glucosidase being immobilized onto alginate beads (*24*). Likewise, the β-glucosidase described herein, and immobilized onto PAM- and TAM-magnetic matrices, retained up to 25–27% of its original activity upon immobilization. The problem has been ascribed to conformational changes upon coupling, which sterically hindered the enzyme, or enzyme was bound in an inactive form by coupling to the functional groups (e.g., $-NH_2$) located at, or near, the active site of the enzyme itself. In the case of a porous matrix, or gel, mass transfer effects have also been reported to affect enzyme activity (*15*). This results in a putative immobilized enzyme activity. Woodward and Wohlpart (*25*), using the rationale of immobilizing β-glucosidase (a glycoprotein) via the attached carbohydrate groups rather than the functional groups on the protein molecule itself, succeeded in coupling active enzymes at high coupling efficiencies ($>81\%$).

The physicochemical properties of the two immobilized β-glucosidase preparations described herein do not differ significantly from those reported for other immobilized β-glucosidase preparations (*15,24–26*), and it appears that the magnetic matrix did not affect the immobilized enzymes' properties, or impede hydrolysis performance. The magnetically-immobilized PAM-β-glucosidase appeared to perform as well as other reported β-glucosidases under reuse conditions (*24–27*) and that loss in hydrolysis performance was mainly attributable to physical loss of immobilized enzyme encountered during the washing and recovery steps. TAM-β-glucosidase, although more active in the hydrolysis of cellobiose and lignocellulose hydrolyzates, was an unstable enzyme preparation resulting in loss of enzyme. The loss was attributable to the unstable nature of the complex formed between the TiO_2 layer and magnetite, which was easily removed from the surface of the magnetite. TiO_2 was

reported to strongly adsorb enzymes (*28*), and its loss from the magnetite resulted in the loss of enzyme from the immobilized matrix. This aspect has also been observed to occur with nonporous glass (*28*). Therefore, it appears that magnetite, unlike glass (*29*), is unsuitable as a matrix for chelation with titanium and subsequent immobilization of β-glucosidases.

Finally, since the enzymatic saccharification of lignocellulosic materials has been technically demonstrated to be enhanced by supplementing cellulase digests with exogenous β-glucosidase (*4,5,11*), a commercial process would become economically viable if an immobilized β-glucosidase preparation was used for this purpose. A convenient form in which an immobilized enzyme preparation could be used in the enzymatic saccharification of lignocellulose would be to use a β-glucosidase immobilized onto a magnetic matrix.

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