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Adsorption of Cellulases on Steam-Pretreated Willow

MATS GALBE, ROBERT EKLUND, AND GUIDO ZACCHI*

Chemical Engineering I, University of Lund,
PO Box 124, S-221 00
Lund, Sweden

ABSTRACT

The adsorption of cellulases on steam-pretreated willow has been measured for 2, 4, and 8 wt% willow and with varying enzyme concentrations (2–100 wt% based on substrate). The enzyme concentration was measured as soluble protein, filter paper activity (FPA), CMC-ase activity, and activity toward willow. The adsorption data were modeled with a Langmuir isotherm. The maximum adsorption capability depends on the method for measurement of the enzyme concentration. The lowest value, 470 mg enzyme/g willow, was obtained for the soluble protein and the highest value, 650 mg/g, for the FPA. For technical applications, a single isotherm can be used for calculation of the adsorption capability of steam-treated willow.

Index Entries: adsorption; cellulases; willow; enzyme recovery.

Abbreviations Used: E_{ads}, adsorbed enzyme (g/L); $e_{ads} = Ea/So$ (g enzyme adsorbed/kg ODM); $e_{ads,max}$, maximum adsorbed enzyme (g enzyme/kg ODM); E_o, initial enzyme concentration (g/L); E_{sup}, enzyme concentration in supernatant (g/L); K, adsorption equilibrium constant (g/L); S_o, substrate concentration (kg/L).

INTRODUCTION

In the enzymatic conversion of cellulose to fermentable sugars, the cost of enzyme is a major fraction of the total hydrolysis cost (up to 80%) (*1*). This necessitates the recovery of the enzymes used in the process.

*Author to whom all correspondence and reprint requests should be addressed.

Several methods have been proposed to recover free enzymes from the hydrolysis solution, such as ultrafiltration (2), enzyme recovery by immobilization on a solid support (3), and aqueous two-phase systems (4). Direct readsorption on fresh substrate is another method that can be employed. Orichowsky et al. (5) made a thorough study of the recovery of enzymes using countercurrent adsorption. Using steam-exploded corn stover as substrate, the enzyme consumption was reduced to 35% compared with hydrolysis without recovery.

Adsorption is preferable to the other methods since it is simple to perform. The success of the method depends on two main factors, the desorption of the enzyme from the solids residue in the hydrolysis and the adsorption capacity of the fresh substrate. The major part of the cellulases is, after hydrolysis, adsorbed to the nondegraded solid residue and has to be desorbed in a suitable way. A thorough investigation was made by Reese (6) in which more than 35 components were screened to evaluate their desorbing effectiveness. The conclusion was that it is possible to recover most of the enzymes, but usually the desorption is proportional to an inactivation of the enzymes. Thus, it is important to find eluting agents that, besides being harmless to the enzymes, are cheap and easy to use.

The purpose of this study was to evaluate the adsorption capacity of steam-pretreated willow. The adsorption of cellulases was evaluated by measuring the quantities of soluble protein, the filter paper activity (FPA), the activity on CMC (CMC-ase), and the activity toward willow in the supernatants in contact with steam-pretreated willow. The adsorption data for varying cellulose and cellulase concentrations have been modeled with the Langmuir isotherm. To estimate the recovery, the enzyme concentration in the hydrolysate must be measured. Some preliminary data on the amount of CMC-ase available for readsorption after hydrolysis, as well as the effect of water as a desorbing agent, are also presented.

MATERIALS AND METHODS

Substrate

The substrate used in this investigation was willow, a fast-growing energy crop with the composition shown in Table 1. The raw material was ground and air dried at room temperature to 94% oven dry material (ODM). It was then passed through a sieve, and the fraction between 1.0 and 3.15 mm was used in the experiments. The material was soaked with water, and the resulting material, at approximately 30% ODM, was steam-treated for 10 min at 222°C. The pretreatment method is described in detail elsewhere (7).

Table 1
Composition of Willow in Percent ODM

	Raw material	After pretreatment
Extractives	7.9	-
Starch	1.9	-
Hemicellulose	24.3	7
Cellulose	33.7	48
Lignin	17.0	42
Raw protein	6.0	-
Ash	3.0	-

Table 2
Protein Content and Activities
in 1 g Enzyme Solution

CMC-ase	150 mg RS/mL min
FPU	60 mg RS/mL min
Willow-ase	3 mg RS/mL min
Protein	40 mg (Coomassie)
	160 mg (Lowry)

Enzyme

The cellulase preparation used was Celluclast 2L (Novo Industries Inc., Copenhagen, Denmark), a cellulase from *Trichoderma reesei.* The CMC-ase activity was measured with CMC-7 (Hercules Inc., Wilmington, DE) as substrate (*8*) and the filter paper activity with Whatman #1 filter paper (*9*). The activity toward willow was determined as described below. The enzyme activities and the protein content are given in Table 2. A cellobiase from *Aspergillus Niger,* Novozyme 188 (Novo), was used in the analysis. The β-glucosidase activity of the cellobiase was 45 μmol *p*-nitrophenol/g min, using *p*-nitro-phenol-β-D-glucopyranoside as substrate (*10*).

Analysis

Four different enzyme activities or concentrations were assessed: activity on CMC (CMC-ase), filter paper activity (FPA), and total protein (Coomassie blue-dye binding assay). A complementary activity, against willow (willow-ase), was also measured. This activity is impossible to use on a comparative basis between different laboratories, but it reflects the true activity toward the raw material used in our ongoing study on the production of ethanol from lignocellulosic materials. From an engineering point of view, this material-dependent activity, therefore is, most valuable.

Activity against CMC was measured with CMC-7 as substrate and the formed reducing sugars analyzed using the DNS method (*11*). Measurement of the FPA was made with an excess of β-glucosidase (0.2% of total) added to the enzyme solutions to be analyzed, a method suggested by IUPAC (*12*). The samples to be tested were placed together with a 1×6 cm strip of Whatman #1 filter paper and then allowed to stand for 1 h at 50°C. The samples were diluted, prior to the hydrolysis, to give a sugar concentration less than 0.2 mg/g solution. A nonlinear standard curve was used to relate the enzyme activity in the sample to the corresponding concentration of original enzyme giving the same activity. In this way, the enzyme activity was expressed as a concentration of original enzyme for direct comparison of all the different activities. Willow-ase was assessed on 300 mg (30% ODM) of a standard pretreated material containing approximately 50 mg cellulose. Samples were allowed to stand for 24 h at 50°C, in capped test tubes, together with an excess of β-glucosidase. The glucose content was then analyzed using the DNS method. The total protein content was, finally, obtained using the Coomassie Brilliant Blue method (*13*).

EXPERIMENTAL PROCEDURE

Water Content in the Pretreated Material

Calculation of the concentration of free enzyme requires that the total free volume of liquid in a sample be known. Some of the total liquid is a constituent part of the fibrous material and may only be separated from it by means of severe pressing and drying. The enzyme concentration in this liquid differs from the concentration in the supernatant. The liquid in the micropores of the cellulosic particles holds no, or small amounts, of enzyme (*14,15*), whereas the liquid in the macropores probably has a very high enzyme concentration since most of the adsorbed enzymes are located there.

The amount of bound liquid was, in this study, estimated using a simple water separator suitable for use with a centrifuge tube. It was found to give satisfactory and reproducible results. The separator is made of plastic with an outer diameter of 15 mm. The bottom part is perforated to allow water to escape. A known amount of wet material was placed in the separator, which was the put into a test tube and centrifuged for 30 min at 9600 rpm. The weight of the centrifuged sample was determined, and the water-holding capacity was calculated.

Adsorption Isotherms

The adsorption of cellulases onto the pretreated material took place in 250 mL flasks at 8°C with a total amount of material, i.e., solids and liquid, of 50 g. Enzyme solutions of various concentrations in a 0.1*M* HAc buffer,

pH 4.8, were prepared the day before the experiment was to be performed. These were stored in a refrigerator overnight at 8°C to ensure that the temperature of the solution was constant during the experiment. The temperature was low to prevent hydrolysis during the adsorption. Different results are found in the literature regarding the influence of temperature on the adsorption. Some investigations (*16*) indicate no, or very weak, temperature dependence, whereas others (*17*) claim a strong temperature dependence. In the latter cases, simultaneous hydrolysis occurred at the higher temperatures (40–50°C), which has to be avoided during enzyme recovery.

The amount of fibrous material was varied between 2 and 8% (wt/wt), to which enzyme solutions in the range 2–100% (wt/wt) of the solid material were added. The enzyme solution was poured over the solid material, quickly shaken, and then placed in a constant-temperature bath. The flasks were either shaken, in a back and forth moving shaker, or stirred with a magnetic stirrer. After 15 min, the samples were filtered through microglassfiber filters and the supernatants analyzed.

In some of the experiments, a 10% enzyme-to-solids solution was contacted with the substrate for times ranging from 1 to 30 min. The flasks were placed in the water bath, half stirred with a magnetic stirrer and the other half not stirred. At the desired time, the supernatant was treated as mentioned earlier.

Determination of CMC-ase Activity in the Hydrolysate

The maximum degree of enzyme recovery is determined by the amount of enzyme available in the solution after hydrolysis. The amount of active enzyme is measured by the determination of the various enzyme activities. This is, however, not a simple procedure since the hydrolysate contains considerable amounts of reducing sugars, and most of the activity assays are based on the formation of small amounts of reducing sugars from various substrates. The hydrolysis products, therefore, must be removed prior to the analysis.

A method using Pharmacia's Sephadex PD-10 gel filtration columns to separate the enzymes from the reducing sugars was adopted. By using this method, the interference of the reducing sugars from the hydrolysis was reduced to a tolerable level. A two-stage procedure, using samples of 2.5 mL and an elution volume of 4 mL in each stage, was found to give a good reproducibility. The glucose concentration was reduced from 50 g/L, which corresponds to the maximum value obtained in the hydrolysate, to 0.85 g/L (i.e., only 2% of the original concentration). The enzyme recovery was 85% when measured as CMC-ase. The enzyme recovery was also measured in terms of total protein content. The peaks for total protein and CMC-ase coincide, which indicates that the various enzymes in the cellulase complex are eluted at the same volumes.

RESULTS AND DISCUSSION

Mathematical Model

The adsorption data were modeled using the model for adsorption and desorption presented by Stuart and Ristroph (*18*)

$$r_a = k_a \cdot C_E \cdot C_b \quad (1)$$

$$r_d = k_d \cdot C_{Eb} \quad (2)$$

where k_a and k_d are the rate constants for the adsorption and desorption reactions respectively, and C_E, C_b, and C_{Eb} are the molar concentrations of free enzyme, adsorption sites on the cellulose, and enzyme-adsorption site complex, respectively. Assuming equilibrium and introducing the definitions relating the theoretical variables to the measureable variables given by Stuart and Ristroph (*18*), the following Langmuir equation is obtained

$$e_{ads} = \frac{e_{ads,max} \cdot E_{sup}}{K + E_{sup}} \quad (3)$$

where $e_{ads} = E_{ads}/S_o$ (g adsorbed enzyme/kg ODM); and E_{sup} = free enzyme in the supernatant (g enzyme/L solution)

Equation (3) is claimed to be useful (*18*) when the cellulose concentration is kept constant and the enzyme concentration is varied. The parameters $e_{ads,max}$ and K are material constants according to the model and, thus, should not be functions of the cellulose concentration while the same cellulosic material is used in the adsorption experiments, i.e., when no hydrolysis occurs. When e_{ads} is plotted vs E_{sup} for different cellulose concentrations, a single isotherm should be obtained if the model is correct.

Adsorption Kinetics

Different results are found in the literature concerning the adsorption rate and the effect of agitation. Lee and Fan (*14*) found that the adsorption of cellulases on 5% Solka Floc was very rapid, with no changes in the amount of soluble proteins in the supernatant after about 2 min. Furthermore, the agitation had no effect on the adsorption when comparing samples with vigorous agitation with samples without agitation. Other studies shows that the adsorption to Whatman filter paper (*19*) and Avicel (*20*) was slow, especially for low cellulose concentrations (< 20 g/L). Tatsumoto et al. (*21*) showed that adsorption to five pretreated aspen substrates was rapid with steady-state conditions, achieved after 10 min, although additional adsorption at a slower rate still occured after 1 h for the steam exploded material.

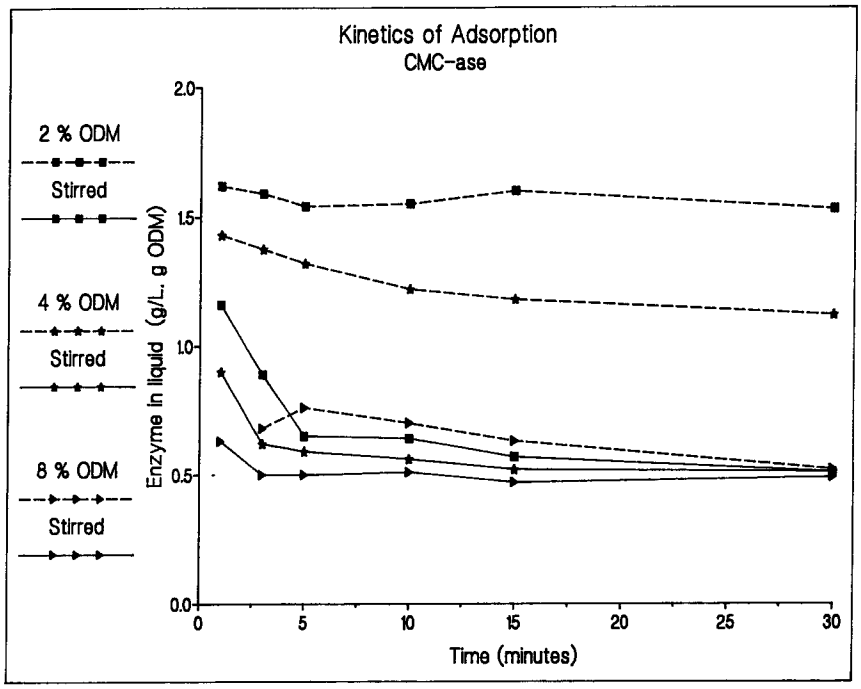


Fig. 1. Influence of agitation on the adsorption rate. Initial enzyme concentration: 10% of ODM. Solid line: stirred; dotted line: no agitation.

The kinetics of the adsorption of cellulases on steam-pretreated willow was measured using 2,4, and 8 wt% ODM with an enzyme concentration of 10 wt% based on ODM. Figure 1 shows the change of the enzyme activity, measured as CMC-ase, in the supernatant vs time for both stirred and unstirred samples. Here, agitation had a significant influence on the adsorption rate; this was most pronounced at low substrate concentrations. For 8% ODM, the samples without agitation reached equilibrium after approximately 30 min, whereas for 2 and 4% ODM, the samples were far from equilibrium after 30 min. For all three substrate concentrations, equilibrium was reached within 15–30 min when the sample was stirred.

The effect of agitation is further shown in Fig. 2, where the adsorption isotherms obtained for stirred samples and samples agitated in a shaker with a 'back and forth' type of motion are compared. The difference between the curves is significant, especially at high enzyme concentrations. Similar curves, as those shown in Figs 1 and 2, were also obtained for all the other enzyme activities measured.

Adsorption Isotherms

In Figs. 3–5, the adsorption isotherms (stirred samples), determined as protein, FPA, and willow-ase, are plotted. The isotherm for CMC-ase is

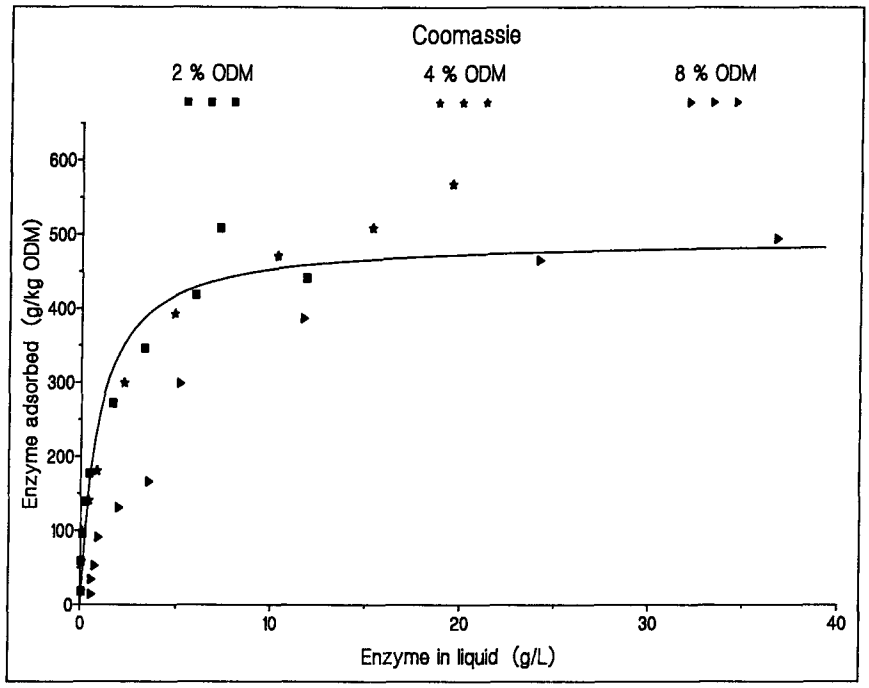


Fig. 2. Influence of stirring on the adsorption isotherms.

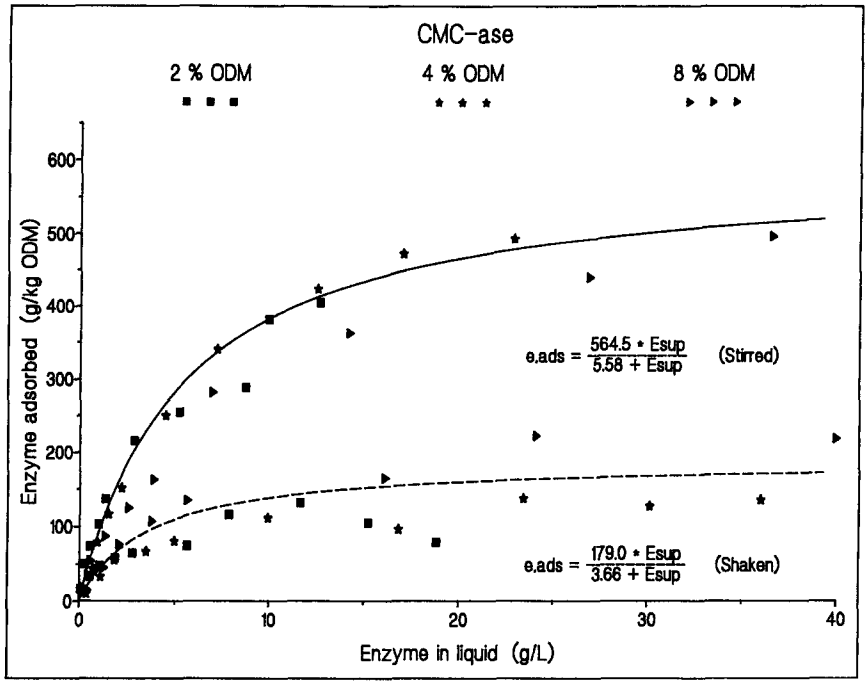


Fig. 3. Adsorption isotherms for soluble protein.

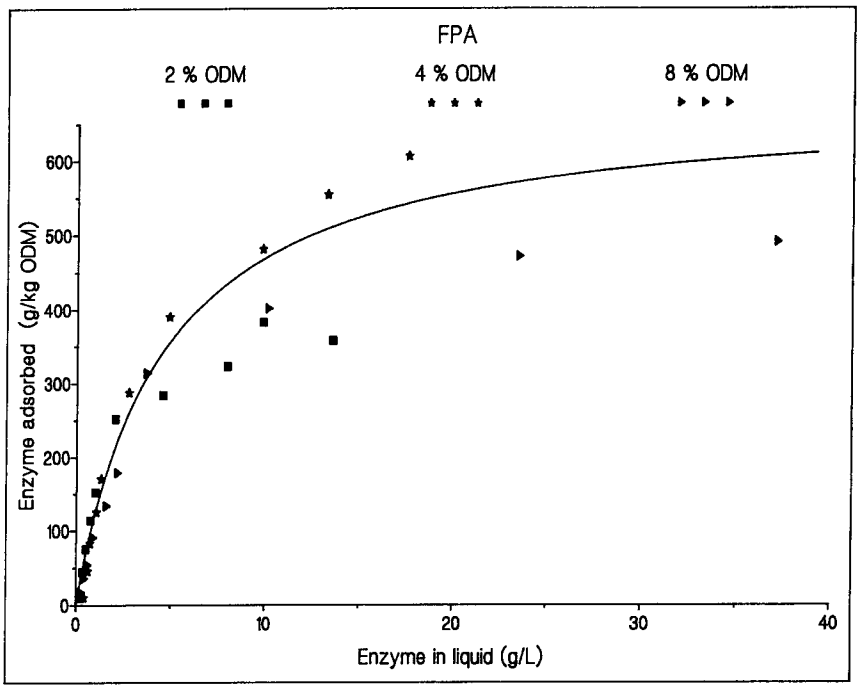


Fig. 4. Adsorption isotherms with enzyme concentration, measured as filter paper activity.

shown in Fig. 2 (the upper curve). The solid lines are obtained with Eq. (3) by a nonlinear least-square fit to the experimental data. The resulting parameters are given in Table 3. The enzyme activities are recalculated to enzyme concentrations for comparison. All the enzyme concentrations correspond to an equivalent amount of original enzyme solution. The concentrations can be easily converted to protein concentration or enzyme activities by multiplication with the corresponding value given in Table 2 (determined using standard methods, as described in the materials and methods section).

The maximum amount of cellulase that can be adsorbed to willow is dependent on the method used for measurement of the enzyme concentration. The highest value, 648 g enzyme/kg willow, was obtained for the FPA, whereas the lowest value, 471 g/kg, was obtained for soluble protein, corresponding to 39 FPU/g willow and 19 g protein (determined with Coomassie)/kg willow, respectively. These values should be considered as model parameters and not be given too much physical interpretation.

The discrepancies between the measured and calculated values at high enzyme concentrations are fairly large. However, the discrepancies are not a serious disadvantage when estimating the adsorption of enzymes in a technical application. The range of interest regarding the enzyme con-

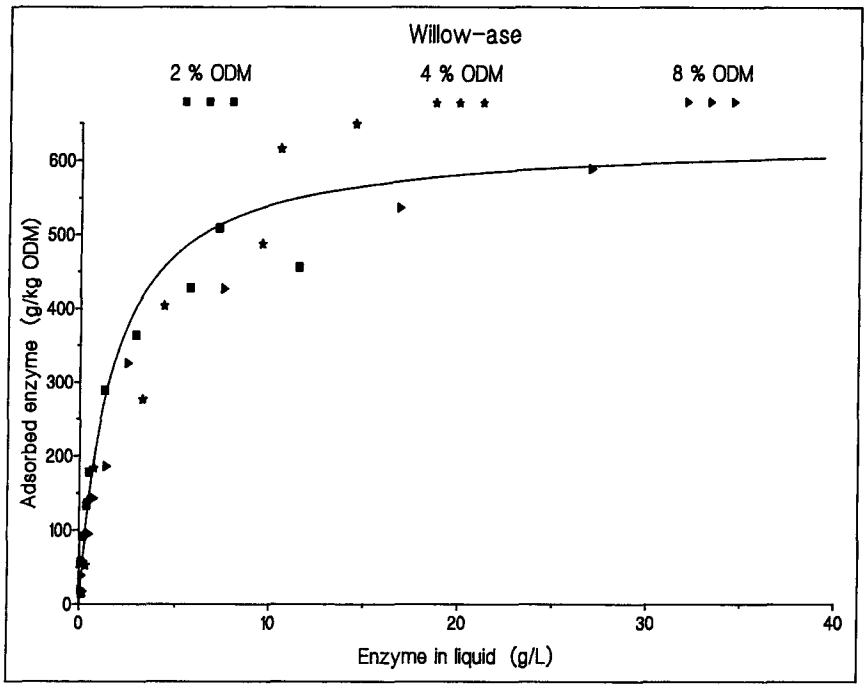
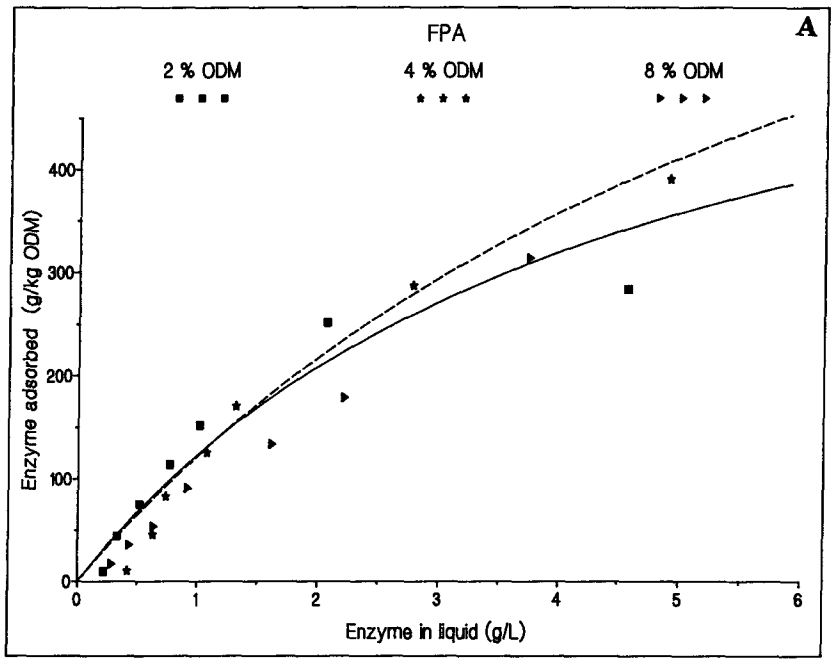


Fig. 5. Adsorption isotherms with enzyme concentration, measured as activity toward willow.

Table 3
Model Parameters

Enzyme conc., measured as	All data points		Data points for $E_0 < 20\%$ of ODM	
	$e_{ads,max}$	K	$e_{ads,max}$	K
Protein	470.7	0.92	364.4	0.58
FPA	647.8	4.58	961.8	7.39
Willow-ase	598.3	1.67	492.1	1.26
CMC-ase	564.5	5.58	452.9	4.23

centration is between 10 and 20 wt% based on the dry substrate, i.e., 6–12 FPU/g ODM. At a concentration of 10 wt% solids, this corresponds to a maximum enzyme concentration in the supernatant of 6 g/L. Figures 6A and B shows the adsorption isotherms, measured as FPA and CMC-ase, when data points above 20% enzyme concentration (based on ODM) are omitted. The solid line is obtained with the model when using all data in the fitting, whereas the dotted line is obtained when the data points above 20% enzyme are omitted.



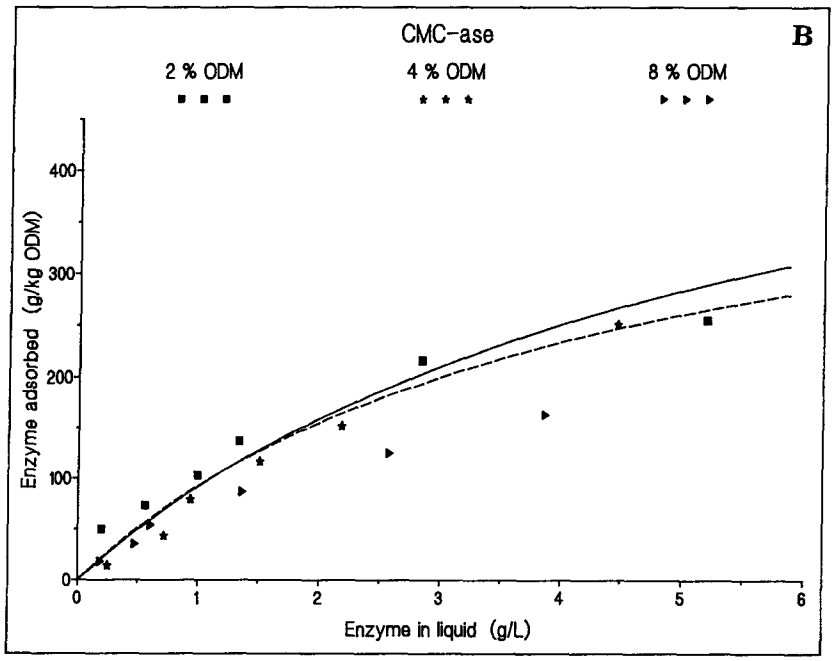


Fig. 6. Adsorption isotherms for enzyme concentrations up to 20% of ODM. Solid line: fitting to all data points; dotted line: fitting to points for initial enzyme concentrations = 2–20% of ODM. (**A**) Enzyme activity, measured as FPA; and (**B**) Enzyme activity measured as CMC-ase.

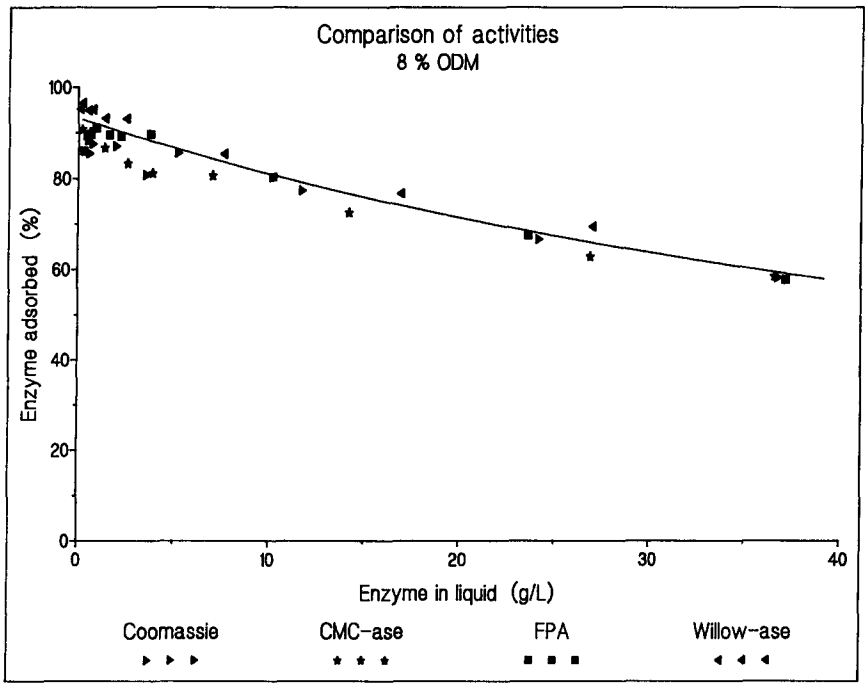


Fig. 7. Adsorption capability, measured as soluble protein, CMC-ase, FPA, and activity toward willow. Line: Calculated with Eq. (3), using the parameters obtained for FPA.

For technical applications, the parameters for the dotted line, given in Table 3, is a better choice. From Figs. 2-6, it is concluded that, for technical applications, the data for varying substrate concentrations may be represented by a single isotherm. This is contradictory to the results of Steiner and coworkers (*20*) when using the same equation for Avicel. The wide scattering of their data may be owing to the fact that the adsorption was measured at 50°C with simultaneous hydrolysis, which is also pointed out by them. The cellulose structure varies for the different samples used since they are hydrolyzed to various extents that may alter the adsorption capability.

At high enzyme concentrations, there is a more pronounced difference between the data for 2 and 4% and the data for 8% substrate. This could be owing to difficulties in the determination of the amount of adsorbed enzymes at higher concentrations, as discussed below.

For use in process simulations, where enzyme recovery by adsorption is one of many process steps, the question arises whether a single model could be used for all activities. We have found that the use of FPA gives a good mean value for calculation of the adsorption capability. Figure 7 shows the amount of enzyme adsorbed as a percentage of the total enzyme

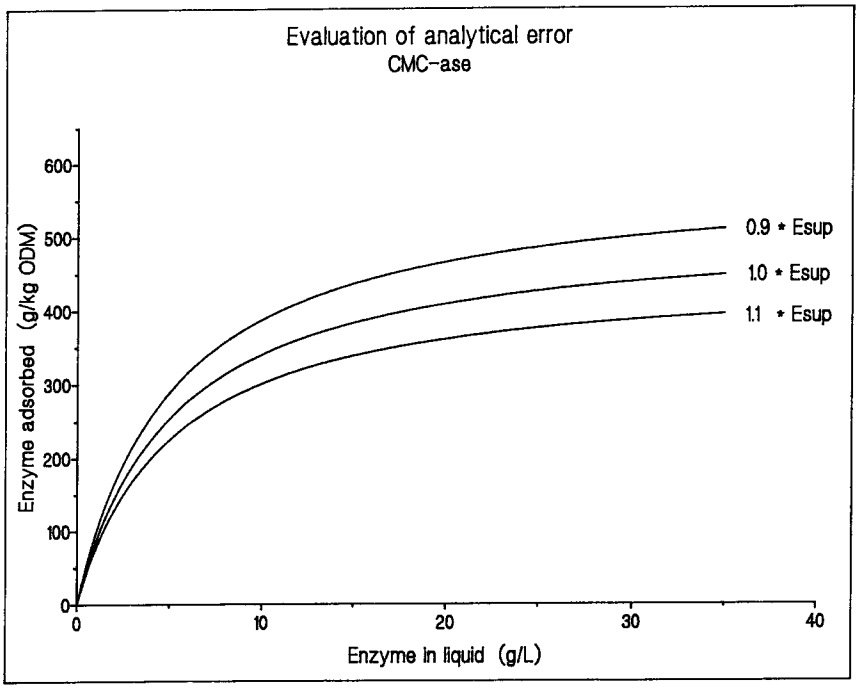


Fig. 8. Influence of analytical errors on the adsorption isotherms.

available measured with the various activities. The solid line is calculated using the model with the parameters for FPA. Similar results are obtained for the other two substrate concentrations.

Error Analysis

The amount of enzymes adsorbed is estimated indirectly by measuring the enzyme activity in the supernatant. At high enzyme concentrations, this involves the subtraction of two large numbers, E_o and E_{sup}. As shown in Fig. 8, an error of $\pm 10\%$ in the analysis results in a final error of about $\pm 15\%$ for the fitted curves. In reality, the experimental points at higher concentrations show larger deviations, with errors up to 25%. This calls for better analysis methods and, perhaps, a different approach than using the enzyme concentration in the supernatant to calculate the degree of adsorption, such as radioactive labeling of the enzymes, which has been used by Kyriacon et al. (22).

Enzyme Recovery from Hydrolysate

The adsorption isotherms can be used to estimate the recovery of enzymes from the hydrolysate. Unfortunately, much of the enzymes are either denatured or very tightly adsorbed onto the solid residue. Our

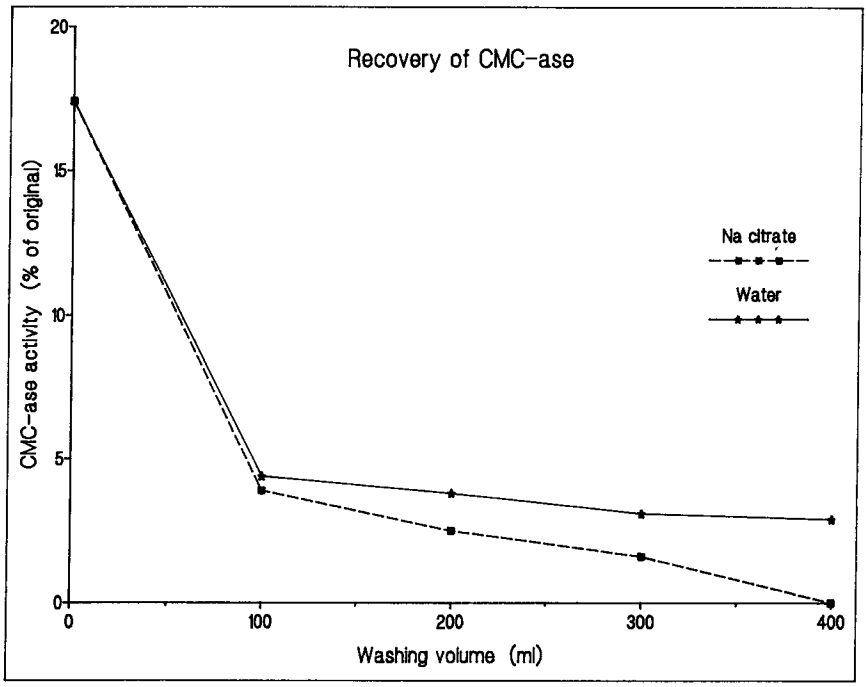


Fig. 9. Desorption of enzymes from residuals of hydrolyzed willow. Enzyme activity measured as CMC-ase.

work on enzyme recovery from the hydrolysate is ongoing, and only some preliminary results are presented here. In Fig. 9, desorption, measured as CMC-ase, with citrate buffer or water as desorbing agents is shown.

About 20% of the original CMC-ase activity can be found in the hydrolysate. Washing with citrate buffer does not liberate much more; the only effect is an exchange of the solution bound to the residue. After four washes, there is no activity in the buffer, and approximately 25% of the original enzyme activity is recovered.

Common tap water seems to be effective as the eluting power; although small, it is obvious even after four washes. Thus, a total of 31% is recovered. It is not, as was the case with the buffer, an effect owing to the exchange of solution. Therefore, it ought to be possible to elute more enzymes in a more effective, continuously working device. Future work will focus on recovery of the enzymes employing direct readsorption after hydrolysis.

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