

## **Cellulase adsorption: measurement of the fraction of covered surface area by XPS**

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### **Introduction**

Cellulases are a consortium of hydrolytic enzymes which adsorb on and hydrolyse insoluble cellulose (Béguin and Aubert, 1994). Adsorption precedes the hydrolytic reaction, and the cellulases activity chiefly depends on it. In turn, the adsorption depends markedly on the cellulose surface area and on the surface properties. Namely, the site specificity of cellulase adsorption, and the influence of the adsorption force on the reaction kinetics are important factors on the enzymes action (Klyosov, 1990). When studying the cellulases adsorption onto different substrates, the amount of enzyme adsorbed per unit of surface area cannot be calculated, unless the accessible area is accurately measured.

XPS is a surface sensitive technique. It was used in this work to analyse the fraction of surface area of the cellulosic fibers with adsorbed enzymes.

### **Methods**

**MATERIALS** - The cellulase from *Trichoderma reesei*, Celluclast, was a kind gift from Novo, Denmark. Three kinds of cellulose were used: Sigmacell type 100; Avicel PH-101; fibrous Whatman CF- 11.

**ENZYMATIC HYDROLYSIS** - The reaction mixture, containing 50mg of cellulose, 3.0ml of 50mM acetate buffer, pH 5.0, with and without Tween 85 at 0.02% (w/v), and 1.0ml of diluted enzyme (final concentration in the reaction mixture ranging from 2 to 250mg ml<sup>-1</sup>), was placed for 18h in a 50ml flask and incubated at 50°C in a end to end shaker. The reaction medium was

then centrifuged (5000rpm, 3min), and the supernatant was analysed for soluble sugars by the DNS method.

ADSORPTION EXPERIMENTS - The suspension containing 50mg of cellulose and 1.0ml of enzyme (with concentrations of 5mg·ml<sup>-1</sup>, 0.1, 1.5mg·ml<sup>-1</sup>) diluted in 50mM acetate buffer pH 5.0, with and without Tween 85 at 0.02% (w/v), in a 2ml ependorf, was incubated for 30min in an orbital shaker (100rpm, 20°C). The particles were then washed as above and prepared for XPS analysis.

XPS ANALYSIS - The washed particles were dried in a SpeedVac, and 0.013m diameter pellets undergoing a pressure of 0.6kg m<sup>-2</sup> for 1min were prepared with a hydraulic press. The pellets were then analysed in an Escalab 200A (VG Scientific - Fisons) XPS analyser equipped with a MgK $\alpha$  radiation source at 1253.6 eV.

#### Theoretical Background

The surface sensitivity of XPS allows the determination of the fractional coverage  $\gamma$  for each analysed pellet, by using the equation:

$$\gamma = I / [I_0(1 - \exp^{-d/\lambda \sin \alpha})] \quad (1)$$

where  $I$  is the N1s photoelectron signal from an overlayer of thickness  $d$  and fractional coverage (monolayer)  $\gamma$ ,  $I_0$  is the intensity for a thick overlayer ( $d = \infty$ ),  $\lambda$  is the attenuation length, and  $\alpha$  is the angle between the surface and the analyser. For a uniform and flat surface, the sampled depth is given by

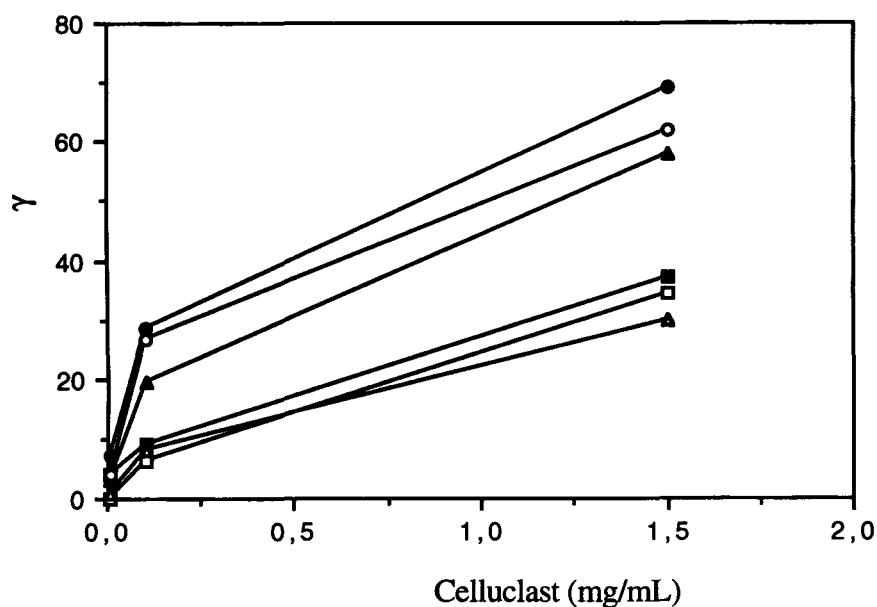
$$d = 3\lambda \sin \alpha \quad (2)$$

and is maximum when  $\alpha = 90^\circ$ . In the case of a substrate with a uniform thin overlayer, the angular variation of intensities enhances the surface sensitivity, and the overlayer thickness can be determined (Andrade, 1983). In order to estimate the  $\gamma$  values of the protein films adsorbed on the cellulose fibres,  $I_0$  was experimentally determined by preparing and analysing a pellet of lyophilized BSA.

### Results and discussion

The theoretical value of N1s intensity ( $I$ ) for the particles with a monolayer of adsorbed cellulases was calculated by equation (1), applying a  $d$  value of  $45\text{\AA}$ , considering the dimensions of the CBH I molecule ( $67\text{-}45\text{-}45\text{\AA}$ ), which accounts for about 60% of the total protein.

The fractional coverage in each experimental case was calculated. The results obtained from these calculations are shown in Figure 1. The values under brackets correspond to the  $\gamma$  value obtained using an exit angle value of  $42^\circ$ .



**Fig.1.** Fraction of surface area covered by the enzymes ( $\gamma$ ) of the several celluloses, assuming an exit angle of  $42^\circ$ ; tw= Tween; Av=Avicel; Sigm=Sigmacell; Wh=Whatman.

The fraction of cellulose surface coverage by the enzymes is higher in the case of Whatman, owing to the comparatively low surface area (Gama *et al*, 1994). The enzymes display higher affinity for Sigmacell than for Avicel, probably due to the lower crystallinity and hence higher wettability of the former. Further experiments would be necessary to verify whether the cellulose

saturation corresponds to the monolayer calculated intensity or not. However, it must be stressed that, due to their geometry and the fitting on the irregular fibres surface, the enzymes are not expected to cover the particles homogeneously, and voids must exist among the molecules when the surface saturation is reached. Therefore, an homogeneous layer of protein on the fibres surface is unlikely. The calculated intensity corresponding to a protein monolayer may therefore have been overexpressed, and, in this case, the real  $\gamma$  values under the experimental conditions of this work are higher.

The rate of the enzymatic hydrolysis for the several celluloses, with and without Tween 85, is presented on Table 1. The surfactant enhanced the enzyme activity on Sigmacell and Avicel, specially at low enzyme concentration, simultaneously reducing its adsorption (Figure 1). The Sigmacell hydrolysis is more markedly accelerated. In the case of Whatman, both the hydrolysis rate and the cellulase adsorption are not so strongly affected by the surfactant.

**Table 1.** Hydrolysis rate ( $\mu\text{g}/\text{mL}\cdot\text{h}$ ) of Avicel (Av), Sigmacell (Sigm) and Whatman (Whatm), with and without surfactant Tween 85 (Surf).

Enz. conc. ( $\mu\text{g}/\text{mL}$ )	2.2	4.4	8.8	17	50	250
Av+Surf	12	25	44	65	117	225
Av	3	13	34	65	115	223
Sigm+Surf	24	46	89	150	278	400
Sigm	3	16	39	104	254	408
Whatm+Surf	7	14	22	38	61	111
Whatm	5	14	29	40	67	103

The Sigmacell hydrolysis by low enzymatic loads ( $2.2\text{mg}\cdot\text{ml}^{-1}$ ) and without surfactant is slow as compared to the rate of What-

man and Avicel hydrolysis (data partially shown on Table 1), and to the relative rate obtained at higher enzyme concentration. This effect can be explained as a consequence of an irreversible adsorption of the enzyme to Sigmacell, or, at least, a slower desorption rate. On the other hand, in the case of Whatman, multiple attacks of the same enzyme molecule on the cellulose surface would be allowed.

These results suggest a cause-effect relationship between the depletion of the enzymes from the fibre surface and the kinetics of hydrolysis. XPS proved to be a powerful technique, probably deserving more attention than it does nowadays in heterogeneous biocatalysis studies. In this paper, the usefulness of XPS as a tool to analyse the cellulose-cellulase interaction was demonstrated.

### References

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