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UTILIZATION OF WASTE CELLULOSE—III*

COMPARATIVE STUDY OF THE ACTIVITY OF THE CELLULASES OF *TRICHODERMA VIRIDE* AND *ASPERGILLUS NIGER* TOWARDS DIFFERENT CELLULOSIC SUBSTRATES

C. DAVID and P. THIRY
Université Libre de Bruxelles, Faculté des Sciences, Campus Plaine 206/1,
Boulevard du Triomphe, 1050 Bruxelles, Belgium

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Abstract—Filter paper, carboxymethylcellulase and β-glucosidase activities have been determined and compared for cellulases originating from *Trichoderma viride* (TV) and *Aspergillus niger* (AN). The formation of glucose and of total reducing sugar has been measured as a function of time for the hydrolysis of cellulose I by the same quantity of FP units from TV, AN or a mixture of both strains. Long term efficiency is lower for AN but an important synergistic effect has been observed for the mixture of the enzymes. This synergistic action has been assigned to a better balance of endo- and exoglucanases and essentially to the addition to TV of thermally stable endoglucanases from AN. The β-glucosidases formed in large quantity by AN have been found to be thermally unstable and susceptible to product inhibition. They do not play any role in the observed synergistic action.

INTRODUCTION

Considerable attention has been focused on the enzymatic hydrolysis of cellulosic substrates in recent years. The concept originally propounded by Reese [1] of a non-hydrolytic factor (so-called C_1) that would carry out a localized loosening of the cellulose chains as a preliminary to hydrolysis by the hydrolytic enzymes (so-called C_x) has never been substantiated and seems to have given way to the more plausible argument that the hydrolysis of native cellulose is the result of the synergistic action of endo- and exoglucanase enzymes.

In this model [2–4], one, or several, endoglucanases (1-4, β-glucan-4-glucanohydrolase) act randomly to produce oligosaccharides and the new chain ends that are produced are then hydrolysed "instantly" by the endwise acting exoglucanases (1-4, β-glucan-4-cellobiohydrolase) in order to prevent reformation of the glucosidic linkage. Finally, a third class of enzymes, cellobiases (β-glucosidases) which are not adsorbed on the substrate [4], are also crucial enzymes for the total saccharification, not only because their product is glucose but also because cellobiose inhibits both endo- and exoglucanases [4–6].

The major obstacle to immediate commercial exploitation of an enzymatic process for the hydrolysis of cellulose is the sharp fall-off in rate as the reaction proceeds. This fall-off has been attributed to two possible mechanisms viz. substrate heterogeneity and product inhibition. The substrate heterogeneity mechanism is derived from the composition of cellulose in amorphous and crystalline regions. When the amorphous sections, readily attacked, have been degraded, the reaction proceeds at a lower rate as the more difficult crystalline sections are hydrolysed [7, 8]. Product inhibition is a phenomenon common to many enzymatic systems and has been demonstrated in the cellulose action [3–6]. The cellulase enzymes produced by the fungus *Trichoderma viride* have been the most extensively studied because of their high activity towards native celluloses. However, *Trichoderma viride* cellulases have been reported to be deficient in the cellobiase enzyme components [6, 9, 10]. *Aspergillus niger* is also known to produce cellulolytic enzymes, in which if exoglucanases are poorly represented, the endoglucanases and β-glucosidases are present in good amount [9].

Our aim is to examine the saccharifying capacity of the enzymes of the two strains, acting alone or together. The addition of the β-glucosidases and/or endoglucanases from *Aspergillus niger* could exhibit synergistic effects with the great amount of exoglucanases originating from *Trichoderma viride*.

EXPERIMENTAL

Determination of sugars

Reducing sugars were determined by the Folin–Wu method [12] and expressed as glucose equivalent. Glucose was analysed by the glucose oxidase method with the "Test combination glucose" supplied by Boehringer Mannheim. δ-Gluconolactone 5×10^{-3} M was added to the tests to inhibit the β-glucosidase activity contained in the reagent.

Determination of filter paper activity

The filter paper activity (FP unity) was found by the method of Mandel [13].

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Determination of carboxymethylcellulose saccharifying activity (CMCase units)

The reaction mixture consisted of 2 ml of 50 mM sodium citrate buffer pH 4.8, containing 25 mg of CMC and 0.5 ml of diluted enzyme solution in the same buffer. After incubation at 50° for a given period (10 min in most cases), the reducing sugars were determined by the Folin–Wu method [12]. The CMCase unit is expressed as μg of reducing sugar in glucose equivalent released per min.

Determination of the cellobiase activity

The reaction mixture consisted of 2 ml of 50 mM sodium citrate buffer pH 4.8 containing 5 mg of cellobiose and 0.5 ml of diluted enzyme solution in the same buffer. After incubation at 50° for a given period (5 or 10 min in most cases), an aliquot (50 or 100 λ) is taken at 0°. The glucose present was then determined by the glucose oxidase from Boehringer in the presence of 5×10^{-2} M of δ-gluconolactone. The reaction was developed for 30 min at room temperature and then the tubes were stored at 0° until measurement. The cellobiase unit was expressed as μg of glucose released/min.

Cellulose

The cellulose used is well characterized (Part I) wood pulp for paper making purpose ground for 60 sec with a type A apparatus from Int. Lab. App. The viscosity average molecular weight measured in Cadoxen and the percent crystallinity determined by X-ray diffraction are respectively 135,000 and 80%.

Cellulases

Trichoderma viride (TV) Onozuka R-10 and Aspergillus Niger (AN) type II were respectively supplied by Kinki Yakult Mfg Co. Ltd and Sigma, St Louis.

RESULTS AND DISCUSSION

Activity of AN and TV—product inhibition

Table 1 gives the activity of AN and TV expressed in FP, CMCase and β-glucosidase (or cellobiase) units. CMCase activity is generally assigned to endoglucanases whereas the saccharification of filter paper needs a contribution of endo- and exoglucanases. These results indicate that AN has a higher β-glucosidase activity but contains less endo- and exoglucanases than TV. It has also been demonstrated [15] that the cellobiase activity of AN is more susceptible to product inhibition (by glucose) than that of TV. Indeed, determination of the cellobiase activity as a function of incubation time has shown that this activity decreases at a much lower concentration of formed glucose in the case of AN.

Kinetics of cellulose saccharification

Figure 1 gives the kinetics of saccharification of cellulose by the same quantity of FP units of the two strains (AN and TV) used either alone or in combination. The rate of saccharification of cellulose by each of the two strains used alone was found to be proportional to enzyme concentration in the range of 0.15–1.5 FP units $\times$ ml^{-1}. The table enclosed in Fig. 1 contains the mean values of the yields obtained for three hydrolysis experiments ($n = 3$) performed during 24 hr. These results show that identical quantities of FP units of TV or AN lead to very different yields of glucose. Indeed, after 48 hr incubation, the glucose formed in the presence of AN is about one third of that obtained in the presence of TV. FP units, which are usually considered as a quantitative measurement of the saccharifying power, have thus only indicative meaning. The yield of glucose indeed depends on the nature of the substrate, on the concentration of enzyme and substrate and on the time of reaction.

Figure 1 also demonstrates that the mixture of both cellulases (AN + TV) gives more glucose (+30%) than when they are used individually. This synergism is not due to the large excess of β-glucosidases brought about by AN. Indeed (Fig. 2), the ratio of reducing sugar to glucose (RS/G) has a similar value which is almost independent of the reaction time, for incubation in the presence of TV alone or in the presence of TV + AN. On the contrary, RS/G increases rapidly with time in the presence of AN alone. This rapid increase indicates a loss of the AN β-glucosidase activity. This loss of activity can be assigned to inhibition by the formed glucose but also to a thermal denaturation of the enzymes.

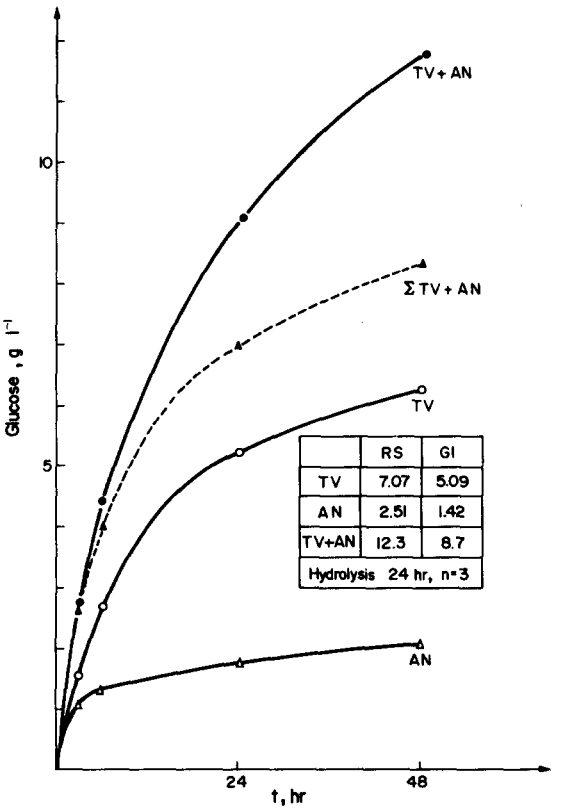

Fig. 1. Kinetics of glucose formation by saccharification of 250 mg cellulose in 10 ml solution at pH 4.8 and 50° in the presence of 0.5 FP units $\times$ ml^{-1} of *Trichoderma viride* (TV) or *Aspergillus niger* (AN) or a mixture of TV and AN.

Table 1. Enzymatic units in *Trichoderma viride* and *Aspergillus niger*

	FP units (u g^{-1})	CMCase units (u mg^{-1})	Cellobiase units (u mg^{-1})
TV	480	200	85
AN	110	69	250

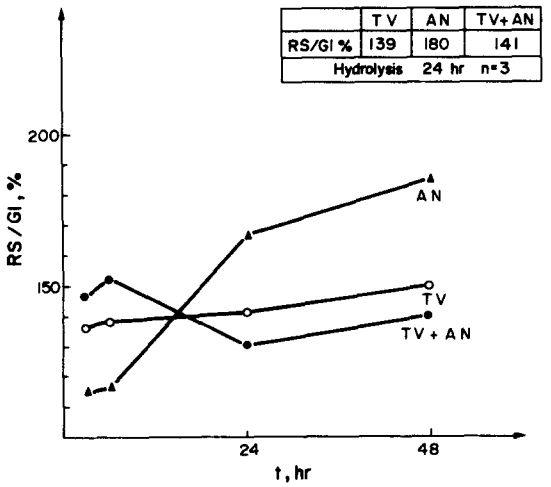

Fig. 2. Reducing sugar to glucose (in mol%) as a function of the time of hydrolysis in the presence of TV, AN or TV + AN (same conditions as in Fig. 1).

Thermal stability of the enzymatic activities

AN and TV have been incubated in the absence of substrate at 45 and 55°. The different activities, measured as a function of time, are given in Fig. 3 (a) and (b). They show that the β-glucosidase activities are more labile than the glucanase activities. Furthermore, the β-glucosidase activity of AN is more rapidly destroyed than that of TV. Actually, the better thermal stability of the glucanases could still be increased in the presence of cellulose substrate on which they are strongly adsorbed unlike the glucosidases [4]. Concerning the glucanase activities, CMCase and FP activities of TV have similar stabilities whereas CMCase of AN is more stable than its FP activity.

The results obtained for the thermal stability of the different activities (Fig. 3) and the synergism observed in the presence of the two strains (Fig. 1) can be explained assuming a different balance of endo- and exoglucanase activities in TV and AN and remembering that both endo- and exoglucanases contribute to FP activity while endoglucanases are only responsible for the CMCase activity. Concerning AN, the thermal stability of exoglucanases seems to control the FP activity since endoglucanases are more stable. For TV, it can be assumed that endoglucanases control the FP activity in the presence of an excess of exoglucanases since the stabilities of FP and CMCase activity are similar. According to this, AN contains an excess of thermally stable endoglucanases and TV an excess of exoglucanases. The synergistic effect observed in the presence of a mixture of the two strains results from a better balance of both components and essentially to the addition of thermally stable endoglucanases from AN to TV which is deficient in this component. The addition of thermally unstable β-glucosidases of AN does not play any role in the observed synergistic action.

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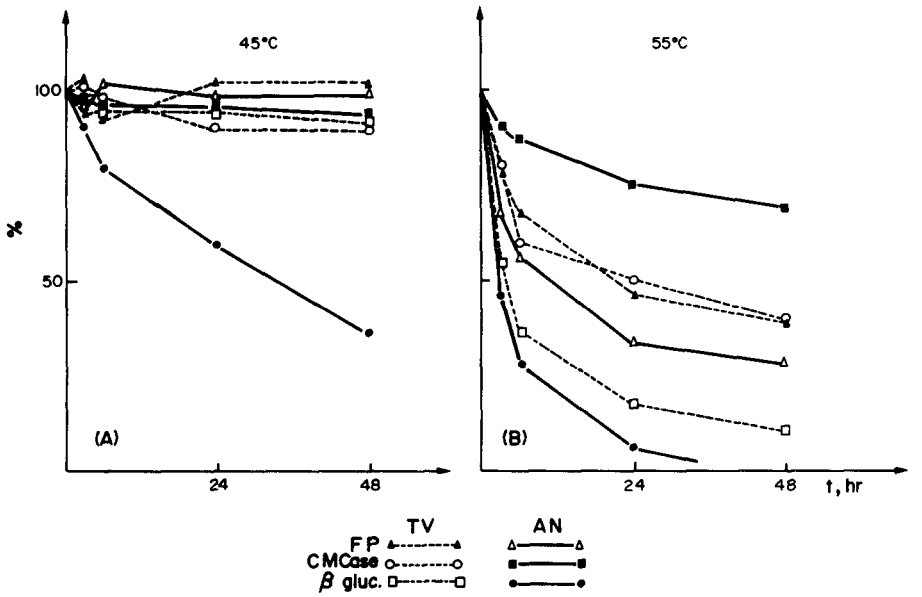

Fig. 3. Thermal stability of TV and AN at 45 and 55°.

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Résumé—Les activités papier filtre, carboxymethylcellulase et β-glucosidase ont été déterminées et comparées pour les cellulases issues de *Trichoderma viride* et d'*Aspergillus niger*. La formation de glucose et de sucre réducteur total a été mesurée en fonction du temps pour l'hydrolyse de cellulose I par une même quantité d'unités papier filtre provenant de TV, d'AN ou d'un mélange des deux souches. Pour les temps d'hydrolyse longs, l'efficacité d'AN est bien inférieure à celle de TV mais, en présence d'un mélange des deux types de cellulases, un effet synergique important est observé. Cet effet synergique a été attribué à une meilleure balance des endo- et exo-glucanases et en particulier à un apport par AN d'endoglucanases thermiquement stables. Les β-glucosidases formées en quantité plus faible par AN sont thermiquement instables et sensibles à l'inhibition par produit. Elles ne jouent aucun rôle dans l'effet synergique observé.