Growth of *Trichoderma viride* on Crude Cell Wall Preparations from Barley

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Trichoderma viride can utilize crude cell wall preparations from barley starchy endosperm as sole source of carbon and energy. In the process β-(1→3)(1→4)-glucan and arabinoxylan are released. The onset of release of the latter preceded that of glucan, consistent with arabinoxylan being encountered and utilized first. The release of several enzymes was observed during growth of *Trichoderma* on this substrate: endo-β-(1→3)(1→4)-glucanase, endo-β-(1→4)-glucanase, endoxylanase, arabinofuranosidase, esterase, carboxypeptidase, and "β-glucan solubilase". It is inferred that each of these activities is necessary for the digestion of this substrate.

Keywords: *Barley; cell wall; endosperm; Trichoderma; enzymes*

INTRODUCTION

The walls surrounding the cells of the starchy endosperm of the barley kernel are largely composed of β-(1→3)(1→4)-glucan (75%), arabinoxylan (20%), and protein (5%), together with traces of cinnamoyl residues, notably ferulic acid (*1*). The β-glucan, and also probably the arabinoxylan, must be efficiently degraded if problems are to be avoided in malting and brewing (*2* and *3*). Although there is extensive knowledge of the factors determining the levels and degradation of (at least) the β-glucan (notably an in-depth appreciation of the endo-β-glucanases which catalyze their degradation (*4*)), there remains a lack of understanding of the exact nature of the walls per se. The architectural configuration of the walls is unknown, despite the fact that an improved understanding of cell wall structure would be of considerable value in facilitating approaches targeted to optimizing degradation of these troublesome entities.

The study of plant cell walls is by no means a trivial undertaking (*5*). Various approaches have been taken to understanding the problem, frequently involving selective digestion protocols.

In this paper we report an alternative strategy which takes advantage of the known ability of enzymes from the fungus *Trichoderma viride* to totally degrade barley β-glucan (*6*). Our approach has been to grow *T. viride* on a medium containing a crude cell wall preparation as sole source of carbon and energy. The assumption has been that the organism will elaborate and secrete those enzymes that it needs to digest this material. By assessing the development of individual enzymes and the release of polymers we hoped to derive some indication of the sequence of stages involved in disruption of these cell walls.

EXPERIMENTAL PROCEDURES

Growth of Fungus. *Trichoderma viride* (NRRL 3652) was stock-cultured by growing for 48 h at 30 °C in a medium comprising (%w/v) KH_2PO_4, 0.2; $(NH_4)_2SO_4$, 0.14; urea, 0.03; $MgSO_4 \cdot H_2O$, 0.03; $CaCl_2$, 0.03; peptone, 0.1; glucose, 1.0; plus trace metal solution (1.0 mL). The latter comprised (mg/100 mL) $FeSO_4 \cdot 7H_2O$, 500; $MnSO_4 \cdot H_2O$, 156; $CoCl_2$, 200; plus 1 mL of 19% HCl (*7*). Mycelium obtained was washed in 0.9% (w/v) NaCl containing 0.1% (w/v) Tween 80, before inoculating into medium (200 mL) in which glucose was replaced by 1% (w/v) crude cell wall preparation. Cultivation was at either 25, 30 or 35 °C for up to 72 h in static Erlenmeyer flasks (500 mL) according to the setup shown in Figure 1. Growth was monitored by quantitation of collected carbon dioxide by subsequent titration with N/50 H_2SO_4 (*8*). Growth was assessed in this way owing to the impracticality of using more familiar means for monitoring growth of a filamentous organism on a very turbid medium. Data are quoted as the integrated quantity of CO_2 released at each successive time interval. All fermentations were performed in duplicate, and all measurements on separate fermentations were also made in duplicate.

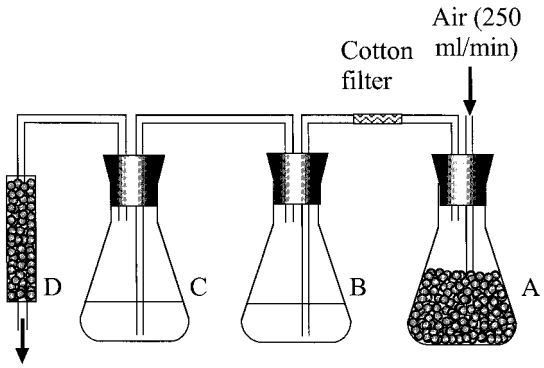

Figure 1. Cultivation of *Trichoderma viride*. The Erlenmeyer flasks (500 mL) contained A, soda lime (to remove CO_2 from the air supply); B, medium (200 mL); C, 0.1N NaOH (to capture CO_2); D, soda lime.

Preparation of Cell Wall Substrate. Barley (var *Morex)* was de-husked, denatured, washed, depleted of starch, and milled according to the procedure of Moore et al. (*9*). This involves extensive washing and attendant removal of anything other than high-molecular-weight and relatively insoluble materials. Analysis of the substrate revealed that it comprised 71% β-glucan, 8% arabinoxylan, 1% each lipid and protein, and the balance ash and residual starch. As the ratio of arabinoxylan to β-glucan is rather lower than that reported for cell walls (*1*) it is presumed that a proportion of the pentosan has

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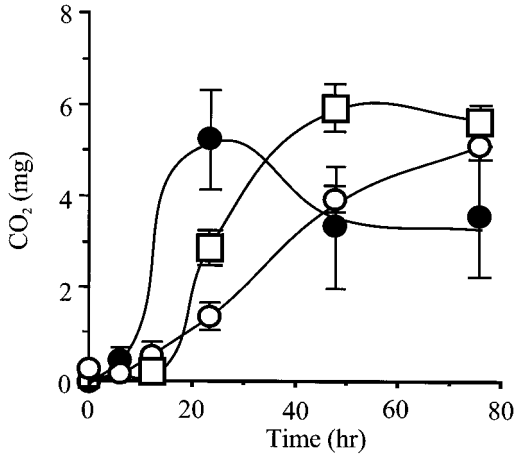

Figure 2. Growth of *Trichoderma viride* on crude barley walls as monitored by CO_2 release: ○, 25 °C; ●, 30 °C; □, 35 °C.

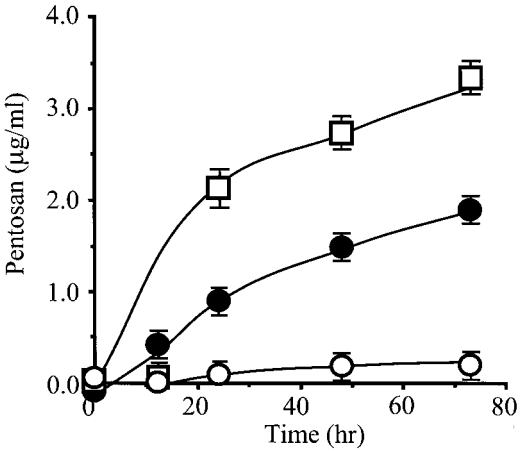

Figure 3. Release of pentosan during growth of *Trichoderma viride* on crude barley walls: ○, 25 °C; ●, 30 °C; □, 35 °C.

been lost during the preparation of the substrate, which might indicate its greater availability to dissolution than that of glucan.

Enzyme Assays. Assays were by published procedures: endo-β-(1→3)(1→4)-glucanase and endo-β-(1→4)-glucanase (*10*); endo-xylanase (*11*); arabinofuranosidase (*12*); esterase (*13*); carboxypeptidase (*14*); and "β-glucan solubilase" (*15*). One unit of activity for each enzyme is defined as the amount of enzyme producing 1 μmol product per minute under the prevailing assay conditions, except for solubilase where one unit is the amount of enzyme that catalyzes the release of 1 mg β-glucan per minute in the standard assay.

Chemical Analyses. Protein was measured by the method of Bradford (*16*) using bovine serum albumin as reference. β-Glucan was assayed according to Bamforth (*17*). Pentosan was quantified by the method of Douglas (*18*).

Chemicals. All chemicals were supplied by Fisher (www.fishersci.com), with the exception of barley β-glucan and the glucose measurement kit which were from Sigma (www.sigma-aldrich.com).

RESULTS AND DISCUSSION

Growth of *T. viride* on Cell Wall Material. The organism grew more rapidly at 30 or 35 °C than at 25 °C (Figure 2). Patterns of release of arabinoxylan, protein, and β-glucan into the medium were different when monitored at the three fermentation temperatures. The release of arabinoxylan was greatest at 35 °C, with very low levels of soluble pentosan appearing at 25 °C (Figure 3). However, in the case of β-glucan,

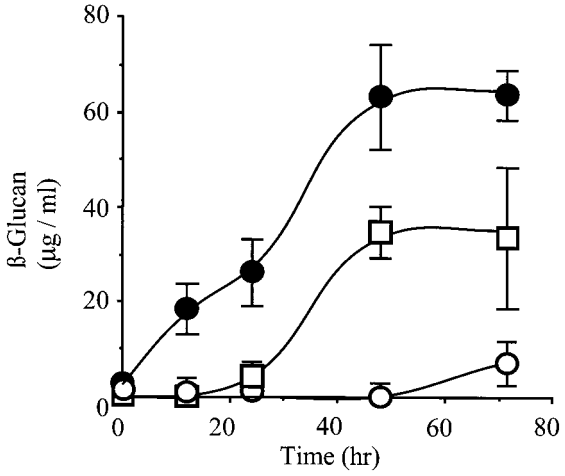

Figure 4. Release of β-glucan during growth of *Trichoderma viride* on crude barley walls: ○, 25 °C; ●, 30 °C; □, 35 °C.

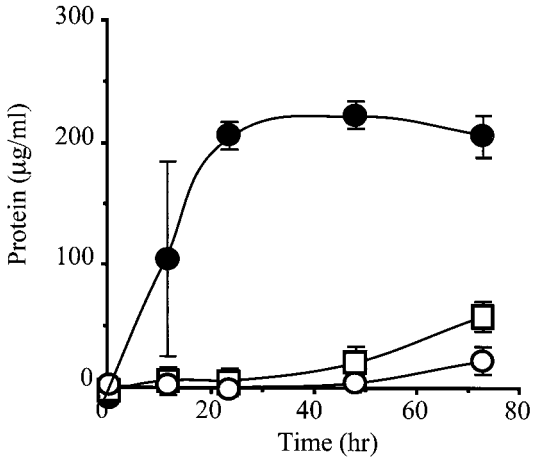

Figure 5. Protein concentration in the medium during growth of *Trichoderma viride* on crude barley walls: ○, 25 °C; ●, 30 °C; □, 35 °C.

solubilization was highest at 30 °C (Figure 4). Most clearly at 25 and 35 °C temperatures the *onset* of release of arabinoxylan preceded that of β-glucan. The levels of protein in solution, which will reflect solubilization and digestion of protein but in particular enzyme synthesis, were substantially greater at 30 °C than at the other two temperatures (Figure 5), suggesting that this temperature is best suited to the metabolism of this organism.

Development of Enzymes by *T. viride* when Growing on Cell Wall Material. It is understood that the development of enzymes in *Trichoderma* that degrade hemicelluloses is subject to glucose repression but also induction by sophorose (*7*) and by hemicellulose substrates and/or their degradation products. For example, xylanase is induced by xylan and xylobiose (*19*). Thus, we have assumed in this study that the elaboration of individual enzymes during the growth of *T. viride* on the crude cell wall preparation reflects the availability of the hemicellulose components that demand those enzymes to digest them.

The temperature dependency of endo-xylanase development mirrors that for pentosan release, with the highest enzyme levels achieved at 35 °C and very low levels developed at 25 °C (Figure 6). Conceivably, advantage might be taken of the very high activity developed at 30 °C, where protein concentrations are relatively low, for the production of xylanase prepara-

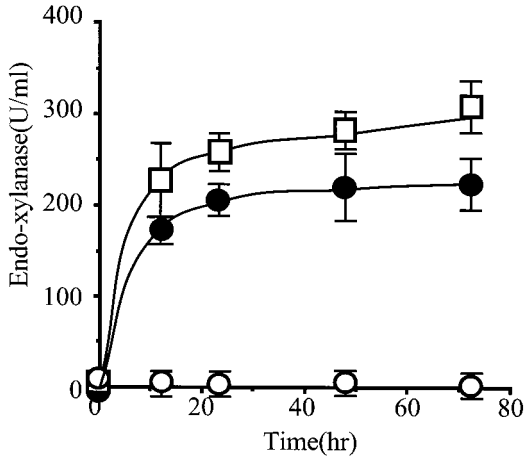

Figure 6. Release of endo-xylanase during growth of *Trichoderma viride* on crude barley walls: ○, 25 °C; ●, 30 °C; □, 35 °C.

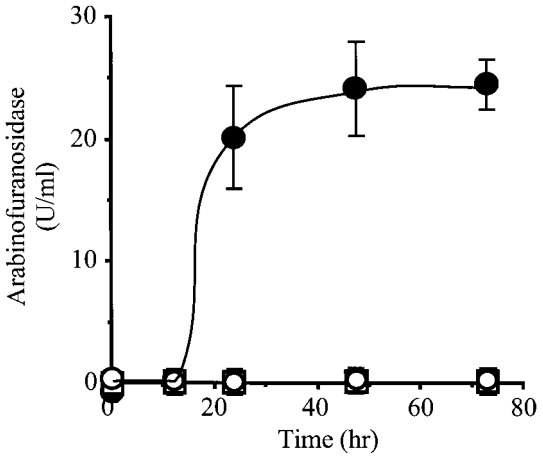

Figure 7. Release of arabinofuranosidase during growth of *Trichoderma viride* on crude barley walls: ○, 25 °C; ●, 30 °C; □, 35 °C.

tions of relatively high specific activity. In contrast to xylanase, detectable levels of arabinofuranosidase develop only at 30 °C (Figure 7), suggesting that it has no obligatory role in the solubilization of arabinoxylan.

Neither the development of endo-β-(1→4)-glucanase nor that of endo-β-(1→3)(1→4)-glucanase exactly mirrors the pattern of release of β-glucan into solution (Figures 8 and 9), although the extent of development of each enzyme in the order 30>35>25 °C certainly matches the extent of solubilization of glucan (Figure 4). In respect of fermentations at 30 °C, there is a closer alignment of glucan release with the development of endo-β-(1→4)-glucanase than with endo-β-(1→3)(1→4)-glucanase. It should be noted that *T. viride* is a cellulolytic fungus, that endo-β-1→4-glucanase is a key component of the cellulase complex (*20*), and that the cell wall substrate may contain some residual cellulose-rich husk material.

It has been suggested that solubilization of glucan may be catalyzed by esterolytic action, either through carboxypeptidase acting in an esterolytic capacity (*15* and *21*), via feruloyl acid esterases (*9*), or general esterases (*22*). Esterase and carboxypeptidase activities do increase in *T. viride* growing on cell wall material (Figures 10 and 11).

Most puzzling of all, however, is the pattern of development of "β-glucan solubilase" (Figure 12). This

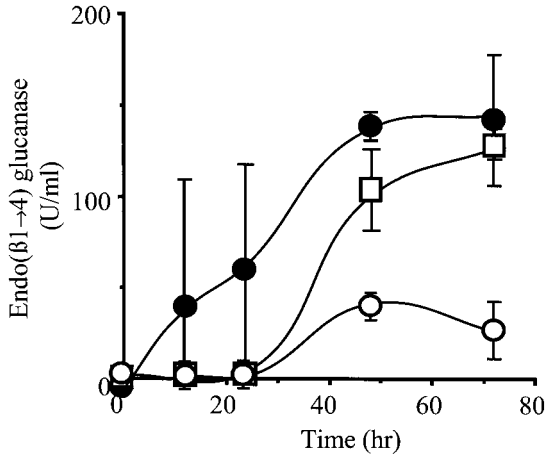

Figure 8. Release of endo-β-(1→4)-glucanase during growth of *Trichoderma viride* on crude barley walls: ○, 25 °C; ●, 30 °C; □, 35 °C.

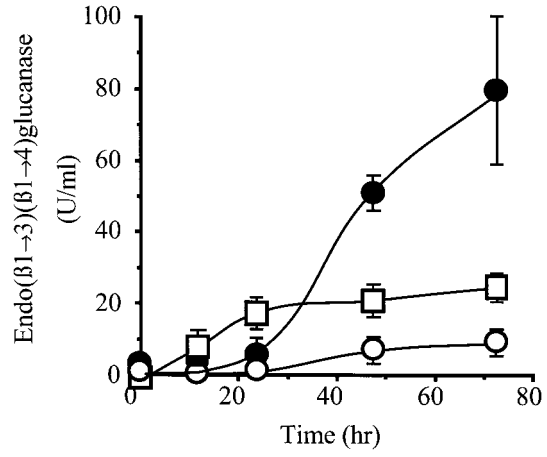

Figure 9. Release of endo-β-(1→3)(1→4)-glucanase during growth of *Trichoderma viride* on crude barley walls: ○, 25 °C; ●, 30 °C; □, 35 °C.

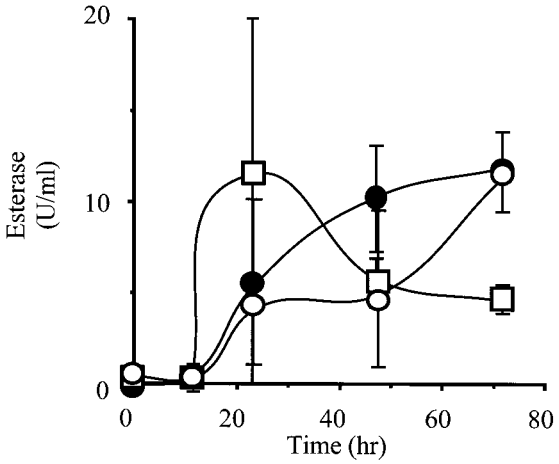

Figure 10. Release of esterase during growth of *Trichoderma viride* on crude barley walls: ○, 25 °C; ●, 30 °C; □, 35 °C.

is the name given to an activity first reported in barley and which is an enzyme(s) capable of solubilizing β-glucan from denatured barley flours of the type used as substrate for culturing *T. viride* in this study. It appears that this enzyme does not develop in cultures growing at 30 °C until after 24 h, and not before the second day when the fungus grows at 25 or 35 °C. It cannot, therefore, contribute to glucan release in the

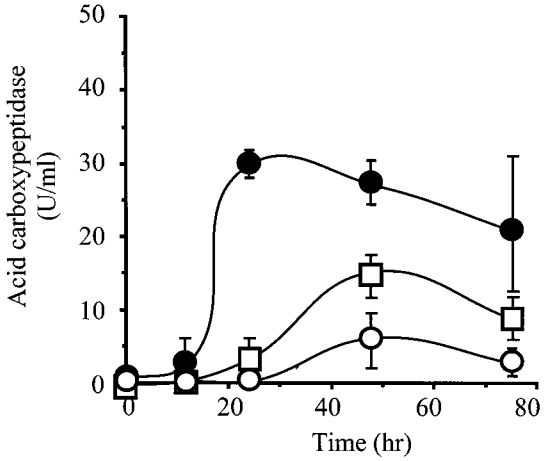

Figure 11. Release of carboxypeptidase during growth of *Trichoderma viride* on crude barley walls: ○, 25 °C; ●, 30 °C; □, 35 °C.

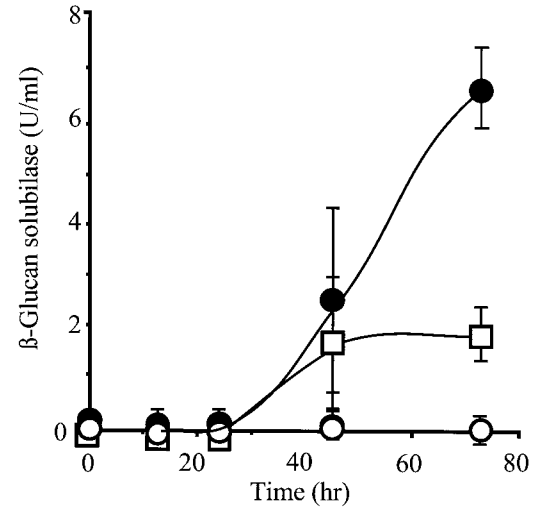

Figure 12. Release of "β-glucan solubilase" during growth of *Trichoderma viride* on crude barley walls: ○, 25 °C; ●, 30 °C; □, 35 °C.

initial stages of culture (Figure 4), though may do so later. Neither is its development pattern similar to that for any of the other enzymes described in this study, suggesting that it is due to another type of activity, as yet unidentified. The solubilase assay is performed at 65 °C, a temperature selected to reflect the conversion conditions in brewery mashes in which glucan release is of great significance.

Quantitative Aspects. The quantities of material released into the culture media are comparatively low. Thus, for example, the crude cell wall preparation comprises approximately 70% β-glucan. The most glucan measured in culture medium was some 80 μg/mL, as compared to an availability of 7 mg/mL β-glucan in the substrate. Some, of course, will be hydrolyzed to sugars, and more will be used in supporting growth of the organism. Using an equivalence of 1 g CO_2 to 0.61 g poly-glucose, then, based on the maximum extent of CO_2 production (Figure 2), we can calculate that rather less than 10% of the glucan has been utilized to support respiration. Clearly, the denatured barley material is not an especially good substrate in support of growth of this organism. Because crude enzyme preparations from this organism are able to totally convert barley β-glucan to glucose (*6*) it is presumed that factors other than the availability of the required hydrolases are limiting growth. Nevertheless, a degree of growth clearly is supported and this work has indicated which sequence of enzymes the organism requires to access the crude cell wall preparation: the release of arabinoxylan appears to precede that of β-glucan.

CONCLUSIONS

Six significant conclusions were reached on the basis of the findings of this study: (1) *Trichoderma viride* will grow on a crude cell wall preparation of barley over the temperature range 25−35 °C, with fastest growth occurring at 30 °C and slowest growth occurring at 35 °C. (2) Growth at the intermediate temperature is characterized by the highest rate of protein production, which is presumably a reflection of a higher degree of enzyme synthesis at this temperature. (3) Despite the very low levels of measurable protein released over the first 24 h of cultivation at 25 and 35 °C, considerable amounts of xylanase (at 35 °C) and some endo-β-(1→3)-(1→4)-glucanase and carboxypeptidase (also at 35 °C) could be detected. This might be exploited for the production of enzyme preparations of relatively high specific activity. (4) The onset of solubilization of pentosan precedes that of glucan, particularly at 25 and 35 °C, consistent with the need to remove at least a portion of pentosan in order to expose glucan. (5) The early development of xylanase appears to promote a solubilization of arabinoxylan into the medium. (6) Glucan solubilization initially appears to reflect the development of esterase, carboxypeptidase, and endo-β-(1→4)-glucanase, and only later to that of endo-β-(1→3)(1→4)-glucanase.

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