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Table of Contents

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Comparison of β-Glucanases Production by *Acremonium* sp. IMI 383068 in Batch and Continuous Culture System

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ABSTRACT

Studies on the possible influence of physical environmental factors on extracellular β-glucanase production by *Acremonium* sp. IMI 383068 were undertaken, in particular to assess the role of varying agitation speed and pO_2 levels, since these have been shown in other studiesto affect the yields of other fungal extracellular enzymes. The use of chemostat cultures in this present study meant that the possible influence of these two parameters on enzyme production could be controlled independently of each other and of growth rate. No other similar data on possible factors affecting fungal (1→3)- and (1→6)-β-glucanases are available in the literature, and therefore other fungal extracellular enzyme systems are used here as comparisons for this discussion.

To investigate the possible relationship between enzyme production and culture morphology, the HGU values or branching frequencies of the fungal hyphae were assessed. Since substantial conidial production also occurred in these cultures of *Acremonium* sp. IMI 383068, it was not possible always to distinguish between mycelial branches that were true vegetative hyphae and those eventually forming the short tapered lateral conidiophores that characterize this strain. Hence HGU determinations include both of these two branch types, except where it was obvious that the branch in question was a conidiophore, adjudged by the presence of emerging conidia from it.

The major findings of this study will be discused here which were mainly engaged to the relationship between production of (1→3)-β-glucanases and the growth of the fungus, where it was growth rate dependent, while that of the (1→6)-β-glucanase was growth rate independent. The production of (1→3)-β-glucanases was shear sensitive and also depended on O_2 availability in the culture medium. Based on the data from both batch and chemostat culture, it was found that there was no clear relationship between branching frequency of the fungal mycelium and β-glucanase production by the fungus.

Keywords: β-glucanases, Acremonium, HGU, batch and chemostat, branching frequency

1.1 Production of β-glucanases by *Acremonium* sp. IMI 383068 in batch and continuous culture

Studies on the possible influence of physical environmental factors on extracellular β-glucanase production by *Acremonium* sp. IMI 383068 were undertaken, in particular to assess the role of varying agitation speed and pO_2 levels, since these have been shown in other studies (Zetalaki & Vas, 1968; Mutakata *et al.*, 1988; Petruccioli *et al.*, 1995; Justen *et al.,* 1998; Barberis & Gentina, 1998) to affect the yields of other fungal extracellular enzymes. The use of chemostat cultures in this present study meant that the possible influence of these two parameters on enzyme production could be controlled independently of each other and of growth rate. No other similar data on possible factors affecting fungal (1→3)- and (1→6)-β-glucanases are available in the literature, and

therefore other fungal extracellular enzyme systems are used here as comparisons for this discussion.

To investigate the possible relationship between enzyme production and culture morphology, the HGU values or branching frequencies of the fungal hyphae were assessed. Since substantial conidial production also occurred in these cultures of *Acremonium* sp. IMI 383068, it was not possible always to distinguish between mycelial branches that were true vegetative hyphae and those eventually forming the short tapered lateral conidiophores that characterize this strain. Hence HGU determinations include both of these two branch types, except where it was obvious that the branch in question was a conidiophore, adjudged by the presence of emerging conidia from it.

1.2 Influence of μ on β-glucanase production

Based on the relationships between enzyme production and biomass formation in batch cultures of *Acremonium* sp. IMI 383068 grown with pustulan as sole carbon source, production of (1→3)-β-glucanase appeared to be growth related while (1→6)-β-glucanase was not. Thus (1→3)-β-glucanase activities increased as biomass levels increased during exponential growth, while (1→6)-β-glucanase activity increases were only detected at a time when biomass production had almost ceased, and then continued into the stationary phase. However, such data are not easily explained, since the (1→3)-β-glucanases, which are growth rate dependent, were not active against pustulan, the sole carbon source employed in the batch culture experiments, under the assay conditions used in this study. These relationships between growth and enzyme production were supported by the chemostat data with scleroglucan as the sole carbon source, which showed clearly that specific activities of both (1→3)- and (1→6)-β-glucanases were affected by changing μ. In particular, (1→3)-β-glucanase specific activities increased as μ increased. Specific enzyme productivity data (q_p) showed that (1→3)-β-glucanase production was clearly growth rate dependent, while (1→6)-β-glucanase production was non-growth dependent, as its specific enzyme productivity remained constant over a wide range of μ.

The different growth dependence manner of (1→3)- and (1→6)-β-glucanases may reflect that these two enzyme activities have different biological roles in the producing organism, but since nothing is known about the ecology of this fungus, what these roles might be is not easily postulated. Many fungi are mycoparasitic and (1→3)-β-glucanases are thought to be important in providing the fungi with the means for degrading parasitised fungal cell walls (Archambault *et al.*, 1998; Vazquez-Garciduenas *et al.*, 1998), but whether *Acremonium* sp. IMI 383068 has this mycoparasitic ability is unknown. However, similar (1→3)-β-glucanases from other fungi have been reported to be active in many processes including fungal morphogenesis, apical growth and also conidiogenesis (Pitson *et al.*, 1993; Fontaine *et al.,* 1997*a*). Not all of these are growth related. The (1→3)-β-glucanases from *Acremonium persicinum* were proposed to play a possible role in the degradation of external β-glucans produced by this fungus to be used as a carbon source, and in the autolysis of hyphal walls (Pitson *et al.*, 1997*c*), roles consistent with their regulation by glucose repression, but again not always obviously growth related. Since *Acremonium* sp. IMI 383068 does not produce any extracellular β-glucan (Pitson *et al.*, 1997*a*), the β-glucanases from this fungus probably have no involvement in degradation of its own glucan for use as a carbon source in this organism, although they may be important in degrading glucans from other sources. The functions of any (1→6)-β-glucanase in fungi generally are less well understood. In *Acremonium* sp. IMI 383068, this enzyme activity was detected only when growth had ceased, which might suggest some roles for it in autolysis or possibly conidiation, except that conidial production always occurred during the growth phase in submerged culture.

Other fungi have been reported to produce growth rate dependent extracellular enzymes, as is the case with the α- amylase from *Aspergillus oryzae* (Morkeberg, Carlsen, & Nielson, 1995) and the glucoamylase from *Aspergillus niger* (Withers *et al.*, 1998; Metwally, 1998; Wongwicharn *et al.*, 1999*b*), consistent with their biological function of degrading extracellular polysaccharides. However, extracellular glucoamylase production by *Fusarium venenatum* from chemostat data (Wiebe *et al.*, 2000) appeared to be a non-

growth associated product. It was suggested that the actual synthesis of this enzyme may in fact be intracellular and growth rate dependent, and the enzyme then only released into the medium upon cell wall autolysis during stationary phase. This seems unlikely, as it does for the (1→6)-β-glucanase from *Acremonium* sp. IMI 383086, since under the chemostat conditions used in these experiments, exponential growth is occurring and mycelial autolysis is not expected. There is some evidence suggesting that different mechanisms of enzyme secretion, growth rate related or not, may exist in *F. venenatum* depending on whether the fungus is grown in batch or chemostat culture (Wiebe *et al.* 2000). Even though any possible intracellular (1→6)-β-glucanase activity of *Acremonium* sp. IMI 383068 was not determined in this study, its activity in *Acremonium persicinum* grown in batch culture was detected both intracellularly and extracellularly (Pitson *et al.*, 1996*a*, 1999).

The chemostat studies also show clearly that changes in β-glucanase activities with changing μ in *Acremonium* IMI 383068 did not always parallel similar variations in its HGU values, which above μ = 0.02 h^{-1} remained constant. Under such conditions where branching frequencies do not change with μ, then hyphal extension rates of the active growth region (Agger *et al.*, 1998), the "active length" (Wongwicharn *et al.*, 1999) or active tips (Amanullah *et al.*, 2002) may be related to changes in levels of production of these enzymes. It is also possible that β-glucanase secretion in *Acremonium* IMI 383068 is related to the extension rate of individual hyphae, and not to the total number of hyphal tips. Tenberge *et al.* (1999) showed with immunogold labelling that the endo-β-glucanase in *Claviceps purpurea* was secreted at hyphal tips, and the glucoamylase in *Aspergillus niger* was also concentrated at hyphal apices (Wosten *et al.*, 1991), shown using green fluorescent protein studies. The results from this present study do suggest that β-glucanase yields in *Acremonium* sp. IMI 383068 are not causally related to hyphal branching frequency, although this has been suggested for several other fungal extracellular enzymes (Johansen *et al.*, 1998; Trinci *et al.*, 1999; Wongwicharn *et al.*, 1999), and the suggestion has been made (Peberdy, 1994) that it might be possible to increase enzyme yields by increasing the frequency of branching, i.e. the number of tips

as sites for protein secretion. In some fungi like *Aspergillus nidulans* and *P. chrysogenum*, HGU changes with μ, often decreasing i.e. becoming more branched, as μ increases (Katz *et al*., 1972; Morrison & Righaletto, 1974; Papagianni *et al* ., 1999), while in other cases μ seems to have no influence on branching frequency (Trinci, 1973), suggesting that different fungi employ different strategies, with possible ecological significance, in response to changes in their growth rates.

1.3 Influence of shear and pO_2 on β-glucanase production

The production of extracellular (1→3)- and (1→6)-β-glucanases by *Acremonium* sp. IMI 383068 was affected markedly by agitation speeds in batch cultures grown with either pustulan or scleroglucan as carbon sources. Specific activities of both enzymes increased with agitation speeds up to 400 rpm, but then a large reduction was recorded with both substrates as agitation speeds increased further (Fig.3.16). These results agree generally with those published for other fungi and other extracellular enzymes, including the cellulase from *T. reesei* (Mukataka *et al.*, 1987; Lejeune & Baron, 1995), glucose oxidase and catalase from *P. variabile* (Petruccioli *et al.*, 1995), glucoamylase from *Aspergillus niger* (Withers *et al.*, 1998) and lysozyme from *Aspergillus niger*(Wongwicharn *et al.*, 1999). However, from these batch culture studies it is not possible to determine whether these changes in enzyme production were caused by a direct influence of shear or indirectly by increasing O_2 availability in the medium, since as agitation speeds increase the k_La of the system would also increase. The data from the present study suggest that the influence of agitation speed is on enzyme production, since no changes in levels of activities of pre-existing (1→3)- and (1→6)-β-glucanases produced with pustulan as a carbon source, were detected after their exposure for prolonged periods to higher agitation speeds of up to 1000 rpm.

Differences in both (1→3)- and (1→6)-β-glucanase production were also observed in *Acremonium* sp. IMI 383068 in response to changes in aeration rate in batch culture, but this effect depended on the carbon source used. When grown with pustulan, a large reduction in specific activities of both enzymes occurred as aeration rates were increased. However with scleroglucan, specific activities of β-glucanases increased as aeration rates

increased. Evidence presented here suggests that the formed enzymes are not oxygen labile either, since no changes in activities of pre-existing (1→3)- and (1→6)-β-glucanases produced in batch culture with pustulan as a carbon source, were detected after their exposure for prolonged periods to higher aeration rates.

The differential effects of aeration rate on enzyme production, which depended on whether scleroglucan or pustulan was used as the carbon source, may reflect differences in O_2 transfer rate in the two media as a result of a reduction in k_La values with the more viscous scleroglucan containing medium. Much larger air bubble sizes would mean a reduction in their interfacial surface areas, but other liquid phase resistance barriers possibly at the hyphal surface may also operate, affecting the balance between O_2 transfer rates to the medium and the cells' consumption rates of O_2. At 0.3 vvm, production of (1→3)-β-glucanase was lower in scleroglucan containing medium compared to that with pustulan. This may indicate that at this level, with scleroglucan containing medium, O_2 is too limiting to support enzyme production, and the rate of O_2 supply is still insufficient compared to the rate of oxygen consumption, while in pustulan containing medium which has a much higher k_La, this aeration rate may be sufficient. However, when the aeration rates increased from 0.3 to 0.6 vvm, measured specific activity of the enzyme with scleroglucan containing medium increased, but with pustulan containing medium it dropped. This may indicate some inhibition of enzyme production which may be from O_2 toxicity at higher DO levels (Halliwell, 1994; KrIener *et al.*, 2000 & 2003; Bai *et al.*, 2003), occurring in pustulan containing media but not in those with scleroglucan. Therefore, there may be a window of k_La values below which oxygen availability is too limiting to support enzyme production, and above which enzyme production is inhibited, as observed in studies with lactase from *Kluyveromyces fragilis* where its specific activities fell at higher k_La (Garcia-Garibay *et al.*, 1987).

It is difficult to distinguish between any direct influence of O_2 availability and an indirect one of the effect of shear on fungal enzyme production (Gibbs *et al.*, 1996). Whether O_2 availability or shear stress alone or together affects production of these glucanases can not be distinguished from the early batch culture data obtained here. Experiments where

pO_2 levels are controlled are crucial to distinguish between them, as used with studies on lactase specific productivity in *K. fragilis* (Barberis & Gentina, 1998), where the influence of pO_2 level was indirect and exerted through changes in agitation speed. When *Acremonium* sp. IMI 383086 was grown at a constant agitation speed and the pO_2 was controlled with a gas mix system, thus minimizing changes to the physical environment in the fermenter, it was clearly shown that production of both (1→3)- and to a lesser extent (1→6)-β-glucanases was affected by changing pO_2 levels. Even though only relatively small changes in specific activities were detected with pustulan grown cultures and changes in controlled pO_2 levels, with scleroglucan it is clear that at 40 % saturation the specific activity of (1→3)-β-glucanase was much higher compared to that achieved at 5 % of saturation. This supports the view that O_2 availability plays a major role in controlling the production of both types of enzyme. Sufficient O_2 in the culture medium is essential for allowing ATP generation, but at too high levels of O_2, an oxidative stress may be imposed upon cultures, possibly mediated through generation of superoxide radicals, as observed in *Aspergillus niger* (Kreiner *et al*., 2000; Bai *et al.*, 2003).

The role of O_2 availability in β-glucanase production by *Acremonium* sp. IMI 383068 becomes clearer from the chemostat experiments where a controlled growth rate and pO_2 levels were employed independently of each other and of shear, allowing the influence of each to be unequivocally determined. Higher production of (1→3)-β-glucanase occurred at the higher levels of 20 % and 40 % compared to 5 % DO saturation. Whether fungi favor high or low levels of pO_2 for enzyme production cannot be generalized since different enzymes appear to behave differently in response to the pO_2 level, depending on the organism and the enzyme. In production of lactase by *K. fragilis,* maximum specific activity was reportedly achieved at 10 % saturation, and then decreased, albeit only slightly, at higher levels (Barberis & Gentina, 1998). However, *Aspergillus niger* (Wongwicharn *et al*., 1999) produced more than double the specific activity of glucoamylase with 50 % compared to 10 % O_2 enrichment of air. A drop in amyloglucosidase yield from *Aspergillus oryzae* because of O_2 limitation also has been reported by Amanullah *et al.* (2000). Even though the possible role of pO_2 in affecting

extracellular enzyme production has not often been pursued in studies with fungi, the organism's response to the presence of O_2 may be explained in terms of their production of catalase, superoxide dismutase and glutathionine peroxidase, which are all thought to be responsible for defending cells against O_2 toxicity (Kreiner *et al.*, 2000&2003; Bai *et al.*, 2002&2003).

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