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ORIGINAL RESEARCH PAPER

Activity-based identification of secreted serine proteases of the filamentous fungus, *Ophiostoma*

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Abstract A general activity probe was synthesized and applied to the supernatant of a filamentous fungus, *Ophiostoma*, culture to identify directly the secreted serine proteases by covalent enzyme labeling. The activity probe contained a chemically reactive group that reacted with, and thus covalently labeled, the serine residues of only active proteases and not heat-inactivated proteases. The activity probe also contained a fluorescent group that allowed for the subsequent visualization of the captured proteases in 1-D gels and their identification by *N*-terminal sequencing. This use of a chemical probe led to the rapid discovery of subtilisin-like serine protease of *Ophiostoma*. Two hypothetical proteins were also captured, with one being a probable endopeptidase K type of protease.

Keywords Enzyme labeling · Filamentous fungi · Fluorescent activity probe · *Ophiostoma* · Secreted proteases

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Introduction

Filamentous fungi can effectively secrete various hydrolytic enzymes into their culture medium. This secretion capacity has been utilized both in industrial enzyme production and in the development of fungi as effective hosts for heterologous production of valuable gene products (Punt et al. 2002; Nevalainen et al. 2005). One group of the main secreted enzymes in fungi are the proteases (Abraham et al. 1995; Hoffman and Breuil 2002, 2004). While proteases as such can find industrial applications, some of their hydrolytic activities also pose a major problem affecting especially the yields of foreign gene products and thus limit their use as surrogate hosts at the industrial scale (van den Hombergh et al. 1997; Nevalainen and Te'o 2003). The ability to profile and identify protease activities responsible for the unwanted degradation remains an issue.

Production of proteases in liquid culture is typically assayed by using azocasein as a general substrate (Lovrien et al. 1985). This preliminary assay may be followed by more specific assays using, for example, commercially available fluorescently labeled peptides capable of differentiating between the main protease classes. However, these assays only provide nonspecific information on the activities without any capability to capture or target a specific family or individual proteases. Specific targeting or profiling of

particular endogenous protease activities would facilitate the design of optimal cultivation conditions and selection of a suitable expression host devoid of side protease activities harmful to the gene product of interest.

Activity-based chemical probes have been developed to capture proteases in their active forms under native culturing conditions for drug discovery (Jeffery and Bogyo 2003; Berger et al. 2004). The probes were reported to label the active residues, such as serine, cysteine or lysine, typically seen in proteases and transferases. The active residue of an enzyme or protein attacks the reactive group of the probe, resulting in a covalent linkage between the active residue of the protein and the probe (Fig. 1). In addition to the reactive group that serves as an activity tag, activity probes typically have three additional components: a linker region, a fluorescent reporter, and an affinity tag for purification of low abundance proteins (Fig. 1). The fluorescent reporter group allows for the physical identification of the tagged protein on gels. The affinity tag allows the protein to be further purified if necessary by affinity purification. The linker physically separates out the different functionalities so they will not interfere with one another's role.

This activity-based approach has been successfully used to identify key enzyme activities in biological systems of the animal kingdom (Jessani and Cravatt 2004). For example, Bogyo and coworkers (Greenbaum et al. 2002) used this strategy to elucidate the important role of cysteine proteases in the host cell invasion process of human malaria parasite, *Plasmodium falciparum*. The *P. falciparum* proteome was subjected to cysteine protease probes, which identified four important protease activities from whole cell lysates.

In this study, the activity-based approach was applied for the first time to investigate the secreted serine proteases from the filamentous fungus *Ophiostoma*. The *Ophiostoma floccosum* strain J2026 MQ 1.2 was derived from *O. floccosum* J2026, an organism known to produce subtilisin-like proteases (Wu et al. 2006). This new approach not only identified the expected serine proteases accurately but also revealed new proteins that have not been previously identified or reported.

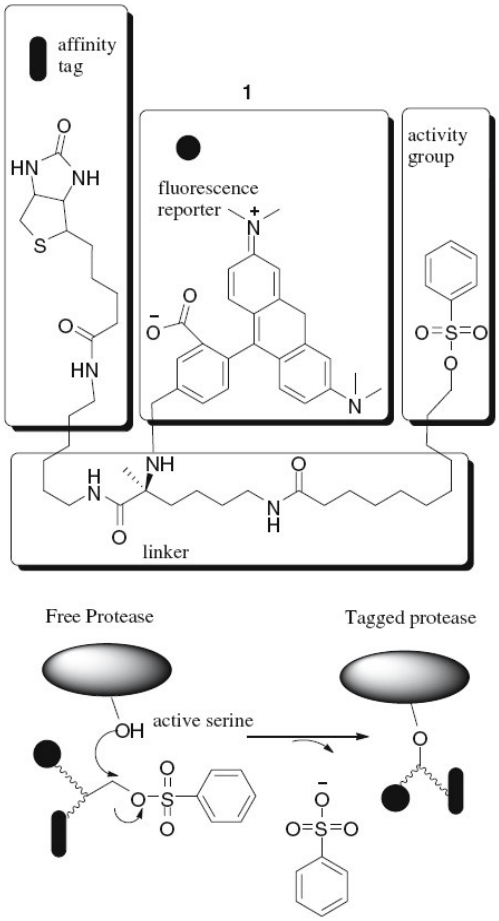

Fig. 1 The anatomy of an activity probe and its labeling of the active serine residue of a protease

Materials and methods

Synthesis and stability test of the activity probe **1**

An activity probe, **1**, was synthesized according to the reported procedure (Adam et al. 2002). HPLC purification was carried out using Prevail C18 analytical or semi-preparative columns (Alltech) in H_2O/acetonitrile (1:1 v/v) with 0.1% TFA to afford the purified probe **1**. Compound **1** was then characterized by LC-MS using a SGE Wakosil C_{18} column (LCMS-2010; Shimadzu, Japan) and fluorescence spectroscopy (LS55 luminescence spectrometer; PerkinElmer, USA) to confirm its synthesis. The mobile phase used in

the LC-MS was a gradient from 70% A/30% B (A: HPLC-grade water with 0.1% formic acid; B: acetonitrile with 0.1% formic acid) to 5% A/95% B in 15 min, followed by a gradient of 95% B for another 10 min at 0.2 ml min^{-1}. Fluorescence scans were carried out for compound **1** at 1 μM in 50 mM Tris/HCl buffer at pH 8. The activity probe **1** (5 μM) was then incubated in buffer conditions for the subsequent protein labeling step (50 mM Tris/HCl buffer at pH 8, with or without 0.5% SDS for 1 h). The probe integrity was examined by HPLC using an Alltech Prevail C_{18} column, 4.6 × 250 mM. The mobile phase was a mixture of acetonitrile/water (1:1 v/v) containing 0.1% TFA at 1 ml min^{-1}.

Fungal strain and culture conditions

Ophiostoma floccosum strain J2026 MQ 1.2 was derived from *O. floccosum* J2026 (kindly provided by Professor Roberta Farrell, University of Waikato, Hamilton, New Zealand) by UV mutagenesis (Bailey and Nevalainen 1981). It was grown in 250 ml conical flasks, containing 50 ml minimal salts (Penttilä et al. 1987) supplemented with 3% (w/v) soluble starch (Mallinckrodt, USA) and 1.5% (w/v) soybean flour. Each flask was inoculated with 10^8 spores harvested from PDA plates using 0.9% NaCl with 0.01% Tween 80. Cultures were filtered and then shaken (250 rpm) at 23–25°C in the dark for 5 days.

Preparation and assay of the harvested fungal protein sample

The culture supernatant (50 ml) was harvested by centrifugation (10,000 × *g*, 4°C for 10 min) and concentrated using a Centriprep YM-3 column (Millipore, USA) to 4 ml. Subsequently, the concentrated sample was desalted using a dialysis tubing (MWCO 10 kDa) in 2 l of a 50 mM Tris/HCl buffer at pH 8 overnight at 4°C. The insoluble material was removed by centrifuging at 10,000 × *g* for 10 min. All samples were kept at 4°C till use. The concentration of the secreted proteins in the culture supernatants was measured using a Bio-Rad protein assay kit.

Assay of total subtilisin-like protease activity

General subtilisin-like protease activity was measured as described previously with some modifications (Markaryan et al. 1996). All reaction mixtures included 0.05 mM of substrate (*N*-Suc-Ala-Ala-Pro-Phe 7-amino-4-methylcoumarin, Sigma), 50 μl sample and 140 μl 0.1 mM Tris/HCl buffer at pH 8.0 containing 5 mM $CaCl_2$. After incubation at 37°C for 10 min, the fluorescence was determined by a fluorimeter at 360 nm excitation and 460 nm emission wavelengths. All assays were carried out in duplicate.

Labeling and detection of excreted proteins after harvest

Protein samples were treated with 5 μM activity probe, **1**, at room temperature. Samples were quenched with one fourth volume of a standard 5× SDS-PAGE loading buffer and separated in a self-casted 12.5% (v/v) SDS-PAGE gel, followed by visualization using a Typhoon laser induced fluorescence scanner (Molecular Technology, Germany). Coomassie blue R250 was used to stain the gel subsequently. Prior to labeling, the samples were denatured by treatment with 10% (w/v) SDS (final concentration of SDS: 0.5% w/v), followed by heating at 90°C for 15 min.

N-Terminal sequencing

Selected protein bands were excised from the 12.5% (v/v) SDS-PAGE gels and their *N*-terminal sequences were determined at the Biomolecular Research Facility, University of Newcastle, Australia. The sequences were analyzed using the blast search engine at the National Center of Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov).

Results

Synthesis and characterization of the activity probe

The activity probe, **1**, was synthesized as previously reported (Adam et al. 2002). An

electrophilic phenyl sulfonate ester, previously reported to covalently label the active sites of enzymes including serine proteases, was linked to a rhodamine and biotin group for visualization and purification. The final probe was characterized by LC-MS. The dominant signal of a doubly charged ion (m/z 590, $C_{62}H_{82}N_8O_{11}S_2 + 2H^+$) confirmed the identity and purity of this probe. The fluorescence characterization of **1** indicated the absorbance peak at 550 nm and the major emission peak at 580 nm. On the basis of this profile, a green laser (532 nm) was selected as the fluorescence excitation source, and the emission wavelength was set at 580 nm for gel visualization. Probe **1** was incubated with or without 0.5% (w/v) SDS for 1 h and analyzed by HPLC. The identical chromatograms confirmed its stability.

The labeling of the secreted proteases by the probe is activity-dependent.

The processed culture supernatant of *O. floccosum* strain J2026 MQ 1.2 provided typically 0.8 mg protein ml^{-1}. The presence of protease secretion was confirmed by the conventional fluorescent peptide assay (Markaryan et al. 1996, data not shown). The samples were then treated with **1** for 40 min for the concentration-dependent study (Fig. 2A). Coomassie Blue staining of the gel confirmed that the total protein loading was consistent in each sample and not affected by any step of the sample denaturing manipulation (Fig. 2B).

Higher background fluorescence was detected when the probe was used at 10 or 20 μM (Fig. 2A, lanes 2 and 3). The labeling of active proteases, however, was significantly decreased if the probe concentration was lowered to 2.5 μM (Fig. 2A, lanes 7 and 8). A probe concentration of 5 μM provided the best signal-to-noise ratio in detecting only the active proteases.

To confirm that the activity probe would label only the active forms of the secreted proteases, samples were treated with SDS and heated to denature the enzymes. The deactivated proteases were then treated with the activity probe to examine if the activity-based labeling would be negated. All denatured protease samples (Fig. 2A and B, lanes 2, 3, 5, and 7) showed only background fluorescence, indicating that the labeling is activity-dependent.

A time-course study to determine the optimal labeling time

Probe **1** was applied at 5 μM and the labeling reaction was quenched at different time points. A negative control, the precursor of probe **1** that does not contain the labeling group, was used instead of probe **1** and examined after 60 min of mixing to provide a background fluorescence reading (Fig. 3A and B).

Protein samples treated with varying concentrations of the negative control (the activity probe without the reactive group for covalent labeling) showed negligible fluorescence on gel as expected (Fig. 3A, lanes 7 and 8). Four proteins were detected by the activity probe in all of the time points (Fig. 3A, lanes 2, 4 and 6). Only background fluorescence was detected in the reactions of the denatured samples with the probe (Fig. 3A, lanes 1, 3 and 5). The Coomassie stain indicated that protein loading was consistent in each lane without nonspecific protein degradation due to sample manipulation (Fig. 3B). The labeling saturated in less than 10 min.

Identification of the proteins labeled by the activity probe

The proteins (Fig. 3A) detected by the probe were *N*-terminally sequenced and their identities are listed in Table 1. This *Ophiostoma* strain has enhanced production of subtilisin-like protease activities, although the specific protein identities are not known (Wu et al. 2006). The sequences were searched using the NCBI database within the fungi kingdom. Of the four proteins selected for *N*-terminal sequencing, protein 2 was matched to a subtilisin-like serine protease found in several *Ophiostoma* species including *O. novo-ulmi*, *O. floccosum* and *O. ulmi* (Hoffman and Breuil 2002). Protein 4 was also matched to a subtilisin-like protease from *Ophiostoma piceae* (Abraham et al. 1995; Hoffman and Breuil 2002, 2004). Proteins 1 and 3 were found to be un-annotated proteins, with protein 3 matched to the probable endopeptidase K from *Neurospora crassa* (Galagan et al. 2003), a member of the same subtilisin-like protease family but under a different sub-family that requires thiol for

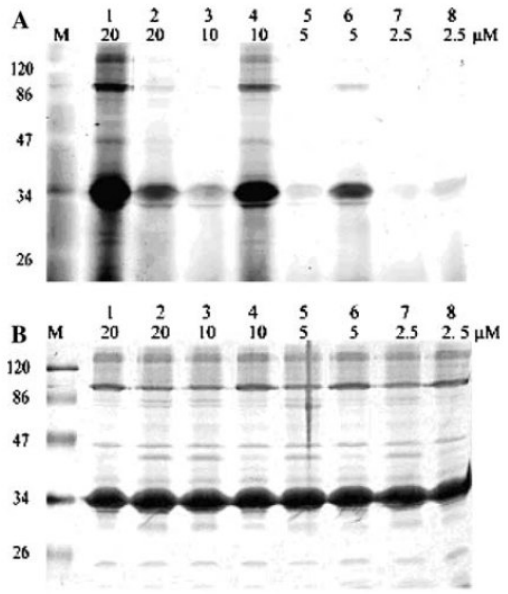

Fig. 2 The effect of probe concentration on the activity-based labeling of the secreted proteome of the *Ophiostoma floccosum* strain J2026 MQ 1.2. (**A**) The fluorescence gel image of proteins treated with the fluorescent activity probe 1, indicating only proteins labeled by the activity probe. (**B**) The same gel stained by Coomassie subsequently, indicating all proteins in each lane. M, molecular weight marker; lanes 1, 4, 6 and 8, samples mixed with 20, 10, 5 and 2.5 μM of probes for 40 min; lanes 2, 3, 5 and 7, denatured samples labeled with 20, 10, 5 and 2.5 μM probes for 40 min. In each lane, 50 μg of proteins was loaded

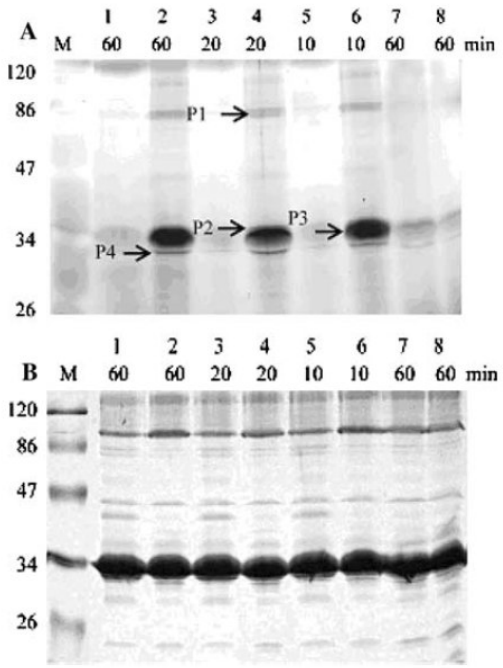

Fig. 3 The effect of the incubation time on the labeling by the activity probe using the secreted proteome of *Ophiostoma floccosum* strain J2026 MQ 1.2. (**A**) The gel fluorescence image of the proteins labeled by the probe. (**B**) The same gel stained by Coomassie subsequently. M, molecular weight marker; lanes 1, 3, and 5, denatured samples incubated with the activity probe for 60, 20, and 10 min; lanes 2, 4 and 6, samples labeled with the activity probe 1 for 60, 20 and 10 min; A probe precursor without the activity labeling group was used to treat the sample for 60 min at two different concentrations (lane 8, 10 μM; lane 9, 5 μM). In each lane, 50 μg of protein was loaded. Proteins P1–P4 refer to the proteins labeled by the probe and used for the *N*-terminal sequencing

activation. No match in the same fungal class could be found for protein 1.

Discussion

Studies into proteases produced by *O. floccosum* have featured enzyme activity assays and SDS-PAGE analyses of proteins secreted into the culture supernatants. Application of an activity probe, **1**, represents a rapid approach to the capture and analysis of proteases by their activities, not just expression, in the supernatants of *Ophiostoma*. The activity probe used in this study contains a sulfonate group that is readily displaced by the active serine residue of a protease. The fluorescent group and the biotin-affinity group of the probe allowed for further manipulation of the tagged proteases for visual-detection and analysis. The biotin group was not exploited in this proof-of-concept study as the captured proteases in this case were easily isolated and purified by the gel process. The biotin affinity tag, however, will be necessary in isolating and enriching for low-abundance proteases in future studies. The multiple functional groups on this probe did not interfere with the activity capturing, indicating that the linker separation was adequate for this application.

The activity probe in this study provided sensitive, accurate and rapid detection of serine protease activities. The background fluorescence

Table 1 The *N*-terminal sequences of proteins in the culture supernatant of *O. floccosum* J2026 MQ 1.2 detected by the activity probe

Protein	Sequence	Identification	Accession number	Matched fungal genus
P1	TFPKASVTVA	Unknown	Unknown	Unknown
P2	AYTTQTGAPW	Subtilisin-like protease	AAL08502.1	*Ophiostoma*
P3	VYDSQAGAGS	Probable endopeptidase K	CAD71122	*Neurospora*
P4	ALTTQSGGTT	Subtilisin-like protease	AAL08508	*Ophiostoma*

in the negative controls (Figs. 2A, 3A) maybe due to the residual protease activities from incomplete heat deactivation. Longer heating time was used but resulted in nonspecific degradation of the proteins (data not shown). The signal-to-background ratio (e.g. Fig. 3A lane 2 vs. lane 1) is clearly high to provide unequivocal read-outs. This also suggests that the detection of the protease activity probe is sensitive. The probe was stable under denaturing conditions, which attributes the lack of labeling in denatured samples solely to the inactivation of the protein activities. The activity labeling required a relatively short amount of time (10 min) and a small amount of probe (5 μM). The identities of the captured proteases revealed in this study are consistent with earlier studies (Galagan et al. 2003; Gao and Breuil 1998; Hoffman and Breuil 2002). Proteins 2 and 4, which had been suggested in our previous work, were found and identified in this study. The probable endopeptidase K-type of proteases, to which protein 3 is matched, are known to be among the excreted protease proteome of fungi (Hoffman and Breuil 2002). All three proteins (2, 3 and 4) have the typical molecular weight (~30 kDa) of secreted fungal proteases. A completely unknown protein around 80 kDa (protein 1) was labeled by the activity probe, suggesting a potentially new enzyme to be added to the secreted proteome of this organism.

In summary, *in vivo* labeling by a small molecule activity probe provided a rapid and efficient detection of active proteases directly in the native secreted fungal proteome. Known and unknown proteins were discovered by this method. The scope of this application for a wider range of fungi with a higher through-put for profiling protease activities is being examined and will be reported in future.

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Expression of a thermostable bacterial xylanase in the filamentous fungus *Ophiostoma floccosum*

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ABSTRACT

Aims: To express a thermostable bacterial xylanase in a novel fungal host.

Methods and Results: A codon-modified *D. thermophilum xynB* gene was expressed under the *O. floccosum* amylase (*amy1*) gene promoter in a mutant strain developed for efficient protein secretion. Expression of the *xynB* gene in a particular transformant, SS41, was studied at the transcriptional level, xylanase activity in the culture supernatant was measured and the active protein visualized by a Zymogram assay. Proteins of the molecular mass of 30 and 27 kDa exhibited thermostable xylanase activity. Expression of the thermostable xylanase also served as a secondary screen for the identification of true *O. floccosum* transformants after antibiotic screening.

Conclusions: An enzymatically-active heterologous bacterial xylanase with biotechnologically attractive properties was successfully produced by *O. floccosum*. Expression of the enzyme provided a confirmatory screen for genetic transformation of a 'hard to transform' *O. floccosum* strain.

Significance and Impact of the study: This is the first example of expression of any foreign secreted enzyme in the newly developed expression host *O. floccosum*, a fungus capable of growing in wood parenchyma cells, thus providing means for *in situ* delivery of selected gene products into wood tissues.

Keywords: *Ophiostoma*, thermostable xylanase, genetic transformation, *amy1*, *xynB*.

INTRODUCTION

A considerable part of the industrial attraction of filamentous fungi originates from their ability to secrete large amounts of proteins into the growth medium. Accordingly, filamentous fungi are being applied as cell factories for the expression of recombinant proteins for various purposes (Punt *et al*. 2002; Nevalainen and Te'o 2003; Adrio and Demain 2003; Bergquist *et al*. 2004; Gerngross 2004; Nevalainen *et al*. 2005). Examples of expression systems currently used are those developed for *Trichoderma reesei* and *Aspergillus niger* var. *awamori* which utilize strong promoters such as *cbh1* (*T. reesei* cellobiohydrolase I) and *glaA* (*A. niger* glucoamylase) (Gouka *et al*. 1997; Penttilä and Limón 2004).

Ophiostoma spp. are economically important organisms that are commonly isolated from blue stain-damaged wood. A successful strategy of inoculating wood chips with a colourless isolate of *O. piliferum*, commercialized as Cartapip^{TM}, not only decreased the triglyceride content in wood by 60-80 % (Farrell *et al*. 1993) but prevented infection by the wild type fungus. This approach enhanced the quality of paper by eliminating pitch, improved the pulping efficiency and reduced the effluent toxicity (Farrell *et al*. 1993). *Ophiostoma* spp. can penetrate wood, establish themselves in the living cells of sapwood, move into axial tracheids, ray parenchyma cells and eventually, the cell lumen. In our recent work, a series of high protein-secreting mutants were generated from *O. floccosum* by UV mutagenesis. One particular mutant (MQ.5.1) with a good secretion ability and low protease production was identified as a candidate for further development as an expression host. Consequently, the promoter region, transcription terminator and the DNA sequence encoding one of the dominant secreted proteins, α-amylase (*amy1*), was isolated and characterized (Wu *et al*. 2006). The α-amylase promoter seemed to be suitable for the expression of recombinant proteins in *Ophiostoma,* which in general, secretes only a small number of different proteins into the culture medium.

Since there are no previous reports on the expression of secreted heterologous gene products in *O. floccosum*, we opted to test the system with a bacterial xylanase enzyme that has industrial potential in pulp bleaching (Morris *et al*., 1998) and fibre modification process. The *xynB* gene of *Dictyoglomus thermophilum* encoding a thermostable xylanase (Te'o *et al*. 2000) was expressed under the *O. floccosum amy1*

gene promoter fused to the α-amylase secretion signal. Xylanase activity in the culture supernatant was monitored by an enzyme activity assay and visualized in a zymogram gel. Successful expression of the recombinant xylanase in *Ophiostoma* facilitates the *in situ* delivery of gene products into wood cells.

MATERIALS AND METHODS

Fungal strains and culture conditions

All chemicals used in this study were from Sigma (USA), unless otherwise specified. The high protein-secreting strain *Ophiostoma floccosum* MQ.5.1 was derived from *O. floccosum* J2026 by UV mutagenesis (Wu *et al*. 2006). Fungi were maintained on potato dextrose agar (PDA) plates. Four different media were used to study the effect of the carbon source on xylanase production. Liquid cultures were performed in 250 ml conical flasks containing 50 ml of minimal salts (Penttilä *et al*. 1987) supplemented with (i) 3 % (w/v) soluble starch (SM); (ii) 3 % soluble starch and 1.5 % (w/v) soybean flour (SSM); (iii) 2 % (w/v) insoluble starch (ISM) or (iv) 2 % (w/v) insoluble starch and 1.5 % soybean flour (ISSM). The pH in all media was adjusted to 6.5. ISSM (medium iv) at pH 5.5 was further used to explore the effect of pH on xylanase production. Each flask was inoculated with 10^8 spores harvested from PDA plates with a 0.9 % (w/v) NaCl-0.01 % (v/v) Tween 80 solution and filtered through a glass funnel (Pyrex, grade 1). Cultures were incubated at 28 °C on a gyrotary shaker at 250 rpm for five days in the dark.

Construction of expression vectors

The heterologous xylanase *xynB* gene was expressed under the α-amylase (AMY1) promoter and secretion signal obtained from *O. floccosum* (Wu *et al*. 2006). In order to construct pOMAY20 (Fig. 1), three DNA fragments consisting of the *amy1* gene promoter, partial α-amylase-coding sequence and the transcription terminator were amplified by PCR using primers listed in Table 1 and MQ.5.1 genomic DNA as a template. The reverse primer amy1nointfu.rev did not contain the two small introns located at the 5'-end of the native α-amylase coding gene. The three amplified gene fragments (around 3.2, 0.8 and 1.5 kb) were recombined by overlap PCR, which was carried out in two steps including a template extension step and a standard PCR (Te'o *et*

al. 2000). The template extension PCR reactions contained 100 ng of the DNA mixture (fragment 1: fragment 2: fragment 3 in Fig. 1 in a ratio of 6:2:2), 25 µM dNTPs, 1 x buffer, 1 unit of Triple Master DNA polymerase (Eppendorf, Germany) and H_2O to 25 µl. Reaction conditions were 1 x (94°C, 2 min), 15 x (94°C, 30 s; 60°C, 30 s; 72°C, 3.5 min). PCR products were purified using a Qiagen PCR purification kit (Qiagen, Germany). Standard PCR reactions contained 2.5 or 5 µl of the PCR product purified from the overlapping PCR step, 25 µmol dNTPs, 100 ng amy1BamHINotI.for primer, 100 ng amy1NotIKpnI.rev primer, 1 x buffer, 1 unit of Triple Master DNA polymerase and H_2O to 50 µl. The reaction conditions were 1 x (94°C, 2 min) and 35 x (94°C, 30 s; 70°C, 20 s; 72°C, 4.5 min) and 1 x (72°C, 7 min). The resulting PCR product was digested with *BamH*I and *Kpn*I, gel purified, and inserted into the plasmid pBluescript SK (Stratagene, USA). The recombinant plasmid was named pOAMY20. Vector pOAMYss was then constructed by replacing the DNA fragment containing the α-amylase gene promoter and partial α-amylase gene in the vector pOAMY20 with a DNA fragment containing the α-amylase promoter and secretion signal sequence. A gene fragment containing the *A. nidulans gpdA* promoter (Punt *et al*. 1990) and the *E. coli hph* gene (Gritz and Davies 1983) was amplified from plasmid pAN7-1 (Wang *et al*. 1999), digested with *BsiW*I and *Mss*I and inserted into the vector pOAMYss digested with the same restriction enzymes. The resulting expression vector was named pOAMYss-*gpdhph* (Fig. 1).

Table 1 Primers used to construct the expression vectors pOAMY20 and pOAMYss.

Primers	5'-3'
amy1BamNot.fwd	CTCAGAGGAT CCGCGGCCGCCATTTGCCGT TGCGTTGTG
amy1nointfu.rev	ATGGTTGATGACACCCTGCCAGCTGCCACCACAGTAGGCTCTATCACCGGTATTGCATGTTGCTGACGTGCTG
amy1nointfu.fwd	GCTGGCAGGGTGTCATCAACCATCTCGATTACATTCAGGGGATGGGCTTCAC
amy1corlinMCSfu.rev	CTATTAGTTTAAACGTACGGCGCGCCTTAAGACCGAGATATCGCGGGTCGCAGCAGTGCACGAGGTGGC
amy1FTfu.fwd	AAGGCGCGCCGTACGTTTAAACTAATAGTGATTGTATTCA AAGTGGAAGG
amy1NotKpn.rev	GTATTGGGTACCGCGGCCGCGTAATAAGTGCTGTAATAGC
gpdBsWiI.for	ACCATGCGTACGAATTCCCTTGTATCTC
hphMssI.rev	GTCGGCGTTTAAACTATTCCTTTGCCCTCG GAC
xynBHindIII.for	GCCTGGAAGCTTGGATATCCAGACGTCTATCACCCTAACC
xynBBamHI.rev	GAGGCCGGATCCTTAAGCTATTAGAAGGTGTTCTGGGTG

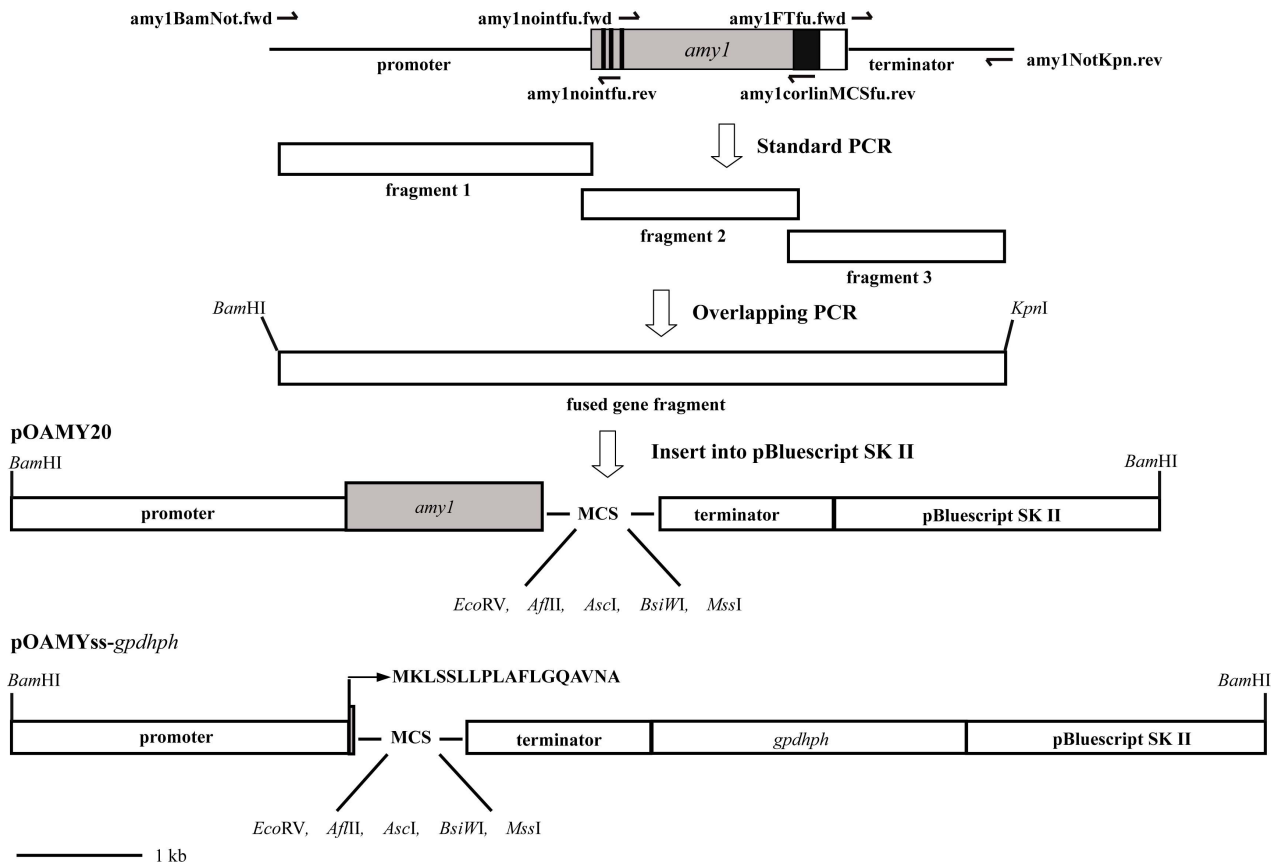

Figure 1 Schematic of the construction of pOAMY20 and pOAMYss-*gpdhph*. *amy1*, the gene encoding α-amylase I. MCS, multiple cloning site. Small black boxes in *amy1* gene in A indicate three introns; the box filled with lines indicates the linker region and the white box shows the starch binding domain of α-amylase I. Fragment 1, the *amy1* gene promoter plus a small portion of *amy1* encoding gene; Fragment 2, a part of *amy1* encoding gene; Fragment 3: *amy1* terminator region. B. MKLSSLLPLAFLGQAVNA, signal sequence of α-amylase.

Amplification of the *xynB* gene and expression in *E. coli*

The codon-modified *xynB* gene from *D. thermophilum* (Te'o *et al*. 2000) was amplified from plasmid pHEN54RQ-*xynB* using primers xynBHindIII.for and xynBBamHI.rev (Table 1) and ligated into pUC19. The recombinant plasmid was transformed into *E. coli* DH5α as described by Chung *et al*. (1988). Xylanase activity of the recombinants was screened as described by Te'o *et al*. (2000). Recombinants producing thermostable xylanase exhibited a clearing halo around the colonies. Plasmid DNA from the *E. coli* colonies containing the active *xynB* gene was isolated, digested with *EcoR*V and *Afl*II and inserted into the *O. floccosum* vector pOAMYss-*gpdhph*. The resulting plasmid was named pOAMYss-*xynBgpdhph*.

Fungal transformation and plate assay for recombinant xylanase activity

The plasmid pOAMYss-*xynBgpdhph* was introduced into *O. floccosum* MQ.5.1 by protoplast transformation. Protoplasts were prepared as described previously (Penttilä *et al*. 1987) with minor modifications. Magnesium sulphate-sodium phosphate buffer containing 10 mg ml^{-1} of lysing enzymes from *T. harzianum* (Sigma, USA) was used to lyse the hyphal walls, resulting in the formation of protoplasts. The protoplasts were separated from the remaining mycelia, first by filtration through Miracloth (Merck, Germany) and then a sintered glass filter (Pyrex, grade 1). Filtered protoplasts were resuspended in 600 µl of 1.2 M sorbitol, 10 mM Tris-HCl, 50 mM $CaCl_2$, pH 7.5 (STC). A portion of the protoplast preparation was removed, diluted and plated onto RM plates (2 % (v/v) glucose, 0.5 % (w/v) yeast extract, 0.5 % (w/v) malt extract, 1 g KH_2PO_4, 1.32 g $(NH_4)_2SO_4$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.5 g, $MgSO_4 \cdot 7H_2O$, 2.5 % (w/v) agar, and 1 M sorbitol made to a liter of MilliQ H_2O at pH 6 as a regeneration control. Transformation was carried out with some modifications to procedures described by Royer *et al*. (1991) and Wang *et al*. (1999). The mixture of protoplasts (4×10^6) and DNA (15 µg) were kept on ice for 20 min and 2 ml of 40 % (w/v) PEG 3350 was added slowly. This mixture was kept at room temperature for a further 20 min. After 4 h incubation, portions were mixed with 2.5 % (w/v) RM agar containing 150 units ml^{-1} of hygromycin B (Merck, USA) and overlaid onto PDA plates containing 150 units ml^{-1} hygromycin B. Following transformation with the plasmid pOAMYss-*xynBgpdhph*, the resulting colonies were transferred directly onto

amylase-induction plates containing 2 % (w/v) insoluble starch, and then incubated at 28 °C for 3 d. After incubation, the plates were overlaid with 0.5 % (w/v) birch wood xylan and 1 % (w/v) agarose and incubated at 70 °C overnight, after which they were flooded with 1 % (w/v) Congo Red (Teather and Wood, 1982). A clearing halo around a colony against the red background indicated expression of an active thermostable Xylanase B (Te'o *et al*. 2000). Selected transformants were inoculated in ISSM (minimal salts, 2 % (w/v) insoluble starch and 1.5 % (w/v) soy bean flour) at pH 6.5 and cultured for 5 d in order to study xylanase production in a liquid medium. Xylanase activity was measured as described below.

Xylanase activity assay

Xylanase activity in the culture supernatants was measured as described by Lever (1973) and Reeves *et al*. (2000) with some modifications. Each reaction contained 50 µl of appropriately diluted culture supernatant and 450 µl of 1 % (w/v) birchwood xylan dissolved in 120 mM Universal buffer, pH 6.5. All assays were conducted at 70°C for 10 min. The reaction was terminated by adding 750 µl of 3,5-dinitrosalicylic acid. In addition, the endogenous proteins in the culture supernatants of untransformed MQ.5.1 and SS41 after 72 h and 120 h incubation were also inactivated at 70 °C for 1 h before the activity assay. Activity units are expressed as nanokatals (nkat) per ml under the assay conditions.

Protein assays

The concentration of total secreted protein in the culture supernatants was measured by using the Bio-Rad protein assay kit (Catalogue number 500-0002) following the manufacturer's instructions, and expressed as mg ml^{-1}.

Isolation of fungal genomic DNA and total RNA

Extraction of genomic DNA from ten selected *O. floccosum* transformants which exhibited potential thermostable xylanase activity was carried out as described by Raeder and Broda (1985), except that the phenol extraction step was performed three times using 2-ml PhaseLock tubes (Eppendorf, Germany). Total RNA was isolated from 72 h- and 120 h-old mycelia as described by Curach *et al*. (2004) with a minor

adjustment whereby mycelia were frozen and ground under liquid nitrogen without washing with cold diethylpyrocarbonate-treated 0.9 % (w/v) NaCl.

Southern and Northern blotting

Southern blotting was carried out using a digoxigenin-labelled (DIG, Roche, Germany) *D. thermophilum xynB* gene fragment (0.6 kb) as a hybridization probe following the manufacturer's instructions. Northern blotting was conducted using the DIG system as recommended by the manufacturer. DIG-labelled *D. thermophilum xynB* gene fragment (0.6 kb), *O. floccosum* actin gene fragment (1 kb) and *O. floccosum amy1* gene fragment (0.3 kb) were used as hybridization probes to detect the transcripts for *xynB*, actin and *amy1*, respectively.

Zymogram activity gel

A zymogram gel assay for xylanase activity was performed essentially as reported by de Faria *et al*. (2002). Proteins in the culture supernatant were separated by 12.5 % (v/v) SDS-PAGE containing 0.1 % (v/v) birch wood xylan as substrate. The gel was rinsed twice with 2.5 % (v/v) Triton X-100 at room temperature for 30 min with agitation. Subsequently, it was soaked in 120 mM Universal buffer pH 6.5 and incubated at 70 °C for 1 h. It was then stained with Coomassie Brilliant Blue (Bio-Rad, USA) and destained in MilliQ H_2O. Finally, the gel was stained with 1 % (w/v) Congo Red to highlight the cleared haloes around the protein bands to visualize xylanase activity.

RESULTS

Expression of Xylanase B in *O. floccosum*

The functionality of the PCR-amplified *xynB* gene was first tested in *E. coli* DH5α before inserting it into the *amy1*-based expression vector pOAMYss-*gpdhph*. Around 100 *E. coli* recombinants were screened using a substrate overlay plate assay as described earlier (Te'o *et al*. 2000). All recombinants exhibited a clear halo around the colonies, indicating expression of an active xylanase. Plasmid DNA containing the active *xynB* gene was then isolated and inserted into plasmid pOAMYss-*gpdhph* for

expression in *Ophiostoma*. The resulting vector pOAMYss-*xynBgpdhph* was introduced into *O. floccosum* MQ.5.1 by protoplast transformation. Around 400 colonies appeared on the selection plates containing 150 µg of hygromycin B after 7d incubation. All 400 colonies were then transferred onto plates containing insoluble starch to induce the *amy1* promoter under which the *xynB* gene was expressed. At this point, a clear halo appeared around each colony after 2 to 3 d incubation due to degradation of starch by endogenous amylolytic enzymes. The plates were then overlaid with 0.5 % (w/v) birch wood xylan and 1 % (w/v) agarose, incubated at 70 ºC overnight and flooded with Congo Red. A clear halo around the colony against the red background indicated expression of an active thermotolerant xylanase under the *amy1* promoter. However, because of the background of underlying haloes originating from starch degradation (see above), it was rather difficult to distinguish whether a halo around each colony after the xylan overlay was a result of degradation of the xylan substrate or background only. It was established that 41 prospective transformant colonies exhibited halos that were considerably larger than those around non-transformant colonies plated as a control. Consequently, ten colonies exhibiting the largest halos were selected for cultivation in a liquid medium to confirm xylanase production and thereby, successful transformation of *O. floccosum* MQ.5.1. Xylanase activity in the culture supernatant of the ten strains was measured and one transformant (SS41), which showed up to 24 times higher xylanase activity than any other strain tested (data not shown) was chosen for further analysis.

Confirmation of the presence of *xynB* gene in the genome of SS41

Southern blotting was performed to confirm integration and also the copy number of the *xynB* gene in the genome of the high xylanase-producing transformant, SS41. Genomic DNA extracted from SS41 and untransformed MQ.5.1 were digested to completion with *Hind*III and hybridized with a DIG-labelled *D. thermophilum xynB* gene fragment (Fig. 2). No signal was detected in the lanes containing the undigested or digested genomic DNA of MQ.5.1. Three bands representing sizes of 6, 3.5 and 3 kb were observed to hybridise when the genomic DNA of SS41 had been digested with *Hind*III. This result indicated that at least three copies of the *xynB* gene were integrated into the genome.

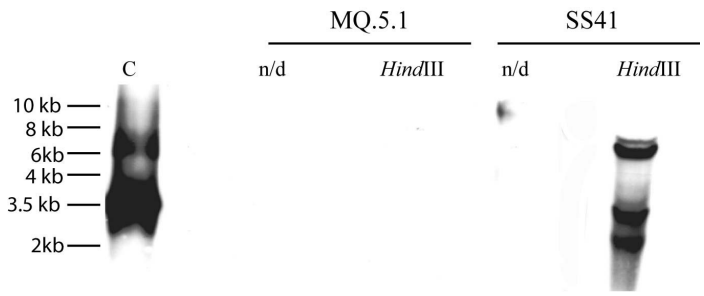

Figure 2 Detection of integration and the copy number of the *xynB* gene in the fungal genome by Southern blotting. C, positive control (3.5 kb gene fragment containing the *amy1* gene amplified from plasmid pOAMYss-*gpdhph*); MQ.5.1, untransformed strain; SS41, high xylanase producing transformant; n/d indicates non-digested genomic DNA.

Effect of the culture medium and pH on Xylanase B production

The transformant SS41 was cultured in liquid media containing different carbon sources under a variety of pH conditions to investigate the effect of culture conditions on xylanase production. The untransformed strain MQ.5.1 was cultured under the same conditions. Xylanase activity was the highest in the culture supernatant of SS41 cultured on ISSM (2 % (w/v) insoluble starch and 1.5 % (w/v) soybean flour) at pH 6.5 (Table 2). Surprisingly, no xylanase activity was detected in the culture supernatant of SS41 in SM (3 % (w/v) soluble starch) and low xylanase activity was obtained in the ISM (2 % (w/v) insoluble starch) medium. Neither the SM nor ISM medium contained soybean flour. Therefore, it appeared that soybean flour was important for the production of xylanase activity. Samples from all cultures were also observed under a light microscope (Olympus, Japan) after a 5-day incubation. Both yeast-like cells and mycelia were present in SM and ISM at pH 6.5 and the cell density was much lower in comparison with cultures grown on the SSM and ISSM media in which mycelia were predominant. Therefore, the physiological growth form seemed to have a profound effect on xylanase production: high xylanase activity was detected in the cultures in which mycelia were prevalent (Table 2).

Table 2 Xylanase activity in unheated culture supernatants of transformant SS41 and untransformed MQ.5.1 under different culture conditions. Cultures were incubated for 5 d at 28 °C. Activity units are expressed as nkat ml^{-1}.

Strain	Culture medium				
Culture pH (ISSM)					
	SM		SSM	ISM	ISSM
5.5	6.0	6.5			
MQ.5.1	0		226	0	134
218	240	150			
SS41	0		662	38	1124
1416	1380	1096			

SM, soluble starch; SSM, soluble starch and soybean flour; ISM, insoluble starch; ISSM, insoluble starch and soybean flour (see Materials and Methods).

Some background xylanase activity was also detected in the unheated culture supernatants of untransformed MQ.5.1 growing in both SSM and ISSM at pH 6.5 (Table 2). In general, ISSM seemed to be a better medium for recombinant xylanase production and was used to study the effect of pH on xylanase yields. Xylanase activity (1416 nkat ml^{-1}) of SS41 was the highest in ISSM at pH 5.5 and lowest (1096 nkat ml^{-1}) in ISSM at pH 6.5 (Table 2).

Expression of the *xynB* gene in *O. floccosum* SS41 on ISSM

The strain SS41 and untransformed MQ.5.1 were incubated in ISSM at pH 5.5 and samples were taken after 72 h and 120 h of growth. Total RNA was extracted and culture supernatants were kept at 4 °C for further analysis. Xylanase activity in the culture supernatants of SS41 and MQ.5.1 was measured at each time point. For SS41, xylanase activity was 458 nkat ml^{-1} and 1618 nkat ml^{-1} after 72 h and 120 h of growth, respectively. The fact that xylanase activity of SS41, produced under the *amy1* promoter, was more than 2.5-fold higher at 120 h compared to 72 h is in accordance with the time-dependent regulation of α-amylase activity (unpublished data) and provided further evidence that the activity detected in SS41 originated from the *xynB* gene expressed under the *amy1* promoter. While xylanase activity of 616 nkat ml^{-1}

and 240 nkat ml^{-1} was also detected at 70ºC assay in the culture supernatant of MQ.5.1 after 72 h and 120 h incubation, it declined over time pointing to another origin. To further explore the case, culture supernatants of MQ.5.1 and SS41 after 72 h and 120 h incubation were heated at 70 °C for 1 h before measurement of the xylanase activity. No activity against birch wood xylan was detected in the heated culture supernatants of MQ.5.1 after 72 h and 120 h incubation whereas xylanase activity in the heated culture supernatant of transformant SS41 remained at 524 nkat ml^{-1} and 1672 nkat ml^{-1}.

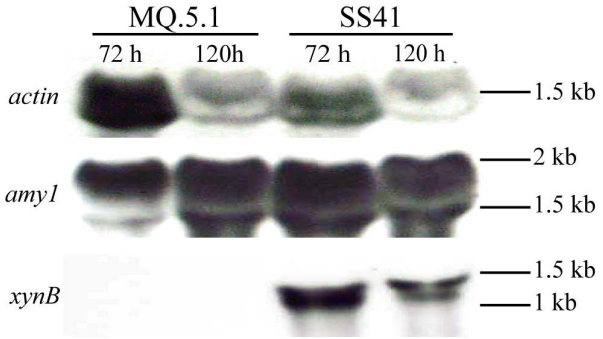

Figure 3 Expression of the *xynB* and *amy1* at the transcriptional level after 72 h and 120 h of growth. Each lane contained 20 μg of total RNA. MQ.5.1, untransformed control; SS41, transformant producing xylanase.

Northern blotting was conducted using the DIG-labelled *D. thermophilum xynB* gene probe to detect expression of *xynB* at the transcriptional level. A DIG-labeled *O. floccosum* actin gene fragment was used as an internal control. Expression of the *amy1* gene was also visualized using a DIG-labeled *amy1* probe. Expression of the *amy1* gene was highly induced after 72 h and 120 h incubation in both SS41 and the non-transformed MQ.5.1. Two dominant transcripts of 1.5 and 1.8 kb were detected in both strains, which was consistent with our previous study (Wu *et al*., 2006). The presence of *amy1* transcripts in the transformant SS41 also indicated that the *xynB* gene had not been integrated into the *amy1* gene locus. No signal was detected in the non-transformed MQ.5.1 using the *xynB* probe. In contrast, two *xynB* transcripts, each around 1 kb were observed from the SS41 mycelia after 72 h and 120 h of growth as expected (Fig. 3). The intensity of the two different-sized *xynB* gene transcripts was similar and there was little difference in the intensity of the signals between the 72 h and 120 h time points (Fig. 3). These results indicated that expression of the *xynB* gene under the regulation of the *amy1* gene promoter was high at the transcriptional level

after 72 h incubation and remained stable until 120 h, following the expression pattern of the *amy1* gene.

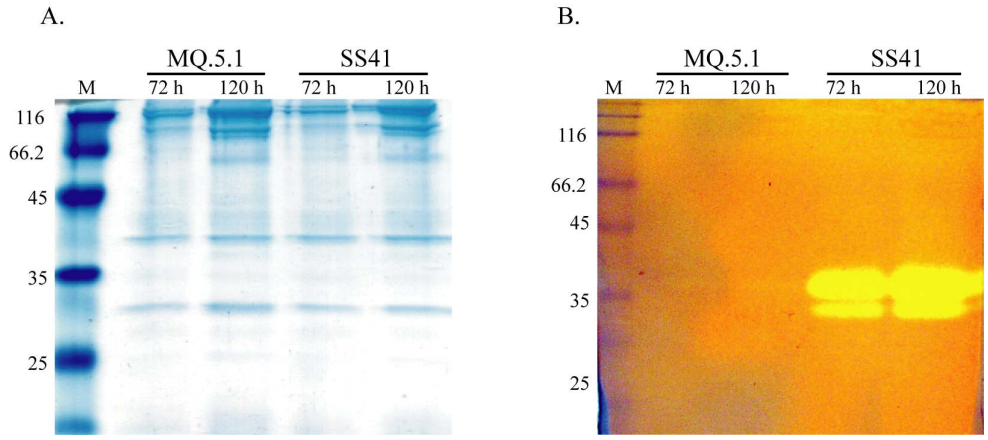

Figure 4 Xylanase activity in the culture supernatants of transformant SS41 and the untransformed MQ.5.1 visualized by a zymogram gel assay of supernatants harvested from cultures after 72 and 120 h of growth in ISSM. A, proteins stained with Coomassie Blue; B, activity gel stained with Congo Red. M, molecular weight marker (Invitrogen, USA).

Zymogram analysis of xylanase activity in the culture supernatant of SS41 revealed two protein bands of approximately 30 and 27 kDa (Fig. 4). The molecular masses of the two proteins were larger than the predicted xylanase B (about 22 kDa) calculated from the *xynB* gene sequence. However, the halo around each band indicated that the recombinant proteins were active. This result was consistent with the considerable xylanase activity detected in the culture supernatant and strong intensity of the two *xynB* transcripts in Northern blot analysis. No xylanase activity was observed from the culture supernatants of MQ.5.1 by zymogram analysis after 72 h and 120 h incubation, further confirming that enzyme activity detected in the culture supernatants of MQ.5.1 after 72 h and 120 h of growth was not originating from *xynB*.

DISCUSSION

Confirmation of genuine transformation of the high protein-secreting mutant strain *O. floccosum* MQ5.1 has proven difficult because of the increasing resistance of colonies to hygromycin B after transformation (unpublished data). Based on our previous observations, it was expected that the majority of the 400 colonies originally growing on the screening plates containing 150 µg of hygromycin B may not be true transformants. Therefore, direct screening of the colonies for expression of

thermophilic xylanase activity provided an option for confirmation of successful transformation. In addition, this approach provided an indication of the levels of xylanase produced by the colonies, based on the size of the hydrolysis halo. In any case, the transformation frequency remained extremely low.

Southern blot analysis revealed that there were at least three copies of the *xynB* gene integrated into the genome of the high xylanase producing transformant SS41. PCR results (data not shown) confirmed that the integration of *xynB* genes had occurred randomly in the genome using the circular plasmid pOAMYss-*xynBgpdhph* as the transforming DNA. Three copies of *xynB* under the native *amy1* promoter produced about 1600 nkat ml^{-1} of xylanase activity into the culture supernatant of SS41. This is a considerable amount of xylanase activity and compares well with the first attempts to produce the XynB enzyme in a well established fungal expression host *Trichoderma reesei* (de Faria et al., 2002) for which the yields have been further increased by six-fold.

Northern blotting revealed two signals of approximately similar size from the SS41 mRNA isolated from mycelia cultivated for 72 h and detected using the *xynB* probe. Generation of the two *xynB* transcripts could be due to differential polyadenylation events as was the case with three *amy1* transcripts generated from the single *amy1* gene locus (Wu *et al*. 2006). The intensity of the *xynB* transcripts in the transformant was strong for both 72h and 120 h cultures, indicating high expression of the *xynB* gene under the regulation of the *amy1* gene promoter. Two protein bands were also observed in the Zymogram analysis although their molecular masses were slightly larger than that predicted for XynB. The most plausible explanation is that the xylanase B protein has been post-translationally modified as there is a potential N-linked glycosylation site at the N-terminus of the protein. However, as expected, the modified xylanase was still active (Bergquist *et al*. 2002).

Xylanase activity in the culture supernatant of the transformant SS41 was hardly detectable when soybean flour was absent from the culture medium. The activity was notably increased when using this complex nitrogen source together with soluble or insoluble starch as a carbon source. Under these conditions, mycelia were the major growth form. A number of studies have indicated that the secretion of proteins destined to the growth medium occurs mostly at the hyphal tips (Conesa *et al*. 2001; Gordon *et*

al. 2000a; 2000b). Accordingly, xylanase activity was expected to be high in the cultures with high proportion of mycelial growth. Variation in pH did not dramatically influence xylanase B production.

The xylanase activity detected in the unheated culture supernatant of the untransformed MQ.5.1 most probably originated from an endogenous xylanase enzyme; it was absent from culture supernatants that were heated for 1 h at 70ºC before assaying for enzyme activity. Xylanase activity has been found in *O*. *ulmi*, *O*. *novo-ulmi* and *O. floccosum* in previously published studies (Binz and Canevascini 1996; Schirp *et al*. 2003), even though most of *Ophiostoma* spp. do not compromise the strength properties of wood.

In conclusion, a heterologous thermostable xylanase B enzyme was successfully expressed in *O*. *floccosum* MQ.5.1 under the endogenous *amy1* gene promoter. Direct screening of thermostable xylanase activity of the potential transformant colonies initially growing on the hygromycin B screening plates also provided a rapid indication of production of a "difficult to express protein" in "a difficult to transform" host strain of *O*. *floccosum*. This fungus is capable of growing in the wood parenchyma cells and therefore provides means for *in situ* delivery of enzymes of interest into wood tissues.

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