Qualitative methods for the determination of lignocellulolytic enzyme production by tropical fungi

Stephen B. Pointing

Fungal Diversity Research Project, Department of Ecology and Biodiversity, The University of Hong Kong, Pokfulam Road, Hong Kong; email: pointing@hkucc.hku.hk

Pointing S.B. (1999). Qualitative methods for the determination of lignocellulolytic enzyme production by tropical fungi. Fungal Diversity 2: 17-33.

A range of qualitative approaches to the assessment of lignocellulose degrading enzyme production are presented, with detailed stepwise methodology for each assay. The advantages, variations and limitations to each method are discussed. Recommendations for the use of these procedures in achieving specific research objectives are given.

Key words: enzyme assay, fungi.

Introduction

There is considerable research effort on the biodiversity, ecology and economic importance of tropical fungi (Hyde, 1997). This includes systematic studies and the screening of fungal isolates for bioactive compounds. Most isolates are from lignocellulose substrates such as wood, grasses, palms and seeds. There is significant interest in the enzymes responsible for lignocellulose degradation in terms of understanding their ecological role, also in the biotechnology potential of enzymes involved in this process (Reddy, 1995).

Lignocellulose is a heteropolymer consisting mainly of three components, cellulose, hemicellulose and lignin (Fengel and Wegener, 1989; Eaton and Hale, 1993). The characteristics of these components are summarised, with the major enzymes responsible for their degradation in Table 1. For recent reviews on lignocellulose degrading enzymes see Eaton and Hale (1993), Reddy and D'Souza (1994) and Thurston (1994).

Qualitative assays are powerful tools used in screening fungi for lignocellulose degrading enzyme production. Such tests give a positive or negative indication of enzyme production. They are particularly useful in screening large numbers of fungal isolates for several classes of enzyme, where definitive quantitative data are not required. The reagents required are all

commonly available and relatively inexpensive. In addition the methodology is straightforward and so assays can be carried out by mycologists with little specialized knowledge of enzymology.

A major drawback in qualitative assays has been the lack of any standardized methodology. Numerous reports in the literature have employed qualitative assays, however many of these studies have used very different protocols. The results range from those with suspect or meaningless data to some excellent findings. Comparison of results between such studies is virtually impossible. The purpose of this paper is to review qualitative methods and suggest standard protocols for assay of the major lignocellulose degrading enzymes. The advantages, variations and limitations to each method are also discussed. The procedures are conveniently grouped into cellulolytic, hemicellulolytic (although in practice xylan is the only substrate used in such assays) and lignin modifying enzyme assays. This is considered the most comprehensive publication to date on qualitative enzyme assay methodology and is primarily intended for use with tropical fungi. The procedures can be adapted for use with marine fungi by adding appropriate concentrations of marine salts. Incubation time and temperature may be adjusted to suit temperate or slow growing strains if necessary.

Table 1. The major components of lignocellulose and fungal enzymes involved in their degradation.

	Cellulose	Hemicellulose	Lignin
% of wood mass	40-50	25-40	20-35
monomer	D-anhydrogluco pyranose	Xylose Mannose plus other pentoses and hexoses	coniferyl alcohol *p*-coumaryl alcohol sinapyl alcohol
polymeric structure	β1-*O*-4 linked linear chains	β1-*O*-4 linked linear chains, with substituted side chains	dehydrogenative polymerisation to an amorphous polymer
Major enzymes involved in degradation	Endoglucanase (E.C. 3.2.1.4) Cellobiohydrolase (E.C. 3.2.1.91) β-glucosidases (E.C. 3.2.1.21)	Endoxylanase β-xylosidase (and other hydrolases)	Lignin peroxidase (E.C. 1.11.1.7) Mn dependant peroxidase (E.C. 1.11.1.7) Laccase (E.C. 1.10.3.2)

Cellulolytic enzyme assays

For qualitative assays it is important to employ uniform inoculation procedures. To obtain inoculum it is recommended that test fungi are cultivated on basal medium supplemented with 0.4 % w/v glucose and solidified with 1.6 % w/v agar. This will limit carry over of nutrients that could interfere with interpretation of assay results. Use a single agar disc cut from the actively growing colony margin of a culture to inoculate each assay medium. Assigning quantitative values to the qualitative assays described here on the basis of reaction intensity should be avoided. If it is essential a scoring system for assays should be as simple as possible with few categories (e.g. no reaction-weak reaction-strong reaction).

Cellulolysis basal medium (CBM) (g l^{-1} in distilled water)

$C_4H_{12}N_2O_6$	5	Yeast Extract	0.1
KH_2PO_4	1	$CaCl_2.2H_2O$	0.001
$MgSO_4.7H_2O$	0.5		

Basal medium may be conveniently stored as a 10 × sterilized stock. It is also possible to simplify the procedure by using less defined basal growth medium such as peptone plus yeast extract, or malt extract. Such basal media used at a concentration of 0.1-0.2 % w/v are sufficient to support cellulose degrading fungi.

Method 1 - Filter paper degradation

Filter paper used in this assay is almost 100 % cellulose. Degradation of this substrate which contains crystalline and amorphous cellulose is indicative of cellulolysis, although the action of individual cellulase complex enzymes cannot be determined.

Protocol

1. Prepare CBM medium, transfer 10 ml aliquots to glass culture bottles and autoclave.
2. Aseptically add one 25 × 5 mm strip of sterile filter paper to each bottle making sure that all filter paper strips are completely submerged.
3. Inoculate with the test fungus and retain uninoculated bottles as controls. Take care that bottle caps are loosely fitted to allow adequate gas exchange.
4. Incubate at 25 C in darkness. Examine daily for 10 days.
5. Assess degradation of filter paper based on increased opacity and physical degradation in comparison with uninoculated controls.

Variations and limitations to the method

This cellulolysis assay uses liquid growth medium and may be useful for strains of aquatic fungi which generally produce higher biomass in liquid culture as compared to agar media. The use of insoluble particulate cellulose substrates is not advised since settling limits their bio-availability, resulting in little or no fungal growth in some cases (Pointing *et al.*, 1999a). The major drawback of using filter paper is in the visual interpretation of degradation. Assessment is very subjective and limits the usefulness of data in comparative studies. Microscopic examination of cellulosic substrates after growth may help to clarify ambiguous reactions.

This method is also useful for assessment of growth on cellulosic waste substrates such as cotton waste and bagasse, or native substrates such as grasses and wood slivers (although it is advisable to leach out wood extractives prior to use since they can inhibit fungal growth).

Method 2 – Cellulose agar clearance (cellulose agar)

This assay uses ball-milled, acid-swollen or microcrystalline cellulose. Incorporation of the cellulose into solid agar media results in an opaque substrate due to the insolubility of the cellulose. Clearance indicates cellulolysis. Positive reactions indicate simultaneous action of all cellulolytic enzymes, although rates of clearance vary according to substrate. Generally microcrystalline cellulose is degraded more slowly than ball-milled or acid swollen cellulose .

Protocol

1. Prepare CBM medium, incorporating 4 % w/v cellulose and 1.6 % w/v agar and autoclave.
2. Aseptically transfer to Petri dishes (cool agar until viscous and gently mix before pouring to ensure uniform distribution of cellulose in the agar medium).
3. Inoculate with test fungus.
4. Incubate at 25 C in darkness and examine daily for 10 days.
5. Assess cellulolysis based on clearance zones of the opaque agar around growing colonies.

Variations and limitations to the method

Recording clearance of cellulose within the growth medium can be difficult to assess, particularly with dense or dark hyphal growth. Variations on this

method have been used by several authors (Rautella and Cowling, 1966; Egger, 1986; Rohrmann and Molitoris, 1992; Paterson and Bridge, 1994).

Method 3 – Dye diffusion from a cellulose-dye complex (cellulose azure agar)

The use of dyed cellulose yields less ambiguous data than either of the above methods since results are more visual. This method also tests for simultaneous action of all cellulase enzymes. Degradation of cellulose results in the release of a bound dye, the vertical migration of which can be observed. This assay can also be used for the simultaneous assessment of lignin modifying enzyme (LME) activity since decolorization of the dye used in this assay has been correlated with LME production (Archibald, 1992; Thorn, 1993). Dye decolorization generally follows migration due to cellulolysis.

Protocol

1. Prepare CBM medium supplemented with 1.6 % w/v agar. Transfer 10 ml aliquots to glass culture bottles and autoclave. Allow to solidify.
2. Prepare CBM medium supplemented with 1 % w/v cellulose azure (azure I dye, C.I. 52010) and 1.6 % w/v agar, autoclave and cool until viscous.
3. Gently mix the agar prepared in step 2 and then carefully aliquot 0.1 ml aseptically on to the surface of the solidified agar as an overlay.
4. Inoculate with test fungus. Also retain uninoculated bottles as controls. Take care that bottle caps are loosely fitted to allow adequate gas exchange.
5. Incubate at 25 C in darkness and examine daily for 10 days.
6. Migration of dye into the clear lower layer indicates cellulolysis. Subsequent dye decolorization indicates LME activity.

Variations and limitations to the method

The cellulose azure assay has been used successfully by several authors (Smith, 1977; Paterson and Bridge, 1994; Pointing, Vrijmoed and Jones, 1998). Cellulose dyed with remazol brilliant blue R (C.I. 61200) may also be used as a substitute for cellulose azure (Ng and Zeikus, 1980). Relatively slow growth of fungi on dyed cellulosic substrates has been reported by Rohrmann and Molitoris (1992) and this may be due to the crystalline content of the cellulose, or some toxic effects of the dye. However good growth rates and clear results have been obtained by the author with a range of tropical fungi using this method. The cellulose azure method is highly recommended as it is the most reliable qualitative assay for cellulolysis.

Method 4 – Dye staining of carboxymethylcellulose agar (CMC agar)

Carboxymethylcellulose (CMC) is a substrate for endoglucanase and so can be used as a test for endoglucanase and β–glucosidase activity. This assay is a good indicator of cellulolytic ability since endoglucanase is generally produced in larger titres by fungi than cellobiohydrolase (Cai, Buswell and Chang, 1994; Buswell *et al.*, 1996; Pointing *et al.*, 1999a), in addition many fungi that successfully degrade cellulose in wood produce no detectable cellobiohydrolase (Eaton and Hale, 1993). After growth of the fungus on CMC a dye is used to differentiate between intact CMC and degraded substrate.

Protocol

1. Prepare CBM medium supplemented with 2 % w/v low viscosity CMC and 1.6 % w/v agar and autoclave.
2. Aseptically transfer to Petri dishes.
3. Inoculate with test fungus.
4. Incubate at 25 C in darkness. When the colony diameter is approximately 30 mm (2-5 days), stain agar plates as follows :
5. Flood the plates with 2 % w/v aqueous congo red (C.I. 22120) and leave for 15 minutes.
6. Pour off stain and wash the agar surface with distilled water.
7. Flood the plates with 1M NaCl to destain for 15 minutes.
8. Pour off destain. CMC degradation around the colonies will appear as a yellow-opaque area against a red colour for undegraded CMC.

Variations and limitations to the method

This assay is a well established procedure and has been used with slight variations (Teather and Wood, 1982; Rohrmann and Molitoris, 1992; Paterson and Bridge, 1994; Pointing *et al.*, 1998). Results are easy to interpret and usually unambiguous, although a control inoculation onto CBM agar lacking CMC is advisable. It is also possible to use a zinc chloride solution for staining in place of the congo red dye (Sass, 1958; Gessner, 1980). Undegraded CMC stains purple with this procedure, while areas around colonies where degradation has occurred will be clear. Dark or densely growing strains where endoglucanase activity is predominantly cell-associated may be difficult to assess using the CMC agar method method.

Method 5 – Esculin plus iron agar (esculin agar)

The hydrolysis of cellobiose to glucose is achieved by β–glucosidase. This enzyme is probably ubiquitous among cellulolytic fungi producing hydrolytic

endoglucanases or cellobiohydrolases. Activity of β–glucosidase can be detected by growth of the test fungus on agar containing esculin (6,7-dihydroxycoumarin 6-glucoside) as the sole carbon source. Splitting of the substrate by the enzyme yields glucose, and a coumarin product that react with iron sulphate to produce a black colour in the growth medium.

Protocol

1. Prepare CBM medium supplemented with 0.5 % w/v esculin, and 1.6 % w/v agar and autoclave.
2. Aseptically add 1 ml of a sterile 2 % w/v aqueous ferric sulphate solution for each 100 ml CBM prepared.
3. Aseptically transfer to Petri dishes.
4. Inoculate with the test fungus and retain uninoculated controls.
5. Incubate at 25 C in darkness. Examine daily over 5 days.
6. A black colour will develop in the medium of colonies producing β–glucosidase.

Variations and limitations to the method

The esculin substrate can be substituted with arbutin (hydroquinone β–D-glucopyranoside) where formation of a black quinone product indicates β–glucosidase activity. In some cases, after an initial positive reaction the test fungus may utilize the black coumarin or quinone products. The growth medium around colonies may then become decolorized. β–glucosidase is predominantly a cell associated or intracellular enzyme in many fungi, so very dark hyphal growth may obscure results.

Hemicellulolytic (xylanolytic) enzyme assays

Relatively little attention has been given to qualitative assays for xylan utilization and few assay procedures have been described. For such assays it is important to employ uniform inoculation procedures. To obtain inoculum it is recommended that test fungi are cultivated on basal medium supplemented with 0.4 % w/v glucose and solidified with 1.6 % w/v agar. This will limit carry over of nutrients that could interfere with interpretation of assay results. Use a single agar disc cut from the actively growing colony margin of a culture to inoculate each assay medium. Assigning quantitative values to the qualitative assays described here on the basis of reaction intensity should be avoided. If it is essential a scoring system for assays should be as simple as possible with few categories (e.g. no reaction-weak reaction-strong reaction).

Xylanolysis basal medium (XBM) (g l^{-1} in distilled water):

$C_4H_{12}N_2O_6$	5	Yeast Extract	0.1
KH_2PO_4	1	$CaCl_2.2H_2O$	0.001
$MgSO_4.7H_2O$	0.5		

The basal medium described here may be conveniently stored as a 10 × sterilized stock. It is also possible to simplify the procedure by using less defined basal growth medium such as peptone plus yeast extract, or malt extract. Such basal media used at a concentration of 0.1-0.2 % w/v should be sufficient to support xylan degrading fungi.

Method 1 – Dye staining of xylan agar (xylan agar)

In this assay xylan utilization is visualized using a simple stain. Positive reactions indicate degradation of the substrate by endoxylanase and β–xylosidase.

Protocol

1. Prepare XBM medium, incorporating 4 % w/v xylan and 1.6 % w/v agar and autoclave.
2. Aseptically transfer to Petri dishes (cool agar until viscous and gently mix before pouring to ensure uniform distribution of xylan in agar medium).
3. Inoculate with test fungus.
4. Incubate at 25 C in darkness. When colony diameter is approximately 30 mm (2-5 days) stain agar plates as follows :
5. Flood plates with iodine stain (0.25 % w/v aqueous I_2 and KI) and leave for 5 minutes.
6. Pour off stain and wash agar surfaces with distilled water.
7. Xylan degradation around the colonies will appear as a yellow-opaque area against a blue / reddish purple colour for undegraded xylan.

Variations and limitations to the method

This staining procedure has been reported as effective with birchwood xylan (Egger, 1986) and it is anticipated that other commercially available xylans (e.g.: oat spelt xylan) would also be suitable.

Method 2 – Dye diffusion from a xylan-dye complex (RBB-xylan agar)

This method for qualitatively assessing xylanolytic activity is based on the same principle as the cellulose azure assay for cellulolysis (and LME production). Here positive reactions indicate degradation of the substrate by

endoxylanase and β–xylosidase. A modified (soluble) xylan (4-O-methyl-D-glucourono-D-xylan) is bound to the dye remazol brilliant blue R (C.I. 61200) to form the substrate RBB-xylan (Beily, Mislovicova and Toman, 1985). Degradation of xylan results in the release of bound dye, the migration of which can be monitored vertically in the agar medium.

Protocol

1. Prepare XBM medium supplemented with 1.6 % w/v agar and transfer 10 ml aliquots to glass culture bottles. Autoclave and allow to solidify.
2. Prepare XBM medium supplemented with 1 % w/v RBB-xylan and 1.6 % w/v agar. Autoclave and cool until viscous.
3. Gently mix the agar prepared in step 2 and then carefully aliquot 0.1 ml aseptically on to the surface of the solidified agar as an overlay.
4. Inoculate with test fungus. Also retain uninoculated bottles as controls (take care that bottle caps are loosely fitted to allow adequate gas exchange).
5. Incubate at 25 C in darkness and examine daily for 10 days.
6. Migration of the dye into the clear lower layer indicates xylanolysis (subsequent dye decolorization indicates LME activity).

Variations and limitations to the method

The effectiveness of this new method among a range of fungal strains is untested. In addition the solubility of the xylan may render this substrate inappropriate for the assay method. However dye diffusion-based assays are generally preferred over other techniques for polyose degrading enzymes due to the ease with which results can be interpreted.

Lignin modifying enzyme assays

For qualitative assays it is important to employ uniform inoculation procedures. To obtain inoculum it is recommended that test fungi are cultivated on basal medium supplemented with 0.4 % w/v glucose and solidified with 1.6 % w/v agar. This will limit carry over of nutrients that could interfere with interpretation of assay results. Use a single agar disc cut from the actively growing colony margin of a culture to inoculate each assay medium. Assigning quantitative values to the qualitative assays described here on the basis of reaction intensity should be avoided. If it is essential a scoring system for assays should be as simple as possible with few categories (e.g. no reaction-weak reaction-strong reaction).

LME basal medium (LBM) (g l^{-1} in distilled water)

KH_2PO_4	1	Yeast Extract	0.01
$C_4H_{12}N_2O_6$	0.5	$CuSO_4.5H_2O$	0.001
$MgSO_4.7H_2O$	0.5	$Fe_2(SO_4)_3$	0.001
$CaCl_2.2H_2O$	0.01	$MnSO_4.H_2O$	0.001

The basal medium may be conveniently stored as a 10 × sterilized stock. It is also possible to simplify the procedure by using less defined basal growth medium such as peptone plus yeast extract, or malt extract. Such basal media have been used in several studies at varying concentrations (Gessner, 1980; Egger, 1986; Niku-Paavola, Raaska and Itavaara, 1990; Rohrmann and Molitoris, 1992; Raghukumar *et al.*, 1994; Pointing Vrijmoed and Jones, 1999b) however, it should be noted that LME production in fungi is generally repressed under conditions of nutrient sufficiency (Reddy and D'Souza, 1994; Eggert, Temp and Eriksson, 1996a). A working strength of 0.01 % w/v peptone and 0.001 % w/v yeast extract in an undefined growth medium is acceptable. For many fungi the presence of a redox mediator in the growth medium significantly enhances LME production (Reddy and D'Souza, 1994; Eggert *et al.*, 1996a). The inclusion of such compounds in a qualitative assay growth medium is not necessary.

Method 1 – staining after growth on lignin agar (lignin agar)

Lignin is not generally used in assays for LME's since several convenient chromogenic substrate analogues exist. Nonetheless it is possible to assay for oxidation of phenolic components in lignin using a staining procedure after growth of a test fungus on agar medium supplemented with lignin. It should be noted however, that most commercially available lignins are not chemically identical to native lignin.

Protocol

1. Prepare LBM medium supplemented with 0.25 % w/v lignin and 1.6 % w/v agar and autoclave.
2. Aseptically add 1 ml of a separately sterilized 20 % w/v aqueous glucose solution to each 100 ml of growth medium prepared.
3. Aseptically transfer to Petri dishes.
4. Inoculate with test fungus.
5. Incubate at 25 C in darkness. After 5-10 days growth (do not allow plates to overgrow) stain agar plates as follows :

6. Flood plate with a 1 % w/v aqueous solution of $FeCl_3$ and $K_3[Fe(CN)_6]$ prepared freshly before use. Phenols in undegraded lignin will stain blue-green, with clear zones around colonies indicating oxidation of phenolic components.

Variations and limitations to the method

This assay can be useful in determining the ability of a fungus to utilize a lignin substrate. The method indicates degradation of phenolic components in lignin. Degradation of the more recalcitrant non-phenolic lignin components however is not indicated by this procedure. Results from this assay should not therefore be used to suggest complete lignin mineralization.

Method 2 – Tannic acid agar (Bavendamm test)

This method is modified from the procedure of Bavendamm (1928) and has been widely used. Due to the development of modern dye based assays this procedure is no longer a preferred test for LME production. The assay provides an indication of overall polyphenoloxidase activity and is not specific to any one LME.

Protocol

1. Prepare LBM medium supplemented with 1.6 % w/v agar and autoclave.
2. Aseptically add 1 ml of a separately sterilized 20 % w/v aqueous glucose solution to each 100 ml of growth medium prepared.
3. Aseptically add 1 ml of a separately sterilized 1 % w/v aqueous tannic acid solution to each 100 ml of growth medium prepared.
4. Aseptically transfer to Petri dishes.
5. Inoculate with test fungus.
6. Incubate at 25 C in darkness and examine plates daily for 10 days.
7. Record LME production as the appearance of a brown oxidation zone around colonies.

Variations and limitations to the method

The tannic acid used in this assay medium may be substituted with gallic acid. The assay relies on production of a coloured product rather than dye-substrate disappearance. The brown colour is similar to many naturally produced fungal pigments and therefore some ambiguity in interpreting results may arise.

Method 3 – Poly-R agar clearance (Poly-R agar)

Decolorization of the polymeric dye Poly-R 478 (Sigma) by fungi has been positively correlated with production of the polyphenoloxidases lignin peroxidase, Mn dependent peroxidase (Boominathan and Reddy, 1992) and laccase (Pointing *et al.*, 1999b). This simple test gives clear results since decolorization of the violet dye is easily observed.

Protocol

1. Prepare LBM medium supplemented with 0.02 % w/v Poly-R and 1.6 % w/v agar and autoclave.
2. Aseptically add 1 ml of a separately sterilized 20 % w/v aqueous glucose solution to each 100 ml of growth medium prepared.
3. Aseptically transfer to Petri dishes.
4. Inoculate with test fungus.
5. Incubate at 25 C in darkness and examine plates daily for 10 days.
6. Record production of LME's as clearance of violet coloured medium.

Variations and limitations to the method

Poly-R agar is the most convenient qualitative assay for LME production among fungi. Positive results can be obtained for peroxidase producing fungi without the addition of H_2O_2 to the medium. There is some ambiguity as to whether all laccases can degrade Poly-R. Several tropical marine fungi have been shown to produce laccase that is not capable of Poly-R decolorization (Pointing *et al.*, 1998), whilst the laccases of many tropical terrestrial basidiomycetes are able to decolorize this substrate (Pointing *et al.*, 1999b; Pointing, 1999).

Method 4 – Azure-B agar clearance (azure B agar)

Decolorization of the dye Azure-B (C.I. 52010) by fungi has been positively correlated with production of lignin peroxidase and Mn dependent peroxidase, however this dye is not a substrate for laccase (Archibald, 1992). This simple test gives clear results when qualitative data on peroxidase-type LME's is required.

Protocol

1. Prepare LBM medium supplemented with 0.01 % w/v Azure B and 1.6 % w/v agar and autoclave.
2. Aseptically add 1 ml of a separately sterilized 20 % w/v aqueous glucose solution to each 100 ml of growth medium prepared.

3. Aseptically transfer to Petri dishes.
4. Inoculate with test fungus.
5. Incubate at 25 C in darkness and examine plates daily for 10 days.
6. Record production of lignin peroxidase and Mn dependent peroxidase as clearance of blue coloured medium.

Variations and limitations to the method

Positive results can be obtained for peroxidase producing fungi without the addition of H_2O_2 to the medium. Degradation of azure dye can also be used to assess peroxidase production with the cellulose azure agar method. This permits an assessment of LME production whilst utilizing a complex carbon source rather than glucose.

Method 5 – ABTS agar

This colourless agar medium turns green due to the oxidation of ABTS (2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) to ABTS-azine in the presence of laccase (Niku-Paavola *et al.*, 1990). Laccase has a confirmed role in lignin degradation (Thurston, 1994; Eggert *et al.*, 1996b), however it is possible that some isoforms may also be involved in general detoxification processes and fruiting body formation.

Protocol

1. Prepare LBM medium supplemented with 0.1 % w/v ABTS and 1.6 % w/v agar and autoclave.
2. Aseptically add 1 ml of a separately sterilized 20 % w/v aqueous glucose solution to each 100 ml of growth medium prepared.
3. Aseptically transfer to Petri dishes.
4. Inoculate with test fungus.
5. Incubate at 25 C in darkness and examine plates daily for 10 days.
6. Record production of laccase as the formation of a green colour in the growth medium.

Variations and limitations to the method

The ABTS substrate can be substituted with 0.005 % w/v α-napthol. A blue colour develops due to laccase activity. Results should be interpreted with some caution since peroxidase enzymes will also oxidize ABTS or napthol in the presence of H_2O_2, which may be generated endogenously. Positive results should only be recorded if a negative reaction is obtained using a peroxidase specific growth medium such as Azure B agar. Some fungi produce very fast

reactions when using the ABTS substrate (within hours of inoculation) and this is probably due to laccase cary-over in the inoculum agar disc.

Method 6 – Well tests for LME's (syringaldazine well test)

There are several reagents which can be used to 'well test' agar growth medium for the presence of LME's. These include ABTS, benzidine (p,p'-diaminophenyl), guaiacol, gum guaiac, α-napthol (1-napthol), pyrogallol (1,2,3-trihydroxybenzene) and syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde). Benzidine is a suspected carcinogen, guaiacol reactions are not specific to LME's and obtaining napthol and pyrogallol in the Asian tropics is problematical. The use of syringaldazine or ABTS for well tests is therefore recommended. Reactions with these substrates are specific to LME's (Harkin, Larsen and Obst, 1974; Niku-Paavola *et al.*, 1990) and easy to interpret.

Protocol

1. Prepare LBM medium supplemented with 1.6 % w/v agar and autoclave.
2. Aseptically add 1 ml of a separately sterilized 20 % w/v aqueous glucose solution to each 100 ml of growth medium prepared.
3. Aseptically transfer to Petri dishes.
4. Inoculate with test fungus.
5. Incubate at 25 C in darkness for 5-10 days. Carry out well tests as follows:
6. Cut wells in the agar growth medium approximately 5 mm in diameter.
7. To test for laccase activity, add a few drops of 0.1 % w/v syringaldazine (in 95 % ethanol) to a well.
8. To test for peroxidase activity, add a few drops of 0.1 % w/v syringaldazine (in 95 % ethanol) plus a few drops of 0.5 % w/v aqueous H_2O_2 solution to a well.
9. Add 95 % ethanol only to a further well as a control.
10. The appearance of a purple colour around each well within 30 minutes indicates enzyme production. The peroxidase result should be regarded as positive only if the laccase test displays a negative or less intense reaction.

Variations and limitations to the method

A 0.1 % w/v aqueous ABTS solution can be used as an alternative substrate. Estimating the relative staining intensity (with either substrate) of laccase and peroxidase reactions is not always easy, especially with very rapid or intense colour development. This can lead to some ambiguity in results.

Table 2. Recommended strategies for qualitative assessment of lignocellulose degrading enzyme production.

General screening for all lignocellulose degrading enzymes	Detailed study of cellulolytic enzymes	Detailed study of hemicellulolytic enzymes	Detailed study of LME's
Cellulose azure agar	Cellulose azure agar	RBB-xylan agar [2]	Azure B agar [3]
RBB-xylan agar [2]	CMC agar		Syringaldazine well test [3]
Poly-R agar [1]	Esculin agar		

1 = The ability of laccase to decolourize Poly R among a wide range of fungi is unknown.
2 = The effectiveness of this method has not been assessed by the author.
3 = Poly-R agar can be substituted as the single assay here if it is known laccase can decolorize the dye.

Method 7 – p-cresol agar

Although not strictly a lignin modifying enzyme tyrosinase is implicated in the detoxification of lignin breakdown products (Eaton and Hale, 1993). The production of tyrosinase can be assayed by the well test procedure using p-cresol (4-methoxyphenol).

Protocol

1. Prepare LBM medium supplemented with 1.6 % w/v agar and autoclave.
2. Aseptically add 1 ml of a separately sterilized 20 % w/v aqueous glucose solution to each 100 ml of growth medium prepared.
3. Aseptically transfer to Petri dishes.
4. Inoculate with test fungus.
5. Incubate at 25 C in darkness for 5-10 days. Carry out spot tests as follows:
6. Cut wells in the agar growth medium approximately 5 mm in diameter.
7. Add a few drops of 0.1 % w/v p-cresol in 0.05 % w/v aqueous glycine solution.
8. The appearance of a red-brown colour around the well indicates a positive result.

Variations and limitations to the method

The colour reaction may take up to 1 day to develop. Tyrosinase is often produced as a cell-associated enzyme.

Summary

A range of qualitative approaches for the assessment of lignocellulose degrading enzyme production have been presented in this paper. Detailed

stepwise methodologies for each assay are described, together with a discussion of the advantages, variations and limitations. These procedures offer a powerful research tool for obtaining data on possible methods of lignocellulose substrate utilization, or the production of commercially important enzymes in the case of xylanases or LME's. They are particularly appropriate for high throughput screening of many isolates due to their simplicity, rapidity and low cost. Ultimately the choice of assay method will depend on growth characteristics of the test organism, availability of reagents and personal preference of the researcher. However as a general guide, combinations of assay for achieving specific research objectives are given in Table 2.

References

Archibald, F.S. (1992). A new assay for lignin-type peroxidases employing the dye azure B. Applied and Environmental Microbiology 58: 3110-3116.

Bavendamm, W. (1928). Uber dad vorkommen und den nachweisvon oxydasen bei holzstorenden pilzen. Z. Pflkrankh. Schulz. 38: 257-276.

Beily, P., Mislovicova, D. and Toman, R. (1985). Soluble chromogenic substrates for the assay of endo-1,4-ß-xylanases and endo-1,4-ß-glucanases. Analytical Biochemistry 144: 142-146.

Boominathan, K. and Reddy, C.A. (1992). Fungal degradation of lignin. In: *Handbook of Applied Mycology. Vol. 4: Fungal Biotechnology* (eds. K.D. Akora, R.P. Elander and K.G. Mukerji). Marcel Dekker, New York, USA: 763-822.

Buswell, J.A., Cai, Y.J., Chang, S.T., Peberdy, J.F., Fu, S.Y. and Yu, H.S. (1996). Lignocellulolytic enzyme profiles of edible mushroom fungi. World Journal of Microbiology and Biotechnology 12: 537-542.

Cai, Y.J., Buswell, J.A. and Chang, S.T. (1994). Cellulases and hemicellulases of *Volvariella volvacea* and the effect of tween 80 on enzyme production. Mycological Research 98: 1019-1024.

Eaton, R.A. and Hale, M.D.C. (1993). Wood, Decay, Pests and Prevention. Chapman and Hall, London, UK.

Egger, K.N. (1986). Substrate hydrolysis patterns of post-fire ascomycetes (Pezizales). Mycologia 78: 771-780.

Eggert, C., Temp, U. and Eriksson, K.E.L. (1996a). The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*; purification and characterization of the laccase. Applied and Environmental Microbiology 62: 1151-1158.

Eggert, C., Temp, U., Dean, J.F.D. and Eriksson, K.E.L. (1996b). A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignins by laccase. FEBS Letters 391: 144-148.

Fengel, D. and Wegener, G. (1989). Wood. de Gruyter, New York, USA.

Gessner, R.V. (1980). Degradative enzymes produced by salt marsh fungi. Botanica Marina 23: 133-139.

Harkin, J.M., Larsen, M.J. and Obst, J.R. (1974). Use of syringaldazine for detection of laccase in sporophores of wood rotting fungi. Mycologia 66: 469-476.

Hyde, K.D. (1997) Biodiversity of Tropical Microfungi. Hong Kong University Press, Hong Kong.

Ng, T.K. and Zeikus, J.G. (1980). A continuous spectrophotometric assay for the determination of cellulose solubilizing activity. Analytical Biochemistry 103: 42-50.

Niku-Paavola, M.L., Raaska, L. and Itavaara, M. (1990). Detection of white-rot fungi by a non-toxic stain. Mycological Research 94: 27-31.

Paterson, R.R.M. and Bridge, P.D. (1994). Biochemical techniques for filamentous fungi. CAB International, London, UK.

Pointing, S.B., Vrijmoed, L.L.P. and Jones, E.B.G. (1998). A qualitative assessment of lignocellulose degrading enzyme activity in marine fungi. Botanica Marina 41: 293-298.

Pointing, S.B., Buswell, J.A., Vrijmoed, L.L.P. and Jones, E.B.G. (1999a). Extracellular cellulolytic enzyme profiles of five lignicolous mangrove fungi. Mycological Research 103: (In press).

Pointing, S.B., Vrijmoed, L.L.P. and Jones, E.B.G. (1999b). Laccase is produced as the sole lignin modifying enzyme in submerged liquid culture by the white-rot fungus *Pycnoporus sanguineus* L. Mycologia 91: (In press).

Pointing, S.B. (1999) An assessment of ligninolytic potential in basidiomycetes from Hong Kong forests. Microbial Ecology 25: (In press).

Raghukumar, C., Raghukumar, S., Chinnaraj, A., Chandramohan, D., D'Souza, T.M. and Reddy, C.A. (1994). Laccase and other lignocellulose modifying enzymes of marine fungi isolated from the coast of India. Botanica Marina 37: 515-523.

Rautella, G.S. and Cowling, E.B. (1966). Simple cultural test for relative cellulolytic activity of fungi. Applied Microbiology 14: 892-898.

Reddy, C.A. and D'Souza, T.M. (1994). Physiology and molecular biology of the lignin peroxidases of *Phanerochaete chrysosporium.* FEMS Microbiology Reviews 13: 137-152.

Reddy, C.A. (1995). The potential for white-rot fungi in the treatment of pollutants. Current Opinion in Biotechnology 6: 320-328.

Rohrmann, S. and Molitoris, H.P. (1992). Screening for wood-degrading enzymes in marine fungi. Canadian Journal of Botany 70: 2116-2123.

Sass, J.E. (1958) Botanical Microtechnique. Iowa State College Press, Iowa, USA.

Smith, R.E. (1977). Rapid tube test for detecting fungal cellulase production. Applied and Environmental Microbiology 33: 980-981.

Thorn, G. (1993). The use of cellulose azure agar as a crude assay of both cellulolytic and ligninolytic abilities of wood inhabiting fungi. Proceedings of the Japanese Academy of Science 69: 29-34.

Thurston, C.F. (1994). The structure and function of fungal laccases. Microbiology 140: 19-26.