Ligninolytic enzymes from tropical endophytic *Xylariaceae*

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This paper focuses on ligninolytic enzyme production by 581 endophytic *Xylariaceae* strains isolated from healthy tropical native plants of northern Thailand. Strain CMUX144 was found to be the best manganese independent peroxidase producer. The effect of pH, temperature, initial glucose concentration and ammonium tartrate concentration on enzyme production by this strain was investigated. An activity of 195 U/l was achieved after cultivation at the optimum condition for 6 days. Enzyme activity reached 292 U/l when the media was supplemented with veratryl alcohol. The decolourization of Poly R-478 at various C/N ratios in media was investigated. Biological decolourisation following 12 days of cultivation was higher than 91%.

Keywords: ascomycetes, enzyme production, manganese independent peroxidase

Introduction

Lignin is a three-dimensional hydrophobic plant polymer derived from the random coupling of phenylpropanoid precursors; coniferyl, sinapyl and *p*-coumaryl alcohols (Eriksson *et al*., 1990). After cellulose, it is the most abundant organic material on earth, making up 20-30% of the dry weight of wood (Abdel-Raheem and Shearer, 2002). Removed from wood pulp in the manufacture of paper, it is used as a binder in particle board and similar products and as a soil conditioner, filler in certain plastics, adhesive for linoleum, and raw material for chemicals such as dimethoxylsulfoxide (DMSO) and vanillin (Eriksson *et al*., 1990).

Although the mechanism of lignin degradation is not yet fully understood, white-rot fungi are thought to be the only known organisms that can completely break down lignin to carbon dioxide and water (Risna and Suhirman, 2002). Laccase, lignin peroxidase (LiP), manganese dependent

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peroxidase (MnP) and manganese independent peroxidase (MIP) are among the major enzymes of white-rot fungi involved in lignin degradation (Moreira *et al*., 1997) and also used in pulp biobleaching, improved digestibility of lignocelluloses and degradation of recalcitrant organo-pollutants such as polyaromatic hydrocarbons, chlorophenols and polychlorinated biphenyls (Grima-Pettenati and Goffner, 1999; Swamy and Ramsay, 1999).

Traditionally white-rot fungi have been thought to include only basidiomycetes such as, *Phanerochaete chrysosporium*, *Trametes versicolor* and *Bjerkandera* sp. (Mester and Field, 1998; Swamy and Ramsay, 1999; de Koker *et al*., 2000). The *Xylariaceae* is a group of ascomycetes with great biodiversity and abundance in tropical Asian forests (Smith and Hyde, 2001). Some xylariaceous taxa, also produce white-rot in hardwoods (Eaton and Hale, 1993; Eriksson *et al*., 1990). Many authors have reported that *Xylariaceae* species are common endophytes in tropical plants (Bayman *et al*., 1998; Fröhlich *et al*., 2000; Rogers, 2000). With the exception of Pointing *et al.* (2003), Investigations into lignin degrading enzyme production in members of *Xylariaceae* however, has not been reported. This is the first report demonstrating the potential of tropical endophytic *Xylariaceae* spp. in producing ligninolytic enzymes.

Materials and methods

Isolation of endophytic Xylariaceae *spp*.

Native plants were collected from Doi Suthep-Pui National Park, Thailand. The isolation of endophytic *Xylariaceae* spp. was carried out by triple surface sterilization according to previous studies (Photita *et al*., 2001; Kumaresan and Suryanarayanan, 2002). Healthy plant tissue parts were collected and washed thoroughly by rinsing in water for 20 minutes. Plant parts were cut into small pieces (10 mm long) and surface sterilized by soaking in 75% ethanol for 1 minute, sodium hypochloride (concentration and time dependent on type of tissue) and 95% ethanol for 30 seconds. All tissue were dried on sterilized filter paper and were then placed on 2%(w/v) malt extract agar with Rose Bengal to slow down the growth of fungi and 50 µg of chloramphenicol or streptomycin sulfate added for inhibit the growth of bacteria. The plates were incubated at 30°C. Hyphal tips of colonies growing out from the plant tissue were transferred to fresh cornmeal agar or malt extract agar or potato dextrose agar slants. Pure cultures were identified according to morphology and sporulation on malt extract agar slants maintained in the Applied Microbiology Research Unit, Department of Biology, Faculty of Science, Chiang Mai University. All strains of endophytic *Xylariaceae* were

grown on PDA plates, incubated at 30°C for 5-7 days and used as inoculum for the experiments.

Screening methods

A single agar disc (0.5 mm diam.) was taken from the leading edge and inoculated on lignin-modifying enzyme basal (LB) medium containing (per liter) 20g glucose, 1g KH_2PO_4, 0.5g $C_4H_{12}N_2O_6$, 0.5g $MgSO_4.7H_2O$, 0.01g $CaCl_2.2H_2O$, 0.01g yeast extract, 0.001g $CuSO_4.5H_20$, 0.001g $Fe_2(SO_4)_3$, and 0.001g $MnSO_4.H_2O$. The polymeric dye Poly R-478 (0.02g) was used as a polyaromatic ligninolytic indicator and the pH was adjusted to 5.5 (Pointing, 1999). Petri-dishes were incubated at 30°C for 5-7 days and during incubation, mycelial growth and decolouration of Poly R-478 was observed. The isolates decolourising the polymeric dye were selected for further study.

Effect of lignin powder and Poly R-478 on enzyme production

Selected isolates of the tropical endophytic *Xylariaceae* were grown on PDA. An agar disc was transferred to a 250 ml Erlenmeyer flask containing 50 ml of LB medium, which included 2 g/l lignin powder or 0.2 g/l Poly R-478. The LB medium was used as a control. The flasks were incubated in darkness on 120 rev/min rotary shaker at 30°C for 12 days. The culture broth was sampled daily for enzyme activity as described below. Each treatment was conducted in triplicate.

Effect of initial pH and temperature on enzyme production

The effect of initial pH was tested by inoculating the selected isolate in LB liquid medium, which was adjusted to pH 3.0-8.0 using 1M HCl or 1M NaOH. The optimum temperature for enzyme production was investigated by incubating triplicate samples of the selected isolates in LB broth at 20, 30, 37, 45°C and room temperature (32-34°C). The flasks were incubated in darkness at 120 rpm on a rotary shaker for 5 days. The culture broth was separated from the mycelium by centrifugation at 10000 rpm for 10 minutes and the supernatant was assayed for enzyme activities.

Effect of glucose and ammonium tartrate concentration on enzyme production

The selected isolate was inoculated into LB broth, which varying glucose (0-5% w/v) and ammonium tartrate concentrations (0-1% w/v). Flasks were incubated in darkness at 120 rpm on a rotary shaker for 5 days. The culture

broth was separated from the mycelium by centrifugation at 10000 rpm for 10 minutes and the supernatant assayed for enzyme activity.

Enzyme assays

A modification of the method of de Jong *et al*. (1994) was used for the ligninolytic enzyme assay. All enzyme activities were determined spectrophotometrically. Laccase, MnP and MIP activity were measured by the oxidation of 2,6-dimethoxyphenol to 2,2′,6,6′-demethxyquinone at 469 nm. LiP activity was measured by the oxidation of veratryl alcohol to veratryaldehyde at 310 nm. The extinction coefficients (ε) used for veratryaldehyde and the dimeric product of 2,6-dimethoxyphenol oxidation were 9300 and 49600 M^{-1} cm^{-1}, respectively (ten Have *et al*., 1998; Moreira *et al*., 1999).

Effect of Tween 80 and veratryl alcohol on enzyme activity

Agar disks from the selected strain were inoculated into 250-ml Erlenmeyer flasks containing 50 ml of LB medium and 2 g/l lignin powder. Some flasks were supplemented with 0.5 ml/l Tween 80, others with 0.25 ml/l veratryl alcohol and the rest with 0.5 ml/l Tween 80 and 0.25 ml/l veratryl alcohol. The medium without Tween 80 and veratryl alcohol was used as controls. Culture flasks were incubated in darkness at 30°C for 12 days. Samples were taken daily to assay for enzymes activity.

Poly R-478 decolorization rate

Poly R-478 dye (polyvinylamine sulfonated backbone with anthrapyridone chromophore) decolourisation rate was measured as the change in the $A520/A_{350}$ ratio per day in a spectrophotometer. Dye was added to the liquid medium as an aqueous solution to a final concentration of 0.2 g/l for the decolourisation study. After inoculation, samples were taken daily and centrifuged at 10000 rev/min for 10 minutes. 0.2 ml of supernatant was diluted with 0.8 ml distilled water. The uninoculated medium was used as a control, while the medium without dye was used as a blank.

Results and discussion

Decolorization of Poly R-478

All 581 tropical endophytic *Xylariaceae* were screened for decolourisation of Poly R-478 and strains CMUX15 and CMUX144 were shown to decolourise Poly R-478 most effectively. Strain CMUX15 caused

rapid decolourisation of the dye after 2-3 days, while complete decolourisation of Poly R-478 by strain CMUX144 was achieved after 5-7 days incubation. The decolourisation of the polymeric dye Poly R-478 was chosen due to its simplicity and reliability as previously demonstrated (Pointing, 1999; Zheng *et al*., 1999; Couto *et al*., 2000; Leung and Pointing, 2002). These two strains were selected for further study.

Effect of lignin powder and Poly R-478 on enzyme production

A greater ligninolytic activity was observed in LB media supplemented with lignin powder, than in cultures with Poly R-478. Strain CMUX15 secreted laccase at 10 U/liter, and strain CMUX144 secreted MIP 105 U/liter. In LB medium supplemented with Poly R-478, 1 U/liter of laccase and 30 U/liter of MIP were produced by strains CMUX15 and CMUX144, respectively (Fig. 1). These results indicated that lignin powder was an inducer of MIP production and strain CMUX144 was selected for further study due to its high MIP activity.

Effect of initial pH and temperature on enzyme production

The optimal pH range for MIP production by strain CMUX144 in LB medium was 4.0-6.5 at 30°C. The highest activity was achieved at pH 5.5 and the activity decreased considerably when the pH of the medium was higher than 6.5 (Fig. 2). Enzyme production by strain CMUX144 at various temperatures following 5 days of incubation is shown in Figure 3. No growth occurred at 37°C and 45°C, while maximum enzyme activity was at 30°C (122 U/liter).

Effect of glucose and ammonium tartrate concentration on enzyme production

Figure 4. illustrates the relationship between glucose-ammonium tartrate concentrations and MIP activities. The highest MIP activity (195.6 U/liter) was achieved at 40 g/l glucose and 0.75 g/l ammonium tartrate, with no activity in medium without glucose and ammonium tartrate. It is possible that *Xylariaceae* CMUX144 was N-unregulated because the addition of ammonium tartrate to the growth medium did not repress MIP activity. This is in accordance with the report of ten Have and Teunissen (2001), who asserted that not all white-rot fungi are N-regulated. Ligninolytic activity in *Bjerkandera adusta* and

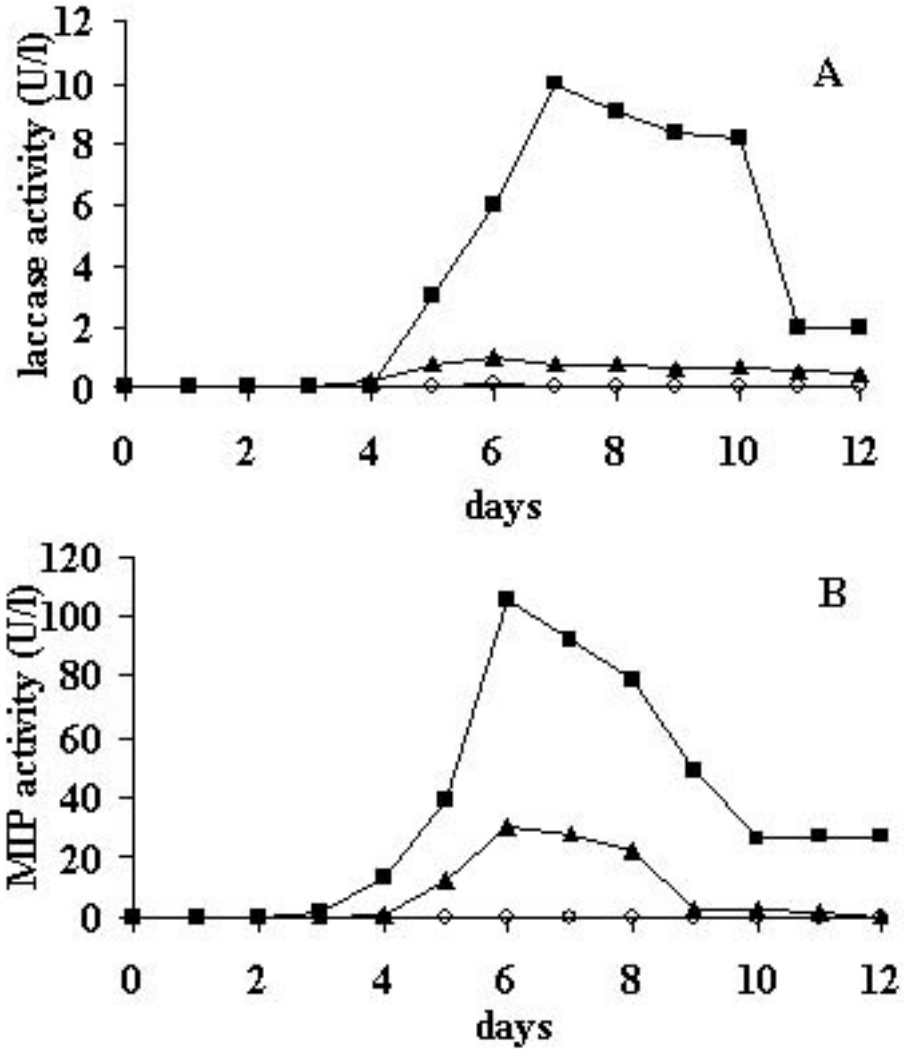

Fig. 1. A) Laccase activity from *Xylariaceae* sp. strain CMUX15 in different medium B) MIP activity from *Xylariaceae* sp. strain CMUX144 in different medium. (o) LB medium, (■) LB medium with lignin powder, (▲) LB medium with Poly R-478.

Coriolus versicolor was not repressed by organic NH_4^+ and/or L-amino acid. In fact, high N media stimulated biomass yields and peroxidase production in N-unregulated fungi.

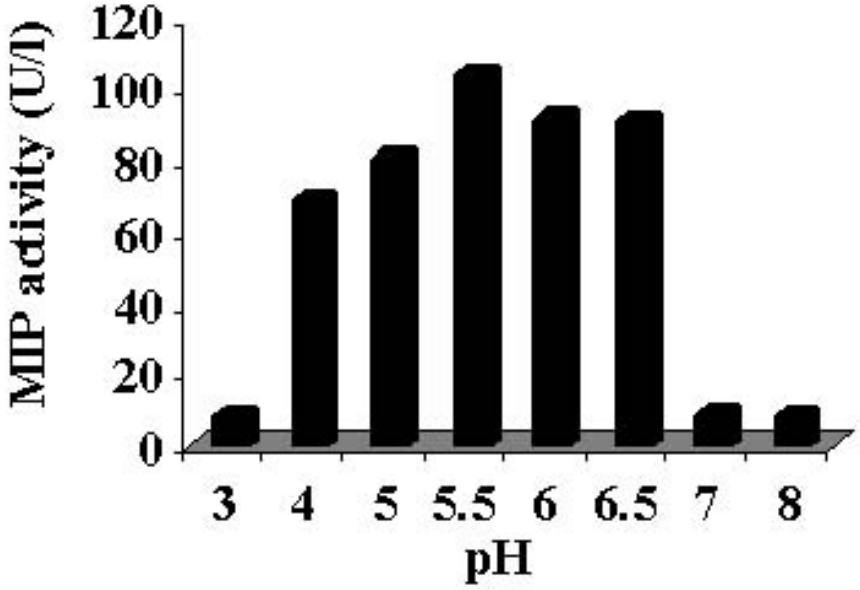

Fig. 2. Effect of pH on MIP production by *Xylariaceae* sp. strain CMUX144.

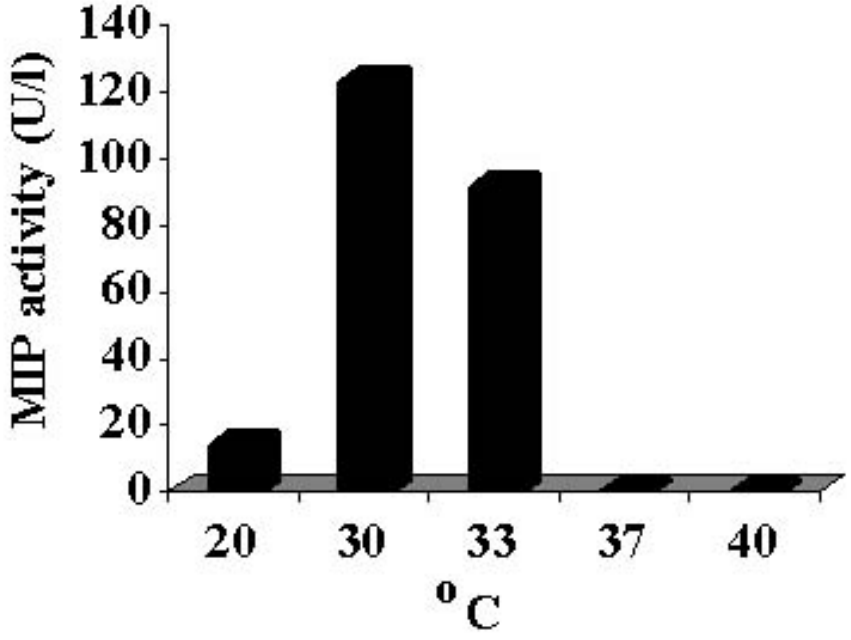

Fig. 3. Effect of temperature on MIP production by *Xylariaceae* sp. strain CMUX144.

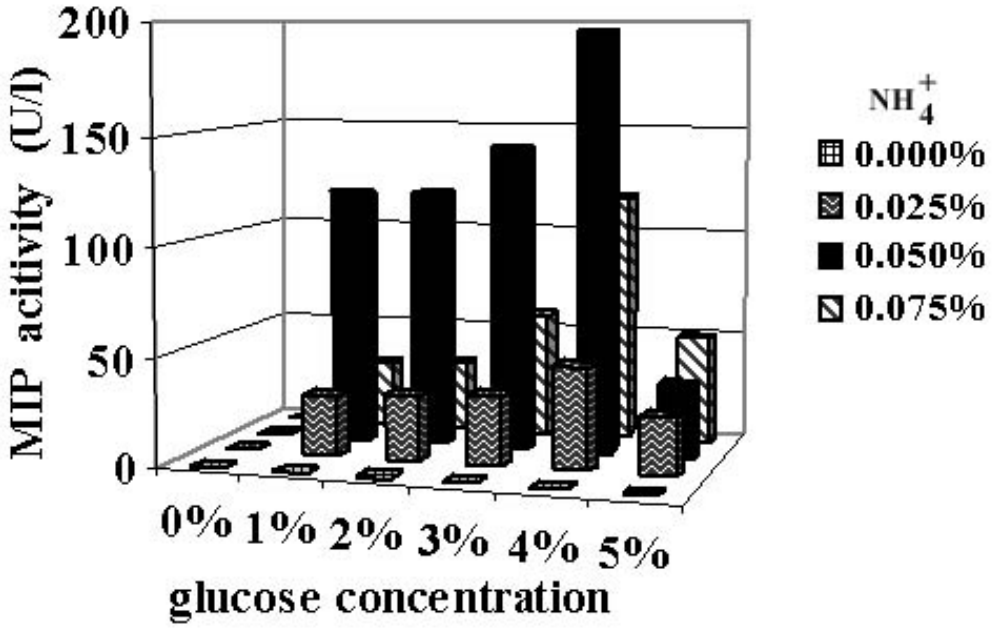

Fig. 4. Relationship between MIP activity and glucose-ammonium tartrate concentration of *Xylariaceae* sp. strain CMUX144.

Effect of Tween 80 and veratryl alcohol on enzyme activity

The mechanism of Tween 80 on ligninolytic enzyme production has not been established. Some authors have suggested that Tween 80 promotes both uptake and exit of compounds from the cell through modification of plasma membrane permeability (Couto *et al*., 2000). In this experiment, it was found that the addition of Tween 80 to the medium did not improve MIP activity. However, when the medium was supplemented with veratryl alcohol, MIP activity was about 1.5-fold higher than that obtained in the control medium (without veratryl alcohol). Furthermore, MIP activity levels were maintained throughout the incubation period. Some authors have suggested that the increase in enzyme production in the presence of veratryl alcohol is due to the fact that it protects the enzyme from H_2O_2 dependent inactivation (Tonon and Odier, 1988).

Decolourisation rate of Poly R-478

The decolourisation rate of the polyaromatic ligninolytic indicator dye, Poly R-478 was measured at 520 and 350 nm, which are the maximum visible

absorbance of Poly R-478 and decolourised Poly R-478, respectively. Strain CMUX144 decolourised Poly R-478 in LB medium, while in LB medium without glucose and/or ammonium tartrate, decolourisation was not observed (Fig. 5). After 7 days of incubation, near complete decolourisation of Poly R-478 was achieved by strain CMUX144, the dye turned from violet to yellow and the dye was adsorbed onto the mycelium. To compare the ability of decolourisation of Poly R-478 by strain CMU144 was similar to *Phanerochaete chrysosporium* (Couto *et al*., 2000).

Further studies will focus on enzyme purification and textile dye decolourisation.

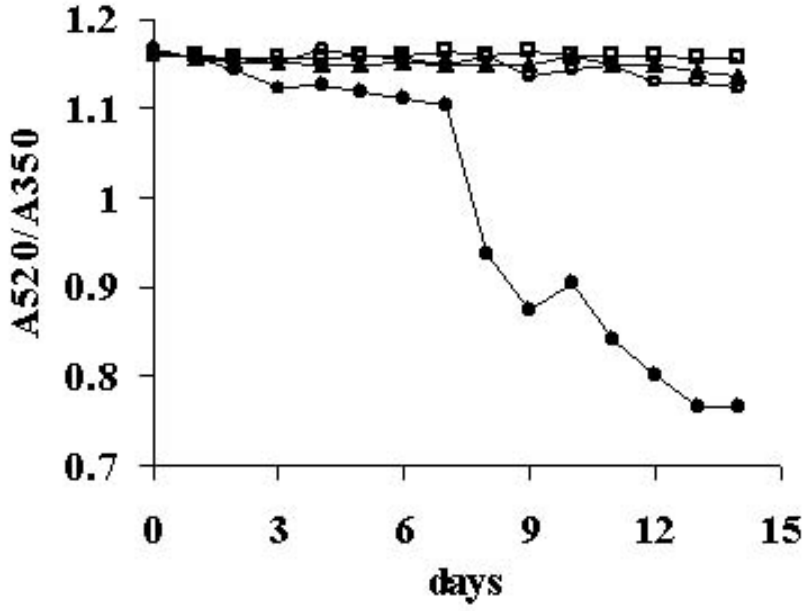

Fig. 5. Poly R-478 decolourisation rate (A_{520}/A_{350}) by *Xylariaceae* sp. CMUX144. (●) LB medium, (o) LB medium without ammonium tartrate, (▲) LB medium without glucose, (□) LB medium without glucose and ammonium tartrate.

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