Thermotolerant wood rotting fungi isolated from northern Thailand and their potential uses in lignin degrading applications

Chartchai Khanongnuch[1*], Nittaya Wanphrut[1], and Saisamorn Lumyong[2] and Takashi Watanabe[3]

[1]Department of Biotechnology, Faculty of Agro-industry, Chiang Mai University, Chiang Mai, 50200, Thailand
[2]Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, 50200, Thailand
[3]Laboratory of Biomass Conversion, Wood Research Institute, Kyoto University, Uji, Kyoto, 611-0011, Japan

Khanongnuch, C., Wanphrut, N., Lumyong, S. and Watanabe, T. (2004). Thermotolerant wood rotting fungi isolated from northern Thailand and their potential uses in lignin degrading applications. Fungal Diversity 15: 187-196.

One hundred and thirteen wood rotting taxa were isolated from northern Thailand. The isolate *C. versicolor* RC3 exhibited highly active growth at 37ºC among 10 thermotolerant isolates including of strains ST40, TP7, TP16, NP14, NP18, NP21, NP26, NP27, 7M and *C. versicolor* strain RC3. Isolate RC3 was found to be *Coriolus versicolor* which degraded Poly R-478 dye at 42ºC. Comparative growth studies on potato dextrose agar revealed that *C. versicolor* strain RC3 had almost the same growth rate as *Phanerochaete chrysosporium* strain ATCC 34541 at 37 and 42ºC, while *C. versicolor* IFO30388 did not. When cultivated in the liquid basal medium containing 0.02% Poly R-478 dye at 37ºC with 150 rpm shaking, *C. versicolor* strain RC3 degraded Poly R-478 dye completely within 5 days. This is more active than the well-researched lignin degrading white rot fungus, *P. chrysosporium*. Addition of 1%(w/v) glucose and 0.2%(w/v) NH_4NO_3 to the medium enhanced dye degradation. *Coriolus versicolor* strain RC3 removed the brown colour from rubber wood chips which changed the lightness from 37.05 to 46.07 of L* value without nutrients. This effect was not found in nutrient rich condition.

Keywords: *Coriolus versicolor*, dye degradation, isolation, Poly R-478

Introduction

Discharges of effluence from pulp and paper mills have been of great concern due to the detrimental impact on the environment (Risna and Suhirman, 2002). Such wastewater contains high levels of toxic pollutants,

*Corresponding author: Chartchai Khanongnuch; e-mail: aiickhnn@chiangmai.ac.th

such as chlorinated phenolic compounds and dioxins (Owen, 1991; Kringatus and Linstron, 1994). These substances are likely to contaminate food chains through bioaccumulation (Bajpai *et al*., 1999). These environmental problems are caused by high usage of chlorine-based chemicals in the bleaching processes. This important step removes lignin and decreases the brown colour in pulp. The use of biological processes to replace or reduce chemical usage in pulping and bleaching has been considered. White rot fungi (basidiomycetes) are reported to be the best lignin degrading fungi. (Gilberton, 1980; Abdel-Raheem and Shearer, 2002; Urairuj *et al*., 2003). Thus, these basidiomycetes are attractive candidates for biological pulping and bleaching. The most well-known white rot fungi, which have been studied extensively to establish for the mechanism of lignin degradation are *Phanerochaete chrysosporium* and *Tramestes versicolor* (Urairuj *et al*., 2003). Generally, most of basidiomycetes have an optimal temperature for growth of about 26-30ºC. There are rare cases of reports of thermotolerant basidiomycetes, which may be more beneficial for utilizing in pulping and bleaching processes and also in other biotechnological applications.

In this paper, we described the new strain of thermotolerant wood rotting fungus *Coriolus versicolor* strain RC3 and investigate it for lignin degrading enzyme production and polymeric dye decolorization with the purpose to apply this organism in the pulp and paper industries.

Materials and Methods

Isolation

Fruit bodies of wood rotting fungi were collected from tropical forests in northern Thailand and induced to form mycelium on the modified LME medium containing of 1g KH_2PO_4, 0.5g $MgSO_4.7H_2O$, 0.01g $CaCl_2.2H_2O$, 0.001g $CuSO_4.5H_2O$, 0.001g $FeSO_4.7H_2O$, 0.001g $MnSO_4.H_2O$, 0.01g yeast extract and 0.02% Poly R-478 dye per litre of water (Pointing, 1999). After incubation at 30ºC, the mycelium formed was transferred to PDA slants as pure cultures for further study. All stock cultures obtained were inoculated on potato dextrose agar (PDA) and incubated at 37ºC for 7 days using *Phanerochaete chrysosporium* ATCC 34541 and *Coriolus versicolor* IFO 30388 as reference strains. The isolates capable of growth at this condition and exhibiting colony diameter of more than 7mm was selected as thermotolerant basidiomycete fungi. The selected isolates were identified as described by the protocol of Ryvarden and Johansen (1980).

Preparation of seed culture and growth study on Poly R-478 agar

To prepare seed mycelium, the mycelia was transferred to PDA and cultivated at 37ºC for 3 days. The growing edge of mycelium was removed using with 0.5 cm diam. cork borer. Growth study on solid medium were performed by placing one piece of seed mycelium on modified LME medium containing 0.02% Poly R-478 dye and incubated at 37, 42 and 45ºC. The radial mycelial growth was measured and Poly R-478 degradation observed by halo formation.

Decolorization of Poly R-478 dye in liquid medium

Five dishes of mycelia were inoculated in 100ml of the modified LME medium containing 0.2g/L of Poly R-478 dye with and without addition of 2g/L of ammonium nitrate and 1%(w/v) glucose. The culture was incubated at 37ºC on 150 rpm of rotary shaker. The culture broth was sampling and centrifuged with 8,000 rpm for 10 minutes to remove the mycelia and determined for the absorbance 520/350 ratio per day by spectrophotometer (Moreira *et al*., 1997).

Investigation for enzyme production on rubber wood chips

Mycelial disks prepared on PDA plates as described above were inoculated on 5g of sterilized rubber wood chips in 250mL Erlenmeyer flasks and 15mL of sterilized distilled water. A second treatment replaced of the sterilized distilled water with 15mL of 4%(w/v) peptone and 4%(w/v) glucose solution. After incubation at 37ºC for 9 days, the enzymes produced were harvested by extraction with 50mL of 0.1M acetate buffer in ice bath for 60 minutes. The extracted solution was separated from the rubber wood chips by filtration with cotton mesh and used as crude enzyme solution for determination of laccase, manganese peroxidase (MnP), manganese independent peroxidase (MIP) and lignin peroxidase (LiP).

Enzyme assay

Laccase, MnP and MIP were determined by oxidation of 2,6-dimethoxy phenol (DMP) (Mester *et al*., 1995). The reaction mixture for the laccase and MnP assay contained 1.0 M sodium tartrate buffer pH 5.0, 4.0 mM DMP, 1mM $MnSO_4$ and 0.1mL supernatant in a final volume of reaction mixture 1mL. The MnP reaction was initiated by adding 1mM H_2O_2 and corrected for laccase and

MIP activity. MIP reaction mixture was the same as MnP, but 1.0 $MnSO_4$ was omitted. LiP activity was determined by oxidation of veratryl alcohol to veratryl aldehyde as described by Leontievsky (1994).

Determination of the colour removal from the rubber wood chips

After the enzyme extraction step, the remaining rubber wood chips were washed with 200 ml of distilled water three times and dried at 70ºC for 12 hours. The dry weight of the rubber wood chips was measured and the colour bleaching was determined by a colour coordination (L*b*a* value) system (De Jong *et al*., 1994), using the Color Quest II machine of Hunter Lab as described in operation manual.

Results and discussion

Isolation and identification of the strains

One hundred and thirteen pure cultures were obtained and tested and 42 of these were capable of growth at 37ºC. Ten isolates had active growth at 37ºC and were also highly active in Poly R-478 degradation (Table 1). The capability to decolorize Poly R-478 has been reported to positively correlate with the production of laccase, lignin peroxidase, and maganese dependent peroxidase (Boominathan and Reddy, 1992; Pointing, 1999). *Coriolus versicolor* RC3 was the only strain capable of the same growth rate at 42ºC as *Phanerochaete chrysosporium* ATCC34541. It also produced lignin-degrading enzymes as it rapidly formed halo on the Poly R-478 agar. The thermotolerant *Coriolus versicolor* RC3 was selected for further studies.

The morphological characteristic of isolate RC3 are given in Table 2 and was identified as *Coriolus versicolor.* Beside *C. versicolor* RC3, isolates 7M and TP16, which were identified as *Pycnoporus* spp. This group of white rot fungi has also been reported to be active laccase producers (Eggert *et al*., 1997). The isolates NP11, NP14, NP28, NP21 and NP27 were gill-like fungi and identified as the *Lenzites* spp. *Lenzites* sp. NP21 was the most active in laccase production up to 2.94U/g substrate after cultivated on the rubber wood chip without any addition of nutrient.

Growth and degradation of Poly R-478 on solid agar by C. versicolor strain RC3

The mycelial growth of *C. versicolor* strain RC3 was studied on Poly R-478 agar at 37 and 42ºC and compared with two reference strains *P.*

Table 1. Growth study and halo formation on Poly R-478 agar by ten of most active isolated wood rotting strains at 37ºC and 42ºC.

Strains	Incubation at 37ºC		Incubation at 42ºC	
	Growth	Halo formation	Growth	Halo formation
Phanerochaete chrysosporium ATCC 34541	+++++	+++	+++++	+++
Coriolus versicolor IFO 30388	*	*	*	*
Coriolus versicolor K 2615	*	*	*	*
Coriolus versicolor K 2912	*	*	*	*
Coriolus hirsutus IFO 4917	*	*	*	*
ST40	++++	++++	*	*
TP7	+++	+++	*	*
TP16	++++	+++	*	*
NP14	++++	+++	*	*
NP18	+++	+++	*	*
NP21	+++	+++	*	*
NP26	++++	+++	*	*
NP27	++++	+++	*	*
7M	++++	+++	*	*
RC3	++++	+++	++++	+++

* None growth; +++ general growth; ++++ high growth; +++++ very high growth

Table 2. The morphological characteristics of *Coriolus versicolor* strain RC3.

Character	Description
Fruiting body	Annual, solitary, latterally fused. Solitary up to 5.5 cm wide, gem broad, 0.1-0.3 (0.4) cm thick, consistency tough and coriaceous when fresh, more hard on drying.
Pileus	Dimidiate with a central attachment, convex, upper surface totally velutinate, often silky, strongly concentrically zoned and suleate in narrow bands. Brownish zone at young and greenish caused from green algae especially near the base when it becomes old. Margin thin, entire, often wavy and pale than the rest of upper surface.
Pore layer	Cream, drying to pale fulous, pore angular 5 per mm, dissepiments eutire and thin tube single-layered up to 1.5-2 mm. Sterile margin broad.
Context	Cream, homogenous, 5 mm thick.
Spore	Cylindrical, slightly curved, hyaline, smooth and thin walled, 2.5-3.1 × 5.1-8.2 μm non-amyloid.
Habitat	On dead leguminous tree (*Delonix regia and Acacia* sp.).
Remark*	Growth at 42ºC in culture.

chrysosporium ATCC 34541 and *Coriolus versicolor* IFO 30388 (Fig. 1). Growth rate of *C. versicolor* strain RC3 was almost the same as *P.*

chrysosporium ATCC 34541 at both 37 and 42ºC, while the growth of *C. versicolor* IFO 30388 did not develop. It took about 5 and 6 days for *C. versicolor* strain RC3 to extend the full agar surface (9 cm diam.) at 37 and 42ºC, respectively, while *P. chrysosporium* ATCC 34541 took only 3 days at both temperatures. This may be due to the lag phase of growth found with *C. versicolor* strain RC3, which occurred for one day after inoculation.

Halo formation due to Poly R-478 degradation by *C. versicolor* strain RC3 was observed after 24 and 48 hours at 42 and 37ºC, respectively, with the halo forming in the centre of the colony (Fig 1, closed and opened arrow). In *P. chrysosporium* ATCC 34541, the halo was observed after 3 days and occurred after full expansion of the mycelium (Fig. 1, closed and opened arrow). This corresponds to the physiological characteristics of *P. chrysosporium* for ligninolytic activity and phenol oxidase, which occurred after 4 days (Kirk, 1981). In Table 1, growth and halo formation by *C. versicolor* strain RC3 were observed at 37 and 42ºC, while it did not occur in strains in the same genus including *C. versicolor* IFO 30388, *C. versicolor* K2615, *C. versicolor* K2912 and *C. hirsutus* IFO 4917. However, the growth and halo formation pattern in *C. versicolor* strain RC3 at room temperature (26-30ºC) was similar to *C. versicolor* IFO30388 (data not shown).

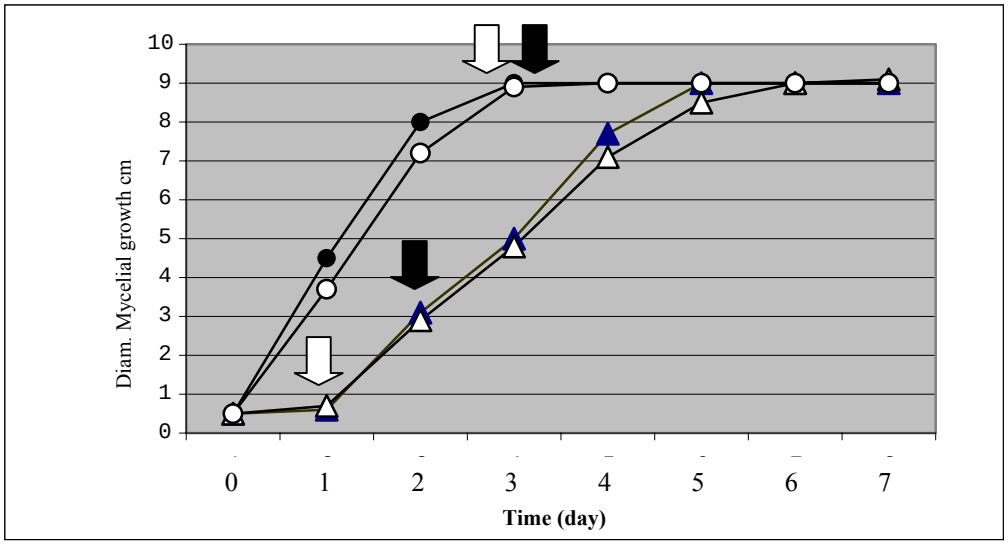

Fig. 1. Mycelial growth and halo formation of *C. versicolor* RC3 (triangle) comparison with *P. chrysosporium* ATCC 34541 (circular). Closed symbol; 37ºC, Opened symbol; 42ºC, Arrow; the day that halo was observed.

Decolorization of Poly R-478 dye in liquid medium

The results of decolorization of 0.02%(w/v) of Poly R-478 suspended in modified LME medium are presented in Table 3. The polymeric dye was degraded completely within 6 days by *C. versicolor* strain RC3, while *P. chrysosporium* did not show an ability to degrade Poly R-478. The addition of 0.2%(w/v) ammonium nitrate as the nitrogen source caused marked enhancing effects on Poly R-478 degradation by *C. versicolor* strain RC3 and the Poly R-478 was removed completely after cultivation for 3 days. The addition of 2%(w/v) glucose as a carbon source had the same effects as ammonium nitrate. This did not occur in *P. chrysosporium* ATCC 34541. These result agree with previous researches where *P. chrysosporium* produced lignin degrading enzymes in conditions with limited nitrogen and carbon sources (Fenn and Kirk, 1981; Boominathan and Reddy, 1992). Moreover, the decolorization of dye by *P. chrysosporium* has been reported to occur only after N-depletion and to be poor in N-rich cultures, with substantial adsorption of the dyes to the mycelium (Sparado *et al*., 1992; Archibald, 1992; Swamy *et al*., 1999).

Table 3. Poly R-478 decolorization during cultivation with 0.2%(w/v) ammonium nitrate in liquid medium.

Strains	Decolorization rate (Δ[A520/A350] day $^{-1}$)	Mycelial dry wieght (mg)	Time used for complete decolorization (days)
P. chrysosporium			
NC-negative	ND	119.9	ND**
NC-positive	ND	125.4	ND
C. versicolor RC3			
NC-negative	0.09	129.2	6
NC-positive	0.20	134.5	3-4

ND; no decolorization of Poly R-478 was observed, NC-negative ; without addition of ammonium nitrate and glucose , NC-positive ; addition of ammonium nitrate and glucose.

Colour removal from rubber wood chips and enzyme production on solid culture

After enzyme extraction as described previously, the remaining rubber wood chips were dried and investigated for colour bleaching. The results are presented in Table 4. When the rubber wood chips was used as the sole carbon and nitrogen source, both *C. versicolor* strain RC3 and *P. chrysosporium* ATCC 34541 showed bleaching activity of 46.1 and 48.3 of the L* value. *C. versicolor* strain RC3 produced LiP and laccase 0.17 and 0.04U/g substrate,

Table 4. Bleaching activity and lignin degrading enzymes produced by *Coriolus versicolor* strain RC3 after cultivated on the rubber wood chips at 42°C for 9 days.

Strains	lightness	Weight loss	Lignin degrading enzymes (U/gram substrate)			
	(L*value)	(%)	LiP	MnP	MIP	Laccase
Phanerochaete	48.3 (-N)	7.4	1.13	-	-	-
chrysosporium	39.2 (+N)	7.8	-	-	-	-
Coriolus versicolor	46.1 (-N)	8.2	0.17	0.02	-	0.04
strain RC3	37.1 (+N)	9.0	0.09	-	-	0.10
Control	37.5	0.7	-	-	-	-

-N ; no addition of glucose and peptone solution
+N ; addition of glucose and peptone solution

respectively. However, laccase was produced at 0.12U/g substrate, while LiP decreased to 0.09U/g substrate when 4%(w/v) glucose and 4%(w/v) peptone solution were added. On the other hand, *P. chrysosporium* ATCC 34541 produced only LiP activity in non-nutrient added condition that was found up to 1.13U/g substrate, while there was no enzyme activity in nutrient rich condition. This is general physiological characteristic of lignin degrading enzyme production by *P. chrysosporium* has previously been observed (Kirk, 1981). These results indicate the difference in physiological responses to nutrient parameters in *C. versicolor* strain RC3 and *P. chrysosporium* ATCC 34541.

The lignin degrading enzymes produced by the fungal strains and colour removal indicates that LiP from *P. chrysosporium* ATCC 34541 may play an important role for bleaching activity as it only one kind of lignin-degrading enzyme found. In case of *C. versicolor* strain RC3, LiP was produced in higher levels in the absence of nutrients and this resulted in higher bleaching activity of rubber wood chips. This enzyme is probably responsible for bleaching activity. However, LiP was reported to be an unimportant enzyme in the biological bleaching and delignification of unbleached kraft pulp by *Trametes versicolor* (Archibald, 1992).

Laccase produced by *C. versicolor* strain RC3 was 0.04U/g substrate and as high as 0.12U/g substrate when nutrients were added. There are many applications of laccase, such as bioremediation of toxic chemical wastes (Glen and Gold, 1983; Swammy and Ramsay, 1999; Mayer and Staples, 2002), pulp and paper industries (Pratima, 1999) and biosensor delvelopment (Kulys and Vidziunaite, 2003). Most commercial laccases are presently produced by white rot fungi, especially *Trametes* spp. or *Coriolus* spp. The thermotolerant property of *C. versicolor* strain RC3 as compared to other species in the same genus is interesting. *Coriolus versicolor* strain RC3 could provide a source of

thermostable lignin degrading enzymes and may be useful in applications described above. However, other characteristics and properties including lignin-degrading enzymes from this strain and the lignin degrading mechanism have to be further investigated. Purification of laccase from *C. versicolor* strain RC3 is in progress.

Acknowledgements

We acknowledge the Thailand Research Fund (TRF) for supporting the research grant and also the JSPS-NRCT Core University Program for providing the chance to work with research staffs of Biomass Conversion Laboratory, Wood Research Institute, Kyoto University. We also acknowledge K.D. Hyde for English corrections and helpful suggestions.

References

Abdel-Raheem, A. and Shearer, C.A. (2002). Extracellular enzyme production by freshwater Ascomycetes. Fungal Diversity 11: 1-19.

Archibald, F. (1992). Lignin peroxidase activity is not important in biological bleaching and delignification of unbleached kraft pulp by *Trametes versicolor*. Applied and Environmental Microbiology 58: 3101-3109.

Bajpai, P., Bajpai, P.K. and Kondo, R. (1999). Biopulping: a less polluting alternative to CTMP. In: *Biotechnology for Environmental Protection in the Pulp and Paper Industry*. Springer, Heidelberg: 29-48.

Boominathan, K. and Reddy, C.A. (1992). Fungal degradation of lignin. In: *Handbook of Applied Mycology. Vol. 4: Fungal Biotechnology*. Marcel Dekker, New York: 763-822.

De Jong, E., Cazemier, A.E., Field, J.A. and de Bont, J.A.M., (1994) Physicological roles of chlorinated aryl alcohols biosynthesis *de novo* by white rot fungus *Bjerkandera* sp. BOS55. Applied and Environmental Microbiology 60: 271-277.

Eggert, C., Temp, U. and Kriksson, K.L. (1997). Laccase is essential for lignin degradation by white-rot fungus *Pycnoporus cinnabainus*. FEBS Letter 407: 89-92.

Fenn, P. and Kirk, T.K. (1981). Relationship of nitrogen to the onset and suppression of ligninolytic activity and secondary metabolism in *Phanerochaete chrysosporium.* Archives of Microbiology 130: 59-65.

Glenn, J.K. and Gold, M.H. (1983). Decolorization of several polymeric dyes by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Applied and Environmental Microbiology 45: 1741-1747.

Gilberton, R.L. (1980). Wood-rotting fungi of North America. Mycologia 72: 1-49.

Gilbertson, R.L. and Ryvarden, L. (1986). *North American Polypores*. Oslo, Norway.

Kirk, T.K. (1981). Physiology of lignin metabolism by white rot fungi. In: *Lignin Biodegradation: microbiology, chemistry and potential applications, Vol. 2,* CRC, Boca Raton: 51-56.

Kringatus, K.P. and Linstron, K. (1994). Spent liquors from pulp bleaching. Environmental Science and Technology 18: 236-248.

Kulys, J. and Vidziunaite, R. (2003) Amperometric biosensos based on recombinant laccases for phenols determination. Biosensor and Bioelectronics 18: 319-325.

Leontievsky, A.A., Myasoedova, N.M. and Golovleva, L.A. (1994). Production of ligninolytic enzymes of white rot fungus *Panus tigrinus*, Journal of Biotechnology 32: 299-307.

Mayer, A.M. and Staple, R.C. (2002). Laccase: new function of an old enzyme. *Phytochemistry* 60: 551-565.

Mester, T., de Jong, E., and Field, J.A. (1995). Manganese regulation of veratryl alcohol in white rot fungi and its indirect effect on lignin peroxidase. Applied and Environmental Microbiology 61: 1881-1887.

Moreira, M.T., Feijoo, G., Sierra-Alvarez, R., Lema, J. and Field, J.A. (1997). Biobleaching of oxygen delignified kraft pulp by several white rot fungal strains. Journal of Biotechnology 53: 237-251.

Nelson, D. and Elisa, E. (2000). Potential applications of oxidative enzymes and phenoloxidase–like compound in wastewater and soil treatment. Applied Catalysis B, Environmental 28: 83-99.

Owens, J.W. (1991). The hazard assessment of pulp and paper effluents in aquatic environment. Environmental Toxicology Chemistry 10: 1511-1540.

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

Pratima, B. (1999). Application of enzymes in the pulp and paper industry. Biotechnology Progress 15: 147 – 157.

Risna, R.A. and Suhirman (2002). Ligninolytic enzyme production by Polyporeaceae from Lombok, Indonesia. Fungal Diversity 9: 123-134.

Ryvarden, L. and Johansen, I. (1980). A preliminary polypore flora of East Africa *Fungiflora*. Oslo, Norway.

Spadaro, J.T., Gold, M.H. and Renganathan, V. (1992). Degradation of azo dyes by the lignin-degrading fungus *Phanerochaete chrysosporium.* Applied and Environmental Microbiology 58: 2397-2401.

Swamy, J. and Ramsay, J.A. (1999). Effects of glucose and NH_4^+ concentrations on sequential dye decoloration by *Trametes versicolor*. Enzyme and Microbial Technology 25: 278-284.

Urairuj, C., Khanongnuch, C. and Lumyong, S. (2003) Ligninolytic enzymes from tropical endophytic Xylariaceae. Fungal Diversity 13: 209-219.

(Received 25 May 2003; accepted 2 December 2003)