J Wood Sci (2008) 54:76–80
DOI 10.1007/s10086-007-0904-x

ORIGINAL ARTICLE

Mariko Takano · Noriko Hayashi · Katsushi Kuroda

Selective staining and visualization of hyphal sheath of a white-rot fungus *Phanerochaete crassa* WD1694 with phloxine B

Received: September 28, 2006 / Accepted: May 14, 2007 / Published online: July 28, 2007

Abstract The hyphal sheath is a morphological feature of many kinds of fungi. Although the fine structures of the sheath have been studied in detail by a number of electron microscopy techniques, the function and physiology of the hyphal sheath are not yet clarified. One reason for this is that the hyphal sheath is a colorless, mucilaginous, and delicate material so that it is not easily identified. We developed a simple method to visualize and identify the hyphal sheath of the white-rot fungus *Phanerochaete crassa* WD1694. The small mycelial pellets in shaken liquid cultures of *P. crassa* WD1694 were stained directly with phloxine B. Both the hyphae and the hyphal sheath that filled the gaps between each of the hyphae were visualized and observed by light microscopy. The stained hyphae were further studied by transmission electron microscopy, atomic force microscopy, and fluorescence microscopy. Based on these observations, we confirmed that the staining of the hyphae was also due to the presence of the hyphal sheath that closely covered the fungal cell wall. These results clearly showed that the hyphal sheath was selectively stained with phloxine B and could be observed and identified by conventional light microscopy.

Key words Hyphal sheath · Slime · White-rot fungus · *Phanerochaete crassa* · Phloxine B

Introduction

The fungal slime or hyphal sheath is a commonly observed morphological feature of many kinds of fungi.[1–4] The fine structures of the hyphal sheath have been studied in detail with electron microscopy.[1–3,5–8] The hyphal sheaths of brown-rot and white-rot fungi are thought to be the site and the pool of fungal enzymes.[2,3] Extracellular enzymes are located in the hyphal sheath as well as at extracellular or intracellular sites.[5,6,9] The hyphal sheath has been suggested to be an important site for the extracellular modification of wood.[2,3,5,6,10,11]

Although the significance of the hyphal sheath in fungal physiology or function has been mentioned in several reports, its full significance has not yet been elucidated.[1–3,6] One of the reasons for this is that the hyphal sheath is not easily isolated and observed. The hyphal sheath is composed of mucilaginous or gelatinous materials and is easily damaged or lost during the sample preparation procedure.[1,4] The hyphal sheath is not always observed with all of the hyphae. Positions and ages of the hyphae affect the presence of the hyphal sheath. The sheath is present only on rapidly growing hyphae, but not on necrotic hyphae.[2] The age of the mycelium may determine whether a sheath is present.[4] In addition to these facts, the hyphal sheath cannot be observed by conventional light microscopy because it is a colorless and mucilaginous material. Accordingly, a simpler method of determining sheath morphology is required for screening or preliminary examination as part of the physiological or biochemical analysis of hyphal sheaths.

We attempted to visualize the hyphal sheath of the white-rot fungus *Phanerochaete crassa* WD1694. Previously, we visualized extracellular peroxidase reaction of *P. crassa* WD1694 with precipitating 3,3′-5,5′-tetra-methylbenzidine, which is a peroxidase substrate.[12] Extracellular peroxidase reaction occurred on the surface of mycelial pellets, and the localization of peroxidase reaction might be caused by the hyphal sheath. The mycelial pellets of shaken liquid culture of *P. crassa* WD1694 were soft and small, which made them available for direct observation by light microscopy. Other white-rot fungi usually produce larger mycelial pellets that are hard and round and require fixation and sectioning, which can cause severe damage to the hyphal sheath. We developed a very simple method to visualize the hyphal sheath of the white-rot fungus *P. crassa* WD1694 and

M. Takano (✉) · N. Hayashi · K. Kuroda
Forestry and Forest Products Research Institute, 1 Matsunosato, Tsukuba, Ibaraki 305-8687, Japan
Tel. +81-29-829-8282; Fax +81-29-874-3720
e-mail: marin@ffpri.affrc.go.jp

Part of this report was presented at the 50th Lignin Symposium, Nagoya, October 2005

succeeded in observing the sheath by conventional light microscopy.

Materials and methods

Fungal strain

The white-rot fungus *Phanerochaete crassa* WD1694 [MAFF420737, *Phanerochaete crassa* (Lev.) Burdsall] was obtained from the culture collection of the Forestry and Forest Products Research Institute.

Culture conditions

Inoculum of *P. crassa* WD1694 was prepared as described previously.[13] Cultivation conditions were as follows, unless otherwise described. A 300-ml Erlenmeyer flask, which contained 100 ml of a medium consisting of 10 g/l glucose, 0.1 g/l $NH_4H_2PO_4$, 0.6 g/l KH_2PO_4, 0.4 g/l K_2HPO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 5 mg/l $CaCl_2$, 0.1 g/l $MnSO_4$, 0.1 g/l yeast extract, and 0.5 g/l Tween 80, was sterilized at 121°C for 20 min and 10 ml of inoculum was added. The flask was incubated on a rotary shaker at 30°C and 100 rpm for each of the test days.

β-1,3-Glucanase treatment

β-1,3-Glucanase was purchased from Wako. The reaction mixture contained 200 units of β-1,3-glucanase, 120 mM β-mercaptoethanol, 150 μl of culture containing the mycelial pellets in 67 mM phosphate buffer (pH 7.5, 1.5 ml). The reaction mixture was incubated for 2 h at room temperature.

Staining

An aliquot including the mycelial pellets was taken from the culture and added to a 20% solution of phloxine B. After incubation at 26°C for 30 min, the mycelial pellets were gently washed by removal of the supernatant and the addition of distilled water. This process was repeated until the supernatant becomes colorless.

Microscopic analysis

Stained mycelial pellets were used directly for light microscopy and atomic force microscopy (AFM). Sample preparation for transmission electron microscopy (TEM) and fluorescence microscopy was conducted as reported previously.[14]

The microscopes used were a Nikon Eclipse TE 2000-U (light microscopy), a Jeol JEM-2000EX (TEM), and a Nano Scope III A (Veeco; AFM). The fluorescence microscope was an Olympus model BX-RFA with a U-MWIG3 cube, a BP 530–550 excitation filter, and a BA570 barrier filter.

Results

Figure 1 shows the results of the staining of *Phanerochaete crassa* WD1694 with phloxine B solution. The cultivation conditions are described in Fig. 1. The shake culture of *P. crassa* WD1694 produced many spherical mycelia of 0.5–1.5 mm in diameter and with many hyphae protruding outward from the sphere. Both the hyphae and the slime of the mycelial pellets were stained. When the mycelial pellets were shaken gently in the staining solution and washed in distilled water, we observed aggregates of slime that had been stained red and had peeled off from the hyphae (Fig. 1A). The slime was not visible without staining.

When the mycelial pellets were dried for 1 h and fixed gently on a plate, the slime that had peeled off from the hyphae was not observed (Fig. 1B); however, in the enlarged images, slime was observed between each hypha (Fig. 1E). When the mycelial pellets on the plate were dried for 4 h, the slime in the mycelial pellet was observed to have shrunk (Fig. 1C). The shrunken slime could be observed without staining (Fig. 1D).

These results indicate that slime is present mainly inside the mycelial pellets and fills the gaps between each of the hyphae. Because the slime was very soft and easily damaged, as has been reported before, the slime peeled off from the hyphae even with gentle mixing in the staining solution, or it shrank during drying on the plate.[1,4]

The degree of staining on the hyphae varied depending on the cultivation period of *P. crassa* WD1694. Hyphal staining was strong and clear during cultivation for 24–72 h, but after 96 h, it had become weak and diminished (Fig. 2). The present result is consistent with the reports that young hyphae have sheaths, but they may be absent on hyphae older than 72 h.[4,15]

β-Glucanase treatment of mycelial pellets of *P. crassa* WD1694 made the staining less distinct (Fig. 3). *Phanerochaete crassa* WD1694 was cultivated for 2 days as described in Fig. 1 and the resultant mycelial pellets were treated with β-glucanase and further stained with phloxine B. Hyphae without β-glucanase treatment were stained magenta, and agglomerates of slime that peeled away from the hyphae were stained red (Fig. 3A). When the mycelial pellets were stained after treatment with β-glucanase, the staining of the hyphae became less effective and no agglomerates of slime were observed (Fig. 3B). It has been reported that fungal slime consists of β-1,3-glucan.[16] The disappearance of the agglomerates of slime and reduction in effective staining of the hyphae are attributable to decomposition of the β-1, 3-glucan by β-glucanase treatment.

The facts that the hyphal staining varied remarkably with and without β-glucanase treatment and with the duration of cultivation suggest that staining of the hyphae was not caused by staining of the fungal cell walls. The stained hyphae of *P. crassa* WD1694 were then observed by TEM (Fig. 4). Examination of cross sections of the hyphae showed that there was a layer of slime around the cell walls. AFM analysis of the same sample revealed the presence of thin layers of slime along the hyphae (Fig. 5).

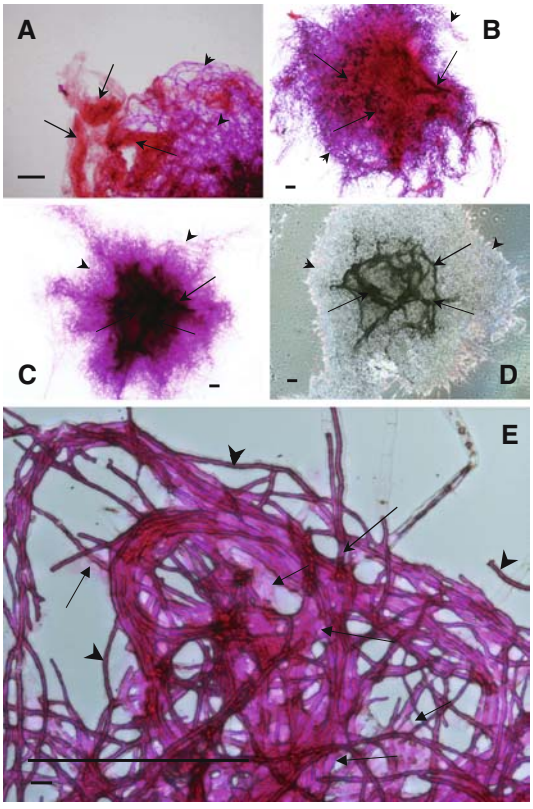

Fig. 1A–E. Staining of *Phanerochaete crassa* WD1694 mycelium with phloxine B. *Phanerochaete crassa* WD1694 was cultivated for 24 h (A, C, D) or 2 days (B, E). Cultivation conditions were as described in Materials and methods except with 1 g/l $NH_4H_2PO_4$ and without Tween 80. **A** Mycelium was shaken gently in phloxine B solution; **B**, **E** mycelium was dried on a plate for 1 h and stained with phloxine B; **C** mycelium was dried on a plate for 4 h and stained with phloxine B; **D** mycelium was prepared as in **C** without staining. *Arrows*, hyphal sheath; *arrowheads*, hyphae. *Bars* 100 μm

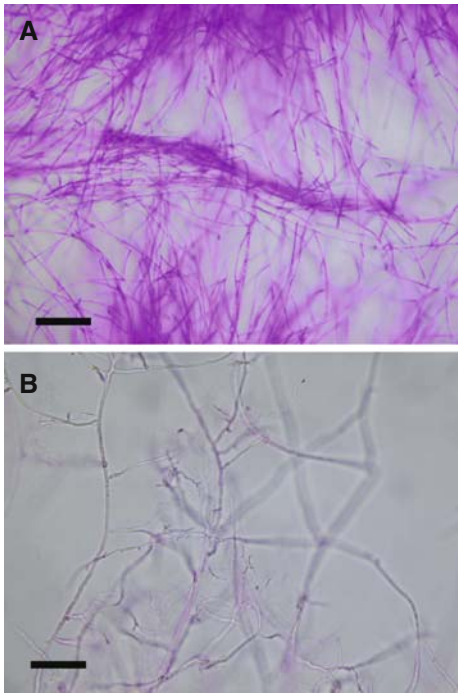

Fig. 2A, B. Effects of cultivation days on the staining of *P. crassa* WD1694 mycelium with phloxine B. **A** Mycelium cultivated for 24 h; **B** mycelium cultivated for 4 days. *Bars* 100 μm

These results showed that the staining of the hyphae with phloxine B was actually the staining of thin slime layer that closely surrounded the surface of the cell wall. This agrees well with the fact that the stainability of the hyphae declined after treatment with β-glucanase. It has been reported that the hyphal sheath is composed of outer and inner layers.[15] We consider that the slime in Fig. 1, which fills the gaps between the hyphae, corresponds to the outer layer, and the slime layer along the cell walls of the hyphae, as observed by TEM (Fig. 4) and AFM (Fig. 5), corresponds to the inner layer.

Sections of phloxine B-stained mycelia were also examined by fluorescence microscopy. Microscopic images of cross sections of the hyphae revealed ring-shaped fluorescent stains (Fig. 6A, C). Ring-shaped images were also observed by visible light microscopy on the same sections and corresponded to the fluorescent parts (Fig. 6B, D). Judging from the thickness of the rings relative to their diameter in the visible light micrograph, we concluded that the rings corresponded to the slime layer in the TEM image in Fig. 4. These results clearly indicate that the staining on the hyphae with phloxine B was caused by the thin layers

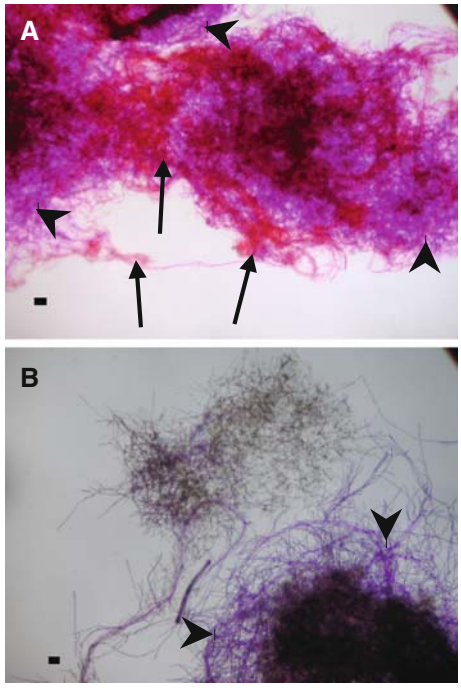

Fig. 3A, B. Effects of β-glucanase treatment on the staining of *P. crassa* WD1694 mycelium with phloxine B. *Phanerochaete crassa* WD1694 was cultivated for 2 days. **A** Without β-glucanase treatment; **B** mycelium treated with β-glucanase. *Arrows*, hyphal sheath; *arrowheads*, hyphae. *Bars* 100 μm

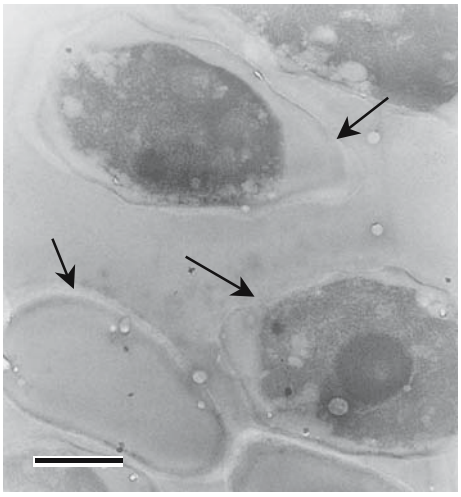

Fig. 4. Cross sections of *P. crassa* WD1694 hyphae observed by transmission electron microscopy. *Phanerochaete crassa* WD1694 was cultivated for 2 days. *Arrows*, hyphal sheath. *Bar* 1 μm

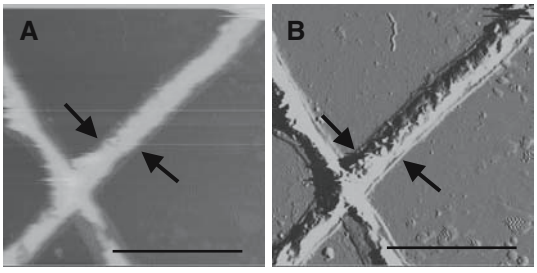

Fig. 5A, B. *Phanerochaete crassa* WD1694 hyphae observed by atomic force microscopy. *Phanerochaete crassa* WD1694 was cultivated for 2 days. **A** Data type was height and the range was 2000 nm; **B** data type was amplitude and the range was 2.0 V. *Arrows*, hyphal sheath. *Bars* 10 μm

of slime along the outsides of the cell walls of the hyphae, which is the inner sheath.

Discussion

The fungal slime or hyphal sheath is a morphological feature that exists outside the hyphae in a variety of fungi.[1–3] Many studies have analyzed the ultrastructure of the hyphal sheath, and in these reports the authors have also speculated on the importance of the hyphal sheath in fungal physiology.[2,3,5,6,10,11]

White-rot fungi have hyphal sheaths, and the ultrastructural distributions of the ligninolytic enzymes in their fungal slime have been reported.[5,6,10,16–18] The hyphal sheaths of white-rot fungi are considered to store, concentrate, and transport extracellular enzymes.[11]

Although the importance of the hyphal sheath in the physiological functions of fungi has been noted in many

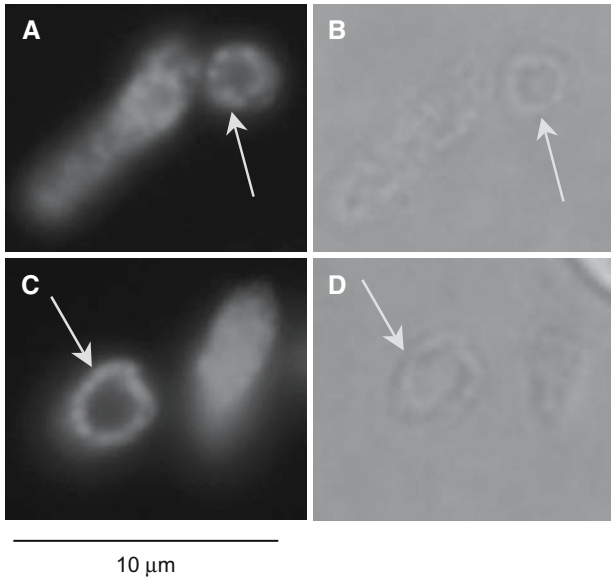

Fig. 6A–D. Cross-sections of *P. crassa* WD1694 hyphae stained with phloxine B. *Phanerochaete crassa* WD1694 was cultivated for 2 days. The same sample was observed by fluorescence (**A**, **C**) and light (**B**, **D**) microscopy. *Arrows*, the stained rings of **A** and **C** corresponded to hyphal sheath of **B** and **D**, respectively

reports, physiological and biochemical analyses of hyphal sheaths have been reported in only a few fungi, and the physiological functions and metabolism of hyphal sheaths have not yet been clarified.[5,6] In most cases, the hyphal sheath has been defined as a fungal structure by electron microscopy.[5–7] However, the need to use electron microscopy simply to confirm the presence of a hyphal sheath is a crucial barrier to the study of this organ's physiology and biochemistry.

In this study, we were able to determine the existence and distribution of the hyphal sheath by conventional light microscopy. The hyphal sheath is a mucilaginous and colorless material and is not easy to distinguish directly by light microscopy.

It has been reported that the hyphal sheath is composed of β-1,3-glucans and is easily damaged by chemical treatment or drying for microscopic analysis.[1,6,19] There are many dyes and methods for staining polysaccharides, such as Schiff's reagent and colloidal iron solution.[20] However, these methods require the use of several steps, acid solutions, or organic solvents, all of which can cause severe damage to the sheath.

We dyed the mycelial pellets with phloxine B without any chemical modification and succeeded in visualizing the fungal slime of *Phanerochaete crassa* WD1694. The analysis also enabled us to understand the distribution of the hyphal sheath in a mycelial pellet. Staining with phloxine B visualized the hyphae and the hyphal sheath among the hyphae. Staining of the hyphae was also due to the presence of the hyphal sheath, which was wrapped tightly around the cell wall. The part of the hyphal sheath among the hyphae was soft, fragile, and easily damaged, as mentioned above. The part of the sheath lying outside the cell wall was thin and

closely attached to the wall; this part was therefore more resistant to the physical damage caused by processes such as mixing and drying.

These results showed the existence of two parts of the hyphal sheath, as has been described previously. Evans et al.[15] reported that the hyphal sheath was composed of two layers. Harrison et al.[21] showed that the hyphal sheath was composed of one layer, although they considered that the inner part might be more condensed and the outer more fibrillar. From these results, we concluded that the part of the hyphal sheath among the hyphae of *P. crassa* WD1694 corresponded to the outer sheath, and the part surrounding the cell wall corresponded to the inner sheath.

Staining of the hyphal sheath was reported previously in *Bipolaris* in which the two layers of the sheath were each stained with a different dye.[15] Phloxine B stained both layers of *P. crassa* WD1694 simultaneously, so that the inner and outer sheath could be stained and distinguished in one step. In addition to this, phloxine B has a strong fluorescence that is apparent even in specimens in which the red staining is not recognizable.

The hyphae treated with β-1,3-glucanase and hyphae grown for 4 days were not stained with phloxine B. The results showed that the inner sheath of these hyphae was absent and the fungal cell wall was not stained with phloxine B. The fungal cell wall consists of chitin and β-1,3-glucans, although the hyphal sheath is mainly constructed of β-1,3-glucans.[22] One of the reasons why the cell wall was not stained with phloxine B is that the concentration of β-1,3-glucans in the cell wall might be much lower than that in the hyphal sheath.

We used phloxine B to stain the hyphal sheath, and we certified the selectivity of its staining of the sheath. Although morphological damage to the outer sheath could not be completely avoided even when the samples were stained directly, the single-step procedure for staining the sheath probably resulted in the least artifacts compared with other methods that require several steps and the use of organic solvents. The inner sheath stained by phloxine B showed no artifacts under observation by light microscopy.

In conclusion, we developed a simple and useful method for distinguishing the hyphal sheath. This method should prove fundamentally useful to studies of the physiology and biochemistry of the hyphal sheath.

References

1. Abu AR, Murphy RJ, Dickinson DJ (1999) Investigation of the extracellular mucilaginous materials produced by some wood decay fungi. Mycol Res 103:1453–1461
2. Palmer JG, Muranis L, Highly TL (1983) Visualization of hyphal sheath in wood-decay hymenomycetes. I. Brown-rotters. Mycologia 75:995–1004
3. Palmer JG, Muranis L, Highly TL (1983) Visualization of hyphal sheath in wood-decay hymenomycetes. II. White-rotters. Mycologia 75:1005–1010
4. Jones EBG (1994) Fungal adhesion. Mycol Res 98:961–981
5. Ruel K, Joseleau JP (1991) Involvement of an extracellular glucan sheath during degradation of Populus wood by *Phanerochaete chrysosporium.* Appl Environ Microbiol 57:374–384
6. Barrasa JM, Gutiérrez A, Escaso V, Guillén F, Martínez MJ, Martínez AT (1998) Electron and fluorescence microscopy of extracellular glucan and aryl-alcohol oxidase during wheat-straw degradation by *Pleurotus eryngii*. Appl Environ Microbiol 64:325–332
7. Ouellette GB, Chamberland H, Goulet A, Lachapelle M (1999) Fine structure of the extracellular sheath and cell walls in *Ophiostoma novo-ulmi* growing on various substrates. Can J Microbiol 45:582–597
8. Connolly JH, Jellison J (1995) Environmental SEM observation of the hyphal sheath and microfibrils in *Postia placenta*. Can J Microbiol 41:433–437
9. Daniel G (1994) Use of electron microscopy for aiding our understanding of wood biodegradation. FEMS Microbial Rev 13:199–233
10. Daniel G, Volc J, Kubatova E, Nilsson T (1992) Ultrastructural and immunocytochemical studies on the H_2O_2-producing enzyme pyranose oxidase in *Phanerochaete chrysosporium* grown under liquid culture conditions. Appl Environ Microbiol 58:3667–3676
11. Murmanis L, Highly TL, Palmer JG (1984) An electron microscopy study of Western Hemlock degradation by the white-rot fungus *Ganoderma appalanatum*. Holzforschung 38:11–18
12. Takano M, Abe H, Hayashi N (2006) Extracellular peroxidase activity at the hyphal tips of the white-rot fungus *Phanerochaete crassa* WD1694. J Wood Sci 52:429–435
13. Takano M, Nishida A, Nakamura M (2001) Screening of wood-rotting fungi for kraft pulp bleaching by the Poly R decolorization test and biobleaching of hardwood kraft pulp by *Phanerochaete crassa* WD1694. J Wood Sci 47:63–68
14. Ishii T, Matsunaga T, Hayashi N (2001) Formation of rhamnogalacturonan II-borate dimmer in pectin determines cell wall thickness of pumpkin tissue. Plant Physiol 126:1698–1705
15. Evans RC, Stempen H, Stewart SJ (1981) Development of hyphal sheaths in *Bipolaris maydis* race T. Canad J Bot 59:453–459
16. Daniel G, Nilsson T, Pettersson B (1989) Intra- and extracellular localization of lignin peroxidase during the degradation of solid wood and wood fragments by *Phanerochaete chrysosporium* by using transmission electron microscopy and immuno-gold labeling. Appl Environ Microbiol 55:871–881
17. Joseleau JP, Ruel K (1992) Ultrastructural examination of lignin and polysaccharide degradation in wood by white-rot fungi. In: Kuwahara M, Shimada M (eds) Biotechnology in pulp and paper industry, Uni, Tokyo, pp 195–201
18. Daniel G, Pettersson B, Volc J, Nilsson T (1990) Spatial distribution of lignin- and manganese peroxidase(s) during degradation of wood and wood fragments by *Phanerochaete chrysosporium* as revealed by T.E.M. immunogold labeling. In: Kirk TK, Chang H-M (eds) Biotechnology in pulp and paper manufacture, applications and fundamental investigations. Butterworth-Heinemann, Stoneham, MA, USA, pp 99–110
19. Bes B, Pettersson B, Lentholm H, Iversen T, Eriksson KE (1987) Synthesis, structure, and enzymatic degradation of an extracellular glucan produced in nitrogen-starved cultures of the white rot fungus *Phanerochaete chrysosporium.* Biotechnol Appl Biochem 9:310–318
20. Hayama M, Momose M (1999) Staining of polysaccarides (in Japanese). In: Mizuguti K (ed) Monthly medical technology, separate volume. Shin senshokuhou no subete. Ishiyaku, Tokyo, pp 136–158
21. Harrison SJ, Moss ST, Jones EBG (1988) Fungal adhesion in aquatic Hyphomycetes. Int Biodeter 24:271–276
22. Nicklin J, Graeme-Cook K, Paget T, Killington R (2001) Protistan microbe – review. In: Instant notes in microbiology (in Japanese). Springer, Berlin Heidelberg New York Tokyo, pp 179–192