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IDENTIFICATION OF WHITE ROT FUNGUS CP9 AND ITS POTENTIAL APPLICATION IN BIOPULPING

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SUMMARY

Wood-rotting fungi represent an important component of forest ecosystems. Among them, white-rot fungi are the most efficient lignin degraders. Biopulping using white-rot fungi in pretreatment of the materials, is one of the solutions to overcome disadvantages of traditional production methods. Today, the isolation and screening of lignin degrading fungi capable for application in biopulping are of keen interest in Vietnam. The use of non–wood, plant fibres in pulp and paper industry, special, agricultural residuces such as rice and wheat straw, sugarcane baggase, cornstalks etc is the new production toward, potential, serving sustainable development. The fungus CP9, which possessed high ligninolytic activity, was identified and studied in pretreatment of rice straw for biopulping. The fruiting bodies of strain CP9 were effuse on trunk. The hymenium was porous and brown white with short tubes, the white mycelia penetrated wood block. The colony was off-white, blossom, irregularly circular. The mycelia were thick and closely bound together. Beside lignin, this fungus could degrade other substrates such as casein, carboxymethyl cellulose and starch. Biological and morphological characteristics of the fungus CP9 suggested its placement in subdivision Basidiomycota. Combined with the results of phylogenetic analysis, which showed 99% similarity of the fungus with species *Leiotrametes lactinea*, our strain was named as *Leiotrametes lactinea* CP9. This fungus could grow well on rice straw under solid state fermentation. Pretreatment of rice straw using *L. lactinea* CP9 was based on the activity of fungal lignin peroxidase and laccase. After 20 days, the residual enzyme activity was of 21.6 and 18.4 nkat/g material for lignin peroxidase and laccase, respectively. Pretreatment significantly improved the quality of straw, as lignin loss of 38% while cellulosic fibers were comparatively well preserved.

Keywords: *biopulping, Leiotrametes lactinea, pretreatment, rice straw, white rot fungi*

INTRODUCTION

Based on the nature of degradation, wood rot fungi can be divided into white-rot, brown-rot and soft-rot fungi. White-rot fungi are the most efficient lignin degrading organisms. They are common inhabitants of trunks and fallen trees and produce extracellular ligninolytic enzymes (lignin peroxidases, manganese peroxidases and laccase) (Husaini *et al.*, 2011). Degradation of lignin is one of the critical factors in many technical processes involving wood such as pulping and bleaching in the paper making. Biopulping, the treatment of plant materials with natural wood decay fungi prior to pulping, was envisioned as a method for saving energy, improving quality of the pulp produced (Chen, 2014) and decrease the environmental hazard caused by the traditional pulping process. The amount of post-harvest rice straw in our country is extremely large, but it has not been efficiency recycled. The use of rice straw or other lignin-containing agro-residues in the pulp and paper industry has increased substantially in order to replace wood resources that are limited. This is the bullish line of production towards serving sustainable development, especially in developing countries.

The tradition taxonomical methods for identifying decay fungi are based on morphological characteristics, i.e. often difficult and time-

consuming. Nowadays, molecular methods are widely used for classification and identification of fungi (Prewitt *et al.*, 2008). The internal transcribed spacer (ITS) region of rDNA fungal containing the ITS 1, 5.8S rDNA gene, and ITS 2. The advantages that make it suitable for molecular identification of fungi are: (i) in fungi, the ITS region is about 600 - 800 bp long and can be readily amplified with universal primers that are complementary to sequences within the rRNA genes; (ii) Having a very high copy number in the genome of fungi, this region is easily amplified from small, diluted, or degraded DNA samples; and (iii) this region is highly variable among morphologically distinct fungal species, but it is conservative within the same species (Gardes, Bruns, 1993). Therefore, morphological characterization, combined with the analysis of phylogenetic relationships, is an effective tool for identification of fungi. In this work, we have combined the morphological characteristics with phylogenetic analysis using ITS1-5.8S-rRNA-ITS2 gene sequences to identify fungus CP9 and evaluated its ability to pretreat rice straw for biopulping.

MATERIAL AND METHODS

Material

The fungus CP9 was collected from the forests of Ninh Binh and screened for its high activity of ligninolytic enzymes.

Methods

Qualitative evaluation of fungal ligninolytic activity

Fragments of thoroughly washed and sterilized pileus were placed in Petri plates containing MEA (malt extract agar) medium with 440 µl/L guaiacol or 100 mg/L Remazol Brilliant Blue R (RBBR). The inoculated plates were incubated at room temperature for 7 days. The presence of extracellular ligninolytic enzmes can be visualized as reddish-brown zones (on guaiacol plates) or clear zones (on RBBR plates) appeared around the fungal colonies.

Morphological and growth characteristics

The fungus was grown on 2% malt MEA at 28°C. From day 2 to the end of cultivation (day 7), the form, size and colour of fungal colonies were observed and recorded. The mycelia were observed under Olympus inverted microscope IX71.

The growth rate of mycelia was determined according to the method described by Schwantes, Salttler (1971): $V = \Delta X/\Delta T$, where V: growth rate of mycelia (µm/h); ΔX: radius of colony (µm); ΔT: cultivation time (hours).

Macroscopic characteristics

The macroscopic characteristics of the fungus were studied by the methods in Trinh Tam Kiet (2011).

Molecular identification

The genomic DNA of the fungus CP9 was extracted using alkaline extraction method (Sambrook, Russell, 2001), and subjected to PCR to amplify the 5.8S rDNA gene using two primers: BF (5'-CTTGGTCATTTAGAGGAAGTAA-3') and BR (5'-CAGGAGACTTGTACACGGTCCA-3') (Gardes, Bruns, 1993). The thermal cyling progam was as follows: intial denaturation at 95°C for 5 min, followed by 30 cycles: denaturation at 95°C for 90 sec, primer annealing at 55°C for 90 sec and extension at 72°C for 2 min, with a final extension step of 72°C for 8 min. The PCR reaction products were examined by electrophoresis in 1% (w/v) agarose gel and the bands stained with ethidium bromide. The PCR products were sequenced by Axil Scientific Pte., Singapore. The sequences of fungus were compared with similar sequences from GenBank using BLAST program for identification of fungal species.

Cultivation and enzyme extraction

Flasks of 250-ml volume containing 50 ml of 2% malt extract broth were inoculated with 3 fungal disks (6 mm diameter) taken from a 4-day culture of the CP9 strain. The fungus was cultivated on a rotary shaker 120 rpm, at 30°C for 5 days to the density of $1x10^6 – 1x10^8$ conidia/ml.

Ligninase production was carried out in plastic jars containing 100 g sterile rice straw soaked with Kirk's medium (pH 4.5) to 60% w/w moisture. The flasks were inoculated with 5% w/w fungal seed culture and incubated in the dark, at room temperature, for 20 days. Extracellular ligninolytic enzymes were recovered by adding 60 ml phosphate buffer pH 5.0 to 3 g pretreated rice straw, and the flask was shaken at 120 rpm for 30 minutes. The filtrate was centrifuged at 10,000 rpm for 5 minutes, and the supernatant was used for enzyme activity assay (Iqbal *et al.*, 2011). Lignin peroxidase and manganese peroxidase activity were assayed by method of Mercer *et al.* (1996). Laccase was measured by method of Cho *et al.* (2003). Acid-

insolube lignin was dertermined according to TAPPI T 222 om-02 method (2002c). The α-cellulose content of rice straw was determined according to TAPPI T 203cm-99 (2009).

RESULT AND DISCUSSION

Ligninolytic activity of fungus CP9

Since ligninolytic enzymes are non-specific regarding substrates, guaiacol and RBBR are widely used as indicators to evaluate ligninolytic activity of microorganisms. On the agar medium supplemented with these indicators, the oxidation of guaiacol or RBBR by ligninase of fungus CP9 can be visualized as 17.0 mm diameter reddish-brown coloration around the fungal colony, or 14.0 mm diameter disappearance of blue colour zone, respectively. As the result, the fungus CP9 showed comparatively high activity of ligninolytic enzymes.

Figure 1. The fungus CP9 in nature.

The result showed that the fungus CP9 was capable of using cellulose and hemicellulose of rice straw for growth. The generative hyphae were septate, with thick wall and clamp connections (Fig. 2). The clamp connections are usually formed during cell division of secondary mycelia. All fungi that produce clamp connections are members of Basidiomycota, but not all Basidiomycota produce clamp connections (Hood, 2006). Hence, the the fungus CP9 was placed into the subdivision Basidiomycota.

The suitable temperature and pH for growth of the fungus CP9 was at 20-30°C and pH 4-7. This fungus fully colonized the Petri dish in 7 days at 30°C. The mycelia growth rate was 191.67 µm/h.

Biological characteristics of the fungus CP9

In nature, the fruiting bodies of the fungus CP9 are fan-shaped, 3.7–7.5 cm diameter and 0.2–0.5 cm thick with wrinkled margin. The upper surface of pileus is smooth, with large, brown concentric zones. The stype is very short so the pileus seems to attach to the wood logs/ dead trees. The hymenium is yellowish, with small and short tubes resembling a honeycomb (Fig. 1).

The fungus CP9 can utilize various substrates due to abundant extracellular enzymes produced such as ligninase, cellulase, protease and amylase. Production of extracellular enzymes by CP9 as measured by diameter of clearing or color reaction zone in mm: amylase: 22 mm; cellulase: 20 mm; protease: 28 mm. In laboratory, this strain had better growth on mineral salt medium (MSL) supplemented with milled rice straw (1% w/v), than on the control medium (not supplemented with rice straw).

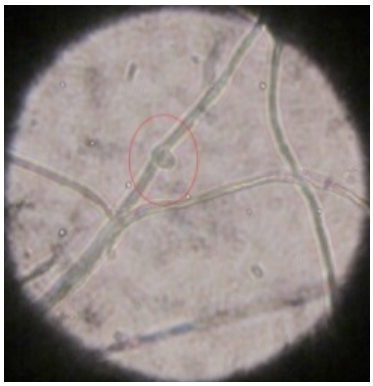


Figure 2. Micrograph of the mycelia (clamp connection shown inside red circle)

The colony was off-white, blossom, and regularly circular with thick mycelia closely bound together.

Molecular phylogenetic analysis of the fungus CP9

The ITS region of fungal rDNA has been successfully applied for identification of wood decaying fungi. The fungal specific primer ITS1 and general primer ITS4 are the earliest PCR primer sets used to amplify fungal ITS regions (White *et al.*, 1990). Nowadays, other specific primers are also available (Jebapriya, Gnanadoss, 2014). Genomic DNA of fungus CP9 was isolated for PCR amplification. The amplified PCR product contained a band of 800 bp in length. Using BLAST program in NCBI database, phylogenetic analysis was done

by CLC DNA workbench 6.6 programme. Similar sequences were retreated for comparison with CP9. The results of phylogenetic analysis are given in Fig. 3. BLAST search of the complete sequence of ITS region and the phylogenetic analysis showed that fungus CP9 was closest (99% similarity) to the species *Leiotrametes lactinea*, hence the isolated fungus CP9 was identified as *L. lactinea* and named as *L. lactinea* CP9. *Leiotrametes* sp. is a tropical species that belongs to the proposed novel genus *Leiotrametes* Welti & Courtec, nested in the *Trametes* clade of the core polyporoid group (Welti *et al.*, 2012). Berrin *et al.* (2012) also reported on *Leiotrametes lactinea* (Accession No. JX082368) collected from tropical forests in Guiana (French).

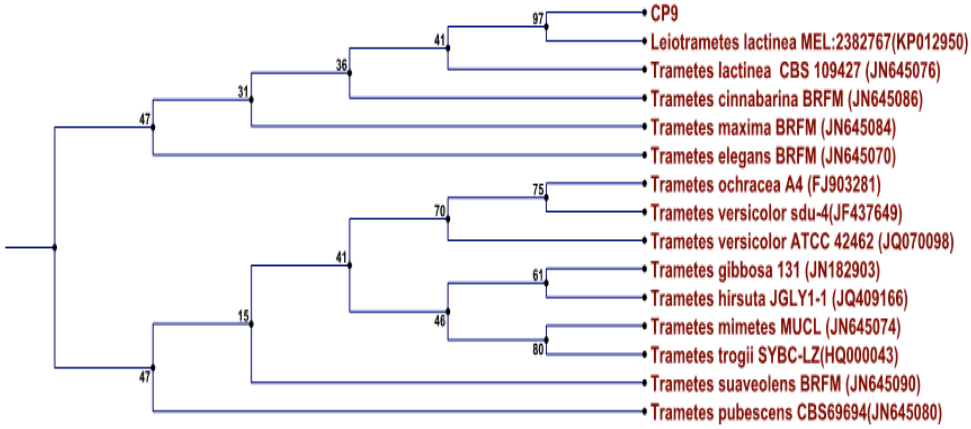
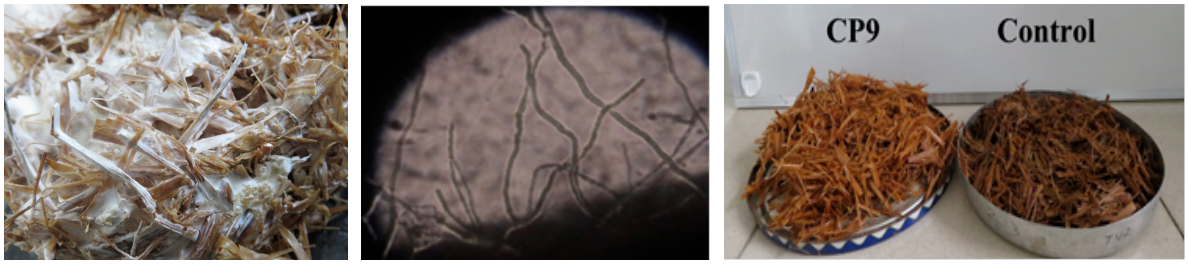


Figure 3. Phylogenetic relationship between the isolate CP9 and other known sequences

Pretreatment of rice traw of *Leiotrametes lactinea* CP9

For biopulping process, selective lignin degradation is required. Thus, only fungi having high ligninolytic enzyme system, yet remarkably low cellulolytic activity are suitable for biopulping (Risdianto, Sugesty, 2015). After 20 days pretreatment of rice straw, *L. lactinea* CP9 already colonized the whole material (Fig. 4A). The residual fungal lignin peroxidase and laccase activity was of 21.6 and 18.4 nkat/g pretreated rice straw. The pretreated rice straw was brighter and softer than untreated material (control) (Fig. 4C), and the quality was significantly improved that favoured biopulping. The lignin content of rice straw was reduced from 22.25% (w/w) before treatment to 13.85%, i.e. lignin loss of 38% by *L. lactinea* CP9. A slight reduction of cellulose was determined, i.e. from initial value of 45.9% (w/w) to 43.6% after pretreatment. Under the same conditions, the ability to degrade lignin from rice straw of *L. lactinea* CP9 equivalent to some fungi, which have announced by Taniguchi *et al.*, (2005). They reported three white rot fungi *Pleurotus ostreatus*, *Phanerochaete chrysosporium* and *Trametes versicolor* that cause 41%, 21% and 37% lignin loss when grown on rice straw for 60 days at 25°C. It can be concluded that *L. lactinea* CP9 has a great application potential in biopulping.



A B C

Figure 4. Delignification of rice straw in solid state culture: A, Fungus CP9 grown on rice straw after 20 days; B, Micrograph of the mycelia on rice straw; C, Pretreated rice straw by CP9 (left) and control sample (right).

CONCLUSION

The fungus CP9 was indentified and assessed of application potential in pretreatment of rice straw for biopulping. Based on morphological characteristics and phylogenetic analysis, this strain is named as *Leiotrametes lactinea* CP9. Atempts to use this fungus in pretreatment of rice straw resulted in comparative improvement of quality of the material. After treatment, the lignin loss of 38% was significant while the loss of cellulose was not (5%). Further research needs to focus on optimization of fungal biomass and enzyme production, as well as development of the pretreatment process.

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REFERENCES

Berrin G, Navarro D, Couturier M, Olivé C, Haon GS, Taussac S, Lechat C, Courtecuisse R, Favel A, Coutinho M, Lesage L (2012) Exploring the natural fungal biodiversity of tropical and temperate forests toward improvement of biomass conversion. *Appl Environ Microbiol* 78(18): 6483–6490.

Chen HZ (2014) *Biotechnology of Lignocellulose: Theory and Practice,* Chemical Industry Press, Springer.

Cho NS, Pashenova N, Hop PTB, Leonowicz A (2003) Purification and characterization of a laccase from *Cerrena unicolor*, *Proceedings of The 4th International Symposium on New horizon of Bioscience in forest products field*, Cheongju, Korea, 19: 19–40.

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Mol Ecol* 2: 113–118.

Hood IA (2006) The mycology of the Basidiomycetes. *Proceedings of a workshop held in Yogyakarta, Indonesia*, Canberra, ACIAR Proceedings 124: 34–45.

Husaini A, Fisol FA,Yun LC, Hussain MH, Sepiah Muid S, Roslan HA (2011) Lignocellulolytic enzymes produced by tropical white rot fungi during biopulping of *Acacia mangium* wood chips. *J Biochem Tech* 3(2): 245–250.

Iqbal HMN, Asgher M, Bhatti HN (2011) Optimization of physical and nutritional factors for synthesis of lignin degrading enzymes by a novel strain of *Trametes versicolor*. *Bio Res* 6: 1273–1278.

Jebapriya GR, Gnanadoss JJ (2014) Screening and molecular characterization of white rot fungi capable of laccase production and dye decolourization. *Int J Life Sci Pharma Res* 4(2): 12–20.

Nishida TK, Kashino Y, Mimura A, Takahara Y (1988) Lignin biodegradation by wood rotting fungi I. Screening of lignin degrading fungi. *Mokku Gakkai* 34: 530–536.

Mercer D, Iqbal H, Miller P, McCarthy A (1996) Screening actinomycetes for extracellular peroxidase activity. *Appl Environ Microbiol* 62: 2186–2190.

Prewitt ML, Diehl SV, McEroy TC, Diehl WJ (2008) Comparison of general fungal and basidiomycete-specific ITS primers for identification of wood decay fungi. *Forest Prod J* 58(4): 66–71.

Risdianto H, Sugesty S (2015) Pretreatment of marasmius sp. on biopulping of fruit bunches. *Mod Appl Sci* 9(7): 1–6.

Sambrook J, Russell DW (2001) *Molecular cloning. A laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Schwantes HO, Salttler PW (1971) Methoden zur messung der Wachstumsgeschwindigkeit von Pilzmycelien. *Oberhess Naturwiss Zeitschr* 38: 5–18.

Taniguchi M, Suzuki H, Watanabe D, Sakai K, Tanaka T (2005) Evaluation of Pretreatment with *Pleurotus ostreatus* for Enzymatic Hydrolysis of Rice Straw. *J Biosci Bioeng* 100: 637–642.

Tappi. T 203cm-99. Alpha-, beta-, gamma-cellulose in pulp. 2009, 7p.

Tappi. T 222om-02. Acid-insolube lignin in wood and pulp. 2002c, 5p.

Trịnh Tam Kiệt (2011) *Nấm lớn ở Việt Nam*, Tập 1 (tái bản lần thứ 2) NXB. Khoa học Tự nhiên và Công nghệ.

Welti S, Moreau P, Favel A, Courtecuisse R, Haon M, Navarro D, Taussac S, Meessen L (2012) Molecular phylogeny of *Trametes* and related genera and description of a new genus *Leiotrametes*. *Fungal Divers* 55: 47–64.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) *PCR Protocols: a Guide to methods and Applications*, Academic Press, New York 315–322.

NGHIÊN CỨU PHÂN LOẠI NẤM MỤC TRẮNG CP9 VÀ KHẢ NĂNG ỨNG DỤNG TRONG SẢN XUẤT BỘT GIẤY SINH HỌC

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TÓM TẮT

Nấm mục là một thành phần quan trọng trong hệ sinh thái rừng, trong đó nấm mục trắng là các vi sinh vật có khả năng phân hủy lignin hiệu quả nhất. Công nghệ sản xuất bột giấy sinh học sử dụng nấm mục trắng trong tiền xử lý nguyên liệu đang là một trong những giải pháp nhằm khắc phục các nhược điểm của công nghệ sản xuất bột giấy truyền thống. Hiện nay, phân lập và sàng lọc các chủng nấm có khả năng phân hủy lignin nhằm áp dụng trong sản xuất bột giấy sinh học là mối quan tâm đặc biệt ở Việt Nam. Việc sử dụng các loại nguyên liệu phi gỗ như các phụ phẩm của nông nghiệp (thân ngô, rơm rạ và bã mía…) thay thế một phần các nguyên liệu gỗ truyền thống trong sản xuất bột giấy đang là sản phẩm mới được hướng tới nhằm phát triển nông công nghiệp bền vững. Trong nghiên cứu này, chủng nấm CP9 được phân lập từ rừng Cúc Phương có hoạt tính phân hủy lignin cao, đã được phân loại, định tên và đánh giá khả năng tiền xử lý rơm rạ nhằm ứng dụng trong sản xuất bột giấy sinh học. Nghiên cứu một số đặc điểm sinh học cho phép xếp chủng CP9 thuộc ngành nấm đảm Basidiomycota. Dựa trên phân tích trình tự vùng gen rDNA phiên mã trong (ITS), chủng nghiên cứu có độ tương đồng 99% với loài *Leiotrametes lactinea* và được đặt tên là *L. lactinea* CP9. Chủng CP9 sinh trưởng tốt trên cơ chất rơm. Sau 20 ngày tiền xử lý rơm với nấm CP9, hoạt tính lignin peroxidase và laccase lần lượt đạt 21,6 và 18,4 nkat/ g rơm. Hàm lượng lignin trong rơm giảm 38%, lượng cellulose hao hụt không đáng kể. Kết quả ban đầu cho thấy chủng CP9 có tiềm năng được sử dụng để tiền xử lý nguyên liệu rơm rạ trong sản xuất bột giấy sinh học.

Từ khóa: *bột giấy sinh học, Leiotrametes lactinea*, *nấm mục trắng, phân hủy lignin, rơm rạ*