

# Delignification of Rice Straw with Ligninase from Novel *Penicillium* sp. strain apw-tt2 for Biopulping

Asri Peni Wulandari, Tika Triyana, and Poniah Andayaningsih

**Abstract**—Study of biodelignification of rice straw was investigated by using a fungus isolated from indigenous the straw. Identification of the fungus was characterized by 18s rRNA sequencing and included in the new species of *Penicillium* sp. strain apw-tt2. Ligninase production and cell growth was observed in incubation condition of Submerged Fermentation (SmF). Optimizations of the enzyme production condition were carried out by altering pH and temperature of the incubation medium. The results showed that the optimum conditions for biopulping are at 40°C and pH 5. High ligninase production was observed at Day 4 with activity 571 U/ml with degraded lignin 66.3%. The quality of pulp showed that the new species is very effective in delignifying rice straw for pulping.

**Index Terms**—*Penicillium* sp., delignification, 18s rRNA, ligninase, biopulping

## I. INTRODUCTION

Indonesia as an agricultural country producing rice straw with a very large amount, however the straw is still regarded as a waste which will ultimately be burned without further utilized.

Delignification is a process separation of lignin from cellulose. Removing lignin by chemical method still widely used mainly as a method of pulping industry and this process is very important in the industry of pulp and paper. Biopulping needed in the industry since delignification process can be environmentally friendly and reduce the cost of production.

Process of making pulp (cellulose fiber) are biologically using ligninolytic microorganism. The most efficient fungi for lignin degradation are included as white rot-fungi which produce ligninase that capable to modify lignin from organic material such as wood, bamboo, or straw. The enzymatic system of ligninase comprising of laccase and peroksidase which has been consider as a useful enzymes for the improvement of biotechnology process for biopulping and biobleaching.

Most researches showed that white-rot fungi as effective lignin decomposer such as *Phanerochaeta chrysosporium* [1], *Agaricus bisporus* [2], *Pleurotus ostreatus* [3], *Bjerkandera adusta* [4], *Curvularia lanata* [5], *Pleurotus sajor-caju* [6],[7]. The soft rot fungi (Ascomycetes) capable to decompose lignin as well. *Aspergillus fumigatus*, *A.*

*japonicas*, *A. niger*, *A. terreus* [8], *Penicillium* sp. [9] and *P. chrysosporium* [10] have the ability to degrade aromatic compounds such as lignin.

Quality of pulp can be seen from the ratio of lignin and cellulose. Pulping process produces pulp of good quality when producing low levels of degradation of cellulose in the presence of high level lignin dissolution. Comparison between biopulping and chemical pulping processes based on the percentage of reduction in lignin content shows the results of each are 44.05% and 56.41%. Several studies have shown that biodegradation of lignin by fungi ligninolytic reduced lignin content by 20 - 30% within 60 days, when delignification performed on sawdust reduced the lignin 19% while the cellulose by 6% for 60 days [11].

For the purpose of producing ligninase, ligninolytic fungi will have a higher activity when incubation condition using submerged fermentation technique or Submerged fermentation (SMF) compared to solid fermentation or Solid State Fermentation (SSF) [12].

The aim of the study was to investigated the potency of straw-indigenous *Penicillium* sp. strain apw-tt2 capable of producing ligninase for biopulping process.

## II. MATERIAL AND METHODS

### A. Rice Straw

Rice straw used in this study were collected from Arboretum, Universitas Padjadjaran, West Java, Indonesia.

Isolate *Penicillium* sp. strain apw-tt2 is a collection of Laboratory of Microbiology, Department of Biology, Padjadjaran University, Indonesia.

### B. Enzyme Preparation and Assay Enzyme

Commercially available Kraft lignin (Sigma) was used throughout this study.

Modification of enzyme preparation and assay enzyme were carried out according to assay ligninase [2], [7]. Crude enzyme extract ligninase was taken (1 ml), centrifuged for 20 minutes at 2000 rpm. The supernatant was taken 0.4 mL then mixed with 0.5 mL of 100 mM acetate buffer pH 5 and 0.1 mL of lignin [2]. This mixture was fed into the cuvette and incubated for 10 min at 30°C. The absorbance of the sample was measured at a wavelength of 383 nm. Observations were made every 24 hours for 10 days. Each sample was tested twice (duplicate). One unit of activity was defined as the ligninase which released for 1  $\mu$ mol product per min or increase in absorbance at 383 nm per min per ml assay mixture at pH 5.

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### C. Identification of Strain

Based on morphological structure, the fungus characterized as genus *Penicillium*. Determination of species identification was done by analysis 18s rRNA. The liquid samples contain of the fungus were centrifuged, the DNA (pellet) was taken and added lyticase. DNA was isolated with the High Pure mushroom Purification Kit (Roche). The total DNA concentration was measured using Eppendorf BioPhotometer plus UV/Vis, then amplified in vitro by PCR was set by giving the program for first denaturation at 94°C for 2 min, 2nd denaturation at 94°C for 1 min, annealing 1 min, and extension 72°C for 1 min and 10 min. 18s rRNA amplicon DNA were analyzed in PT Genetic Science (Singapura). Consensus sequences (about 1165 bp) from each OTU (Operational Taxonomic Unit) were compared with other 18s rRNA genes in Gen Bank (NCBI).

Consensus sequences (about 1165 bp) from each OTU (Operational Taxonomic Unit) were compared to other 18s rRNA genes in Gen Bank (NCBI) using BLAST search from NCBI and aligned to closely related sequences identified from the BLAST search. The phylogenetic tree was inferred using the Clustal method. The ready sequenced data for related taxa were available at (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

### D. Delignification of Rice Straw

Delignification of the straw was carried out in 250-ml Erlenmeyer flask containing 1 g of the straw and 10 ml of cell-free supernatant from 3 day old of culture. Flasks were incubated at the pH 5 and temperature 40°C.

Control contained sterile medium instead of culture supernatant.

### E. Dependence of Enzyme Activity on Temperature and pH Optimization

To examine the temperature optimum of the reaction ligninase, the enzyme reaction was performed at various temperatures ranging from 30°C, 35°C, and 40°C. pH optimum was analyzed by measuring the activity remaining after incubation using different buffer with pH 4-7.

### F. Lignin Determination and Cellulose Determination

Lignin (%) and cellulose (%) were calculated from ADF Method, for the gravimetric method of lignin and cellulase determination [2], [7], [13].

## III. RESULTS AND DISCUSSION

### A. Identification of Strain

The 18S rRNA gene sequence from fungus was determined and based on BLAST sequence comparison, confirmed that the isolated strain apw-tt2 was *Penicillium* sp.

The result of the analysis shows that the *Penicillium* is a new species that has the closest genetic relation with *Penicillium decumbens* and *Penicillium expansum* (Fig.1), with the highest percent identitif against *Penicillium expansum* 99.6% and 99.3% against *Penicillium decumbens*., with 99% homology.

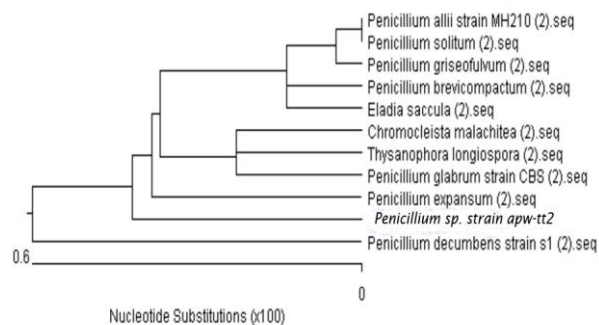


Fig. 1. Phylogenetic tree based on 1165-bp of the gene 18s for *Penicillium* strain sp. apw-tt2 determined using Clustal Method based on allignment of 1165-bp 18s rRNA.

### B. Production of Ligninase and Growth of Fungi

Fig. 2 show that biomass increased markedly during the first 4 days and then showed a declining trend during the biodelignification. In Fig.3 the ligninase activity peaked at Day 4. The results indicated there is a pattern alignment enzyme production of ligninase and cell growth of *Penicillium* sp. strain apw-tt2.

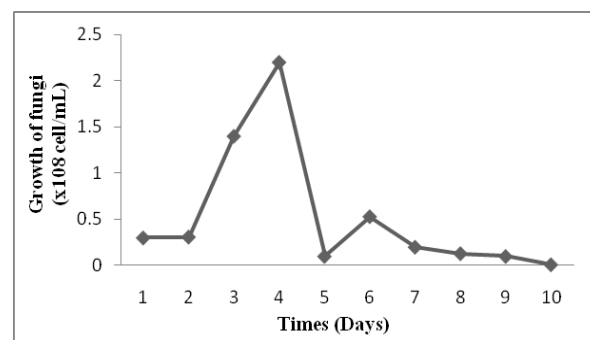


Fig. 2. Growth of *Penicillium* sp. strain apw-tt2 during delignification of rice straw in conditions pH 5, 30°C

### C. Dependence of Enzyme Activity on Temperature and pH Optimum

The effect of pH on the ligninase activity are shown in Fig.4. Determination profile pH by measuring the enzyme activity at various pH from 4 - 7. The maximum ligninase activity was observed at pH 5, increasing pH for condition of incubation showed reducing of the activity of the enzyme.

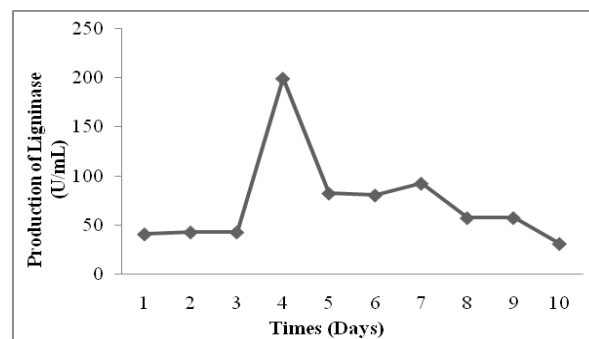


Fig. 3. Production of ligninase during biodelignification of rice straw using *Penicillium* sp. strain apw-tt2 in conditions pH 5, 30°C

The optimum temperature for maximum ligninase production by *Penicillium* sp. strain apw-tt2 was found to be 35°C on Day 4 with an activity of 571 U/ml. Given that the enzymatic reaction will be increased with higher temperatur, indicated that in this case the stability of ligninase remained at

40 °C, therefore the addition of temperatures above 40°C may still determinate.

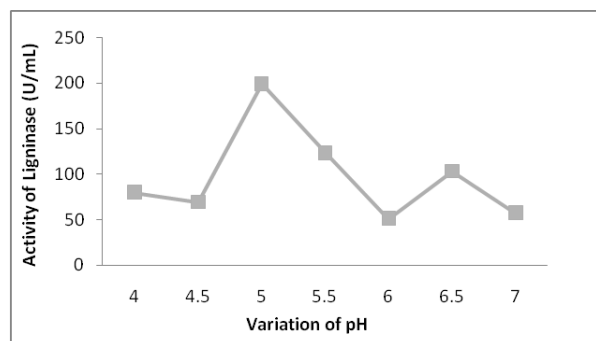


Fig. 4. Ligninolytic enzyme activity in *Penicillium* sp. strain apw-tt2 under variation of pH at 30°C

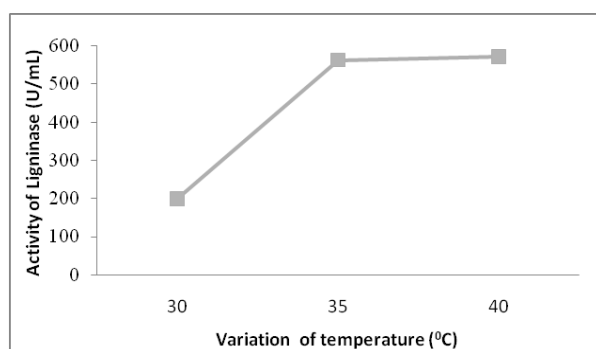


Fig. 5. Ligninolytic enzyme activity in *Penicillium* sp strain apw-tt2 under different temperature condition at pH 5

#### D. Analysis of Pulp

Delignification selectivity can be determined from the comparison of the degradation of lignin and cellulose during the process of delignification. Biodelignification of rice straw for four days by *Penicillium* sp. strain apw-tt2 resulted in decreased levels of 66.3% lignin, cellulose degradation was detected only 28%.

#### E. Analysis Scanning Electron Microscope (SEM)

SEM confirmed that penetration of fungal mycelium on straw and analyzed the changes in lignin degradation. Structural fibers were observed at 200 x magnification (Fig. 6.) and the control (straw without fungus) which may indicated that the structure of the cell wall are still visible piled and rich of the lignin

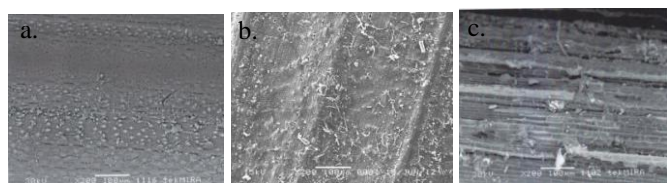


Fig. 6. Photomicrograph SEM (a): Control (straw without fungus); (b) delignification 4 days; (c) delignification 10 days.

While the straw that had been treated for 4 days by the fungus suggests that the cell wall has been degraded and the fibers begin to appear on the cell surface that is dominated by the growth of mycelium. To show the fungal activity on straw delignification for 10 days, lignin degraded on the cell structure of the straw which lignin component dwindling and the growing mold spores have penetrated into the interior of the cell wall.

## IV. DISCUSSION

The results of this study indicate that the *Penicillium* sp. strain apw-tt2. is an indigenous fungus from rice straw showed potential to become ligninase producer. Initial study indicated there was high activity of ligninase that qualitatively proven by clear zone test (data not shown). Quantitatively, by assay ligninase during the incubation process showed there was consistency in the ability to produce high ligninase.

The novelty of this study is when the results of DNA sequencing of *Penicillium* sp. strain apw-tt2 and according to the phylogenetic tree dendrogram showed that an isolate from rice straw is a new species that have DNA character which located between *Penicillium decumbens* and *Penicillium expansum*.

Some studies biopulping include the *Penicillium* sp. KSt3 produce ligninase activity 3.32 U / mL [14]. Incubation using immobilized polyurethane foam, the production of lignin-degrading enzyme Lignin (Lip), manganese peroxidase (MNP), and laccase (Lac) by the white-rot fungus *Bjerkandera adusta* showed the maximum activity of the enzymes were 450, 370, 100 U / ml [10]. *Pycnoporus coccineus* showed that levels of lignin can be degraded as much as 26.9%, and the enzyme lignolytic activity detected 23 U / mL within 60 days [15]. In this study, at the optimum conditions *Penicillium* strain apw-tt2 the lignin was degraded 66.3% and reduction of cellulose that was detected only 28%. Pulping process would be good if only slight damage the cellulose in the straw, but the dissolution of lignin in high level [16], therefore the use of *Penicillium* strain apw-tt2 can be designed in process biopulping to produce good quality paper.

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