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SHORT COMMUNICATION

The Lignocellulolytic Activity and Ability to Produce Indole Acetic Acid Hormone of Fungal Inoculant Isolated From Spent Mushroom (*Agaricus* sp.) Substrate

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The main problem in soil conservation is the lack of carbon source from organic material. Rice straw from spent mushroom substrate (SMS) can be used as organic fertilizer to supply organic carbon for soil. It can also improve soil structure and increase macro-elements and micro-elements required by plants. This research focused on analyzing lignocellulolytic activity and Indole Acetic Acid (IAA) concentration produced by 14 fungal strains isolated from rice straw that had previously been used as substrate for champignon (*Agaricus* sp.). Four fungal strains were isolated (JPF 2, JPF 5, JPF 13, and JPF 14) and then characterized. JPF 14 isolate had the highest laccase activity (1.767 U mL^{-1}) and produced the highest concentration of indole acetic acid (IAA) hormone (6.78 mg mL^{-1}). JPF 13 isolate had the highest amylase activity (0.746 U mL^{-1}). JPF 2 isolate had the highest cellulase activity (0.502 U mL^{-1}). JPF 5 isolate had the highest xylanase activity (0.560 U mL^{-1}). Based on their ability to grow on certain pH and temperature, isolate JPF 2 was classified as mesophylic-acidophylic microbe, isolates JPF 5 and JPF 14 were psycrophylic-acidophylic microbes, and isolate JPF 13 was psycrophylic-alkalophylic microbe.

Key words: Champignon, indole acetic acid hormone, lignocellulolytic fungi, rice straw, spent mushroom substrate

Jerami padi merupakan salah satu substrat yang kaya lignoselulosa dan memiliki potensi untuk dapat diolah menjadi pupuk organik. Penambahan kompos jerami akan menambah kandungan bahan organik tanah, sehingga dapat mengembalikan kesuburan tanah. Tujuan penelitian ini adalah seleksi dan karakterisasi jamur pendegradasi lignoselulosa dan penghasil hormon asam indol asetat (IAA) yang diisolasi dari jerami padi limbah budidaya jamur Champignon (*Spent Mushroom Substrate*). Hasil yang diperoleh menunjukkan bahwa dari 4 isolat jamur lignoselulolitik terpilih (JPF 2, JPF 5, JPF 13, dan JPF 14), isolat JPF 14 memiliki aktivitas enzim lakase tertinggi yaitu sebesar 1.767 U mL^{-1} dan mampu menghasilkan hormon IAA (asam indol asetat) dengan kadar tertinggi yaitu 6.78 mg mL^{-1}. Isolat jamur JPF 13 memiliki aktivitas enzim amilase tertinggi dengan nilai 0.746 U mL^{-1}. Isolat jamur JPF 2 memiliki aktivitas enzim selulase tertinggi yaitu sebesar 0.502 U mL^{-1}. Isolat jamur JPF 5 memiliki aktivitas enzim xilanase tertinggi dengan nilai 0.560 U mL^{-1}. Selain itu berdasarkan pertumbuhan pada suhu dan pH tertentu, isolat jamur JPF 2 dapat dikelompokkan sebagai mikroba mesofilik-asidofilik, isolat jamur JPF 5 dan JPF 14 dapat dikelompokkan sebagai mikroba psikrofilik-asidofilik, isolat jamur JPF 13 dapat dikelompokkan sebagai mikroba psikrofilik-alkalofilik.

Kata kunci: hormon asam indol asetat, jamur Champignon, jamur lignoselulolitik, limbah jerami padi

Rice straw is a by-product of rice production in Indonesia. It becomes a problem because it contains lignocellulose compound that is very difficult to decompose (Arantes *et al.* 2012). On the other hand, it can potentially be converted into organic fertilizer. During harvesting months, rice straw is produced in huge quantity and cannot be optimally utilized. In the fact, rice straw can be used to increase soil organic nutrient by composting process. Long term utilization of rice straw compost can recover soil fertility because it contains lignocelullolytic microbes as bio-remedy agents (Chang *et al*. 2012). Rice straw compost contains phosphate (6.86 ppm), carbon (35.83%), water (35.83%), nitrogen (1,57%), phosphor (0.02%), iron (4.04 ppm), and zinc (0.09 ppm). Based on this composition, each ton rice straw compost contains soil nutrition equivalent to 41.2 kg Urea and 4.5 kg SP36 (Liu *et al*. 2012). It can substitute more than half of chemical fertilizer stock. Lignocelullolytic enzymes such as laccase, cellulase, and xylanase can be produced by lignocelullolytic fungi isolated from spent mushroom substrate that had been used to grow champignon (*Agaricus*) (Dixon and Webb 1979). The lignocellulolytic fungi have a main role of converting bio-mass of rice straw into compost. So, they can degrade and use lignin, cellulose, and hemicelluloses respectively as carbon source and hydrogen source for

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growth (Dewi 2002).

Cellulase is an enzyme that hydrolyzes β-1,4 glucoside chain on cellulose and derivatives into glucose. It has a multi-enzyme system consisting of endoglucanase (EC.3.2.1.4), selobiohydrolase (EC.3.2 .1.91), and β-glucosidase (EC.3.2.1.21) (Ahamed 2008; Bhat 1997). Xylanase is an enzyme group which can hydrolyze polymer xylan into xylose. It can be classified into β-xilosidase, exoxylanase, and endoxylanase. Exoxylanase can cut the chain of xylan polymer at the end of reduction, so it can produce xylose as the main product as well as other short chain oligosaccharides (Da Silva *et al.* 2005, Goddess 2002, Linko *et al.* 1984). Laccase (benzendiol: oxygen oxidoreductase, EC 1.10.3.2) is an extracellular enzyme that uses oxygen to carry out oxidation reaction in aromatic and non aromatic compound. It belongs to oxidase enzyme class which requires metal ion. The Laccase enzyme requires oxygen and produces water as one of the side products. The main substrate of laccase is lignin and oxidation reaction will not produce hydrogen peroxide compound (Couto *et al.* 2006; Couto *et al.* 2007; Kirk and Farrel 1997; Kruus 2000). Lignin peroxidase, and Manganese peroxidase are two ligninase enzymes which can degrade lignin in addition to the laccase enzyme (Jeffries 1994, Kruus 2000).

Fungi isolated from spent champignon substrate not only can produce lignocellulolytic enzyme, but also Indole Acetic Acid (IAA) hormone to induce and increase plant growth (Aryantha *et al*. 2002). IAA is an endogenic auxin hormone that is available in plant body. It is a regulatory growth hormone that was first found as the main signal for plant growth (Ahmad *et al*. 2005). It is formed in the root and crops meristem tissue. Moreover, it can push elongation cell in coleoptil and plant internode. Elongation cells especially exist in vertical turn near the amplification cell. The IAA hormone also has a role in activating cell component, producing cell wall, and reorganizing into matrix whole cell (Bric *et al*. 1991, Levean *et al*. 2004). The IAA hormone produced by fungi will be absorbed by plant, that the plant grow faster and bigger (Arshad and Frakenburg 1991). Based on previous research, so this study will concern to selecting fungi isolates from spent Champignon substrate which can produce high concentration IAA hormone and high activity of lignocellulolytic enzyme.

Moreover, this research focused on producing organic fertilizer starter inoculant made of lignocellulolytic fungi isolated from spent champignon substrate which can produce IAA hormone. The starter inoculant was then used to produce compost from rice straw as spent mushroom substrate. Concentration of IAA hormone and activity of laccase, cellulase, and xylanase enzymes from fungal isolates were used as the parameters for selection and characterization process.

Fungi isolated from spent champignon substrate were grown on potato dextrose agar (PDA) medium. PDA medium contained 400 g potato extract boiled in 1 L of aquadest, 7 g bacto agarose, and 20 g dextrose. Next, the fungal isolates were grown in 50 ml Potato Dextrosa Broth (PDB) medium (37 °C, 72 h, 120 rpm min^{-1}) to analyze the lignocellulolytic activity.

Extraction process was done using buffer phosphate 0.2 M (pH 7) with ratio of culture : buffer phosphate (1:2) (v/v). Then, it was centrifuged using Kubota High Speed Refrigerated Centrifuge model 6500, capacity (6 x 500 mL), and dimension (500 x 740 x 940 mm) with parameter (20 000 x g, 10 min, 0-4 °C). Crude lignocellulolytic enzyme and IAA hormone were isolated on supernatant fraction. Next, it was filtered using Whatman filter paper grade 41 (diameter porous 20-25 μm) to get crude extracts of laccase, cellulase, xylanase enzymes, and IAA hormone.

Assay of laccase enzyme activity was conducted based on previous report (Bourbonais and Paice, 1990). Three hundred seventy five μL sample of crude extract containing laccase enzyme was mixed with 375 μL (1.8 mM) ABTS (2,2-azinobis-3-ethylbenzthiazoline-6-sulfonate) in sodium acetic buffer (pH 4.5). The mixture was then incubated in water bath at 37 °C for 10 min. Enzymatic reaction was stopped using 250 μL 1 % (b/v) SDS (sodium dodecyl sulphate). Then, it was measured by using Spectrophotometer UV-Vis (λ=420 nm). One International Unit of laccase enzyme activity was defined as the total laccase enzyme that can oxidize 1 μmol ABTS each minute.

Assay of cellulase and xylanase enzyme activity was conducted based on previous report (Miller 1959). One hundred twenty five (125) μL sample of crude extract containing cellulase enzyme was mixed with 0.5 % (b/v) CMC (carboxy methyl celullose) liquid substrate in 0.05 M acetic acid buffer pH 5. Then, It was incubated in water bath at 37 °C for 10 min. Later, 500 μL DNS (3,5-dinitro salisilic acid) was added. Then, the mixture was heated in water bath (100 °C, 5 min) to stop enzymatic reaction. Before measurement using Spectrophotometer UV-Vis (λ= 540 nm), 5 mL aquadest was added to dilute th sample. One International Unit of cellulase enzyme activity was defined as the total μmol product of glucose as the

results of cellulase enzyme hydrolysis each minute. For xylanase, 150 µL crude xylanase extract was mixed with 0.5% (b/v) suspension of beechwood xylan in 0.05 M acetic acid buffer pH 5. Then, the mixture was incubated in water bath at 37 °C for 10 min. Later, 200 µL DNS (3,5- dinitro salisilic acid) was added. Then, the mixture was heated in water bath (100 °C, 5 min) to stop enzymatic reaction. Two mL aquadest was added to dilute the sample before being measured with Spectrophotometer UV-Vis (λ=540 nm). One International Unit of xylanase activity was defined as the total µmol xylose produced from hydrolysis by xylanase enzyme each minute.

Assay of indole acetic acid (IAA) hormone was conducted based on previous report (Gordon and Webber 1950). Zero point five mL crude extract of IAA hormone was mixed with 1 mL Salkowski reagent (0.5 M $FeCl_3.6H_2O$ in H_2SO_4). The mixture was then incubated in dark room for 30 min until color is formed. Then, the absorbance was measured using Spectrophotometer UV-Vis (λ=530 nm). ConcentrationIAA hormone was calculated by linear regression analysis using IAA standard curve. The standard curve was made using varying concentration of IAA from 0.005 mg mL^{-1} to 0.050 mg mL^{-1}.

In general, all 14 fungal isolates showed low cellulase and xylanase activities. Nevertheless, their laccase activities and concentrations of IAA hormone differ from one another. Fungal isolate JPF 2 had the highest cellulase activity (0.502 U mL^{-1}) (Table 1). This results was higher than cellulase activity expressed by *Aspergillus* sp. (0.04 U mL^{-1}), *Fusarium* sp. (0.05 U mL^{-1}), *Aspergillus flavus* (0.34 U mL^{-1}), and *Botryotrichum* sp. (0.0098 U mL^{-1}) (Table 1) as reported by Kerem *et al*. (1992). However, it was still lower than the celllulase activity showed by *Trichoderma reesei* (1.66 U mL^{-1}) and *Aspergillus niger* (1.69 U mL^{-1}) (Table 1) grown on rice straw medium (Ahamed and Vermette 2008).

Fungal isolate JPF 5 showed the highest xylanase activity (0.560 U mL^{-1}). It was still lower than that of *Aspergillus niger* grown on rice straw medium, of which the activity was 15.33 U mL^{-1} (Da Silva *et al*. 2005). The highest laccase activity was shown by fungal isolate JPF 14 (1.767 U mL^{-1}), followed by fungal isolate JPF 4 (1.691 U mL^{-1}), of which the activity was still higher than that of *Omphalina* (1.162 U mL^{-1}) isolated from empty bunch of palms (Kruus 2000). Nevertheless, the activities shown by the fungal isolates were lower than that of *Marasmius* sp. (4.560 **U**.mL^{-1}) and *Pleurotus ostreatus* (4.394 U mL^{-1}), which were isolated from industrial pulp paper (Couto and Toca Herera 2007; Kirk and Farrel 1997).

Fungal isolate JPF 14 not only showed the highest

Table 1 Cellulase, xylanase, laccase activities and IAA hormone concentration expressed by 14 fungal isolated from spent champignon (*Agaricus* sp.) substrate

Fungal isolate	Cellulase activity (U mL^{-1})	Xylanase activity (U mL^{-1})	Laccase activity (U mL^{-1})	IAA hormone concentration (mg mL^{-1})
JPF 1	0.340	0.412	0.988	5.34
JPF 2	0.502	0.476	0.654	4.23
JPF 3	0.344	0.464	1.467	5.17
JPF 4	0.380	0.493	1.691	5.48
JPF 5	0.423	0.560	0.756	5.20
JPF 6	0.353	0.458	0.935	4.00
JPF 7	0.315	0.431	0.965	3.91
JPF 8	0.438	0.527	0.969	5.64
JPF 9	0.387	0.533	1.113	5.53
JPF10	0.302	0.453	1.603	3.98
JPF 11	0.385	0.508	0.403	4.09
JPF 12	0.473	0.541	0.889	6.11
JPF 13	0.243	0.479	1.281	4.84
JPF 14	0.371	0.482	1.767	6.78

laccase activity, but also produced the highest concentration of IAA hormone (6.78 mg mL^{-1}) in comparison to the other fungal isolates. The second best IAA producer is JPF 12 with IAA hormone concentration 6.11 mg mL^{-1}. All 14 fungal isolates isolated from spent champignon substrate produced with high concentrations of IAA hormone between 3.91 mg mL^{-1} and 6.78 mg mL^{-1}. This was much higher than the amount produced by *Rhyzobium* sp. (14.40 µg mL^{-1}), which formed symbiosis with plant root, and *Pseudomonas putida* (1.1225 µg mL^{-1}) (Levean and Lindow 2004; Ahmad *et al*. 2005).

Fungal isolates having high activity of lignocellulolytic enzyme and IAA hormone were characterized by measuring the biomass dry weight at varying temperature and pH.m Cultivation was performed on shaker incubator (72 h, 100 rpm min^{-1}) with pH variation from 4.0 to 8.0 (with interval pH 1.0) and temperature variation between 20 °C and 60 °C (with interval temperature 10 °C). After incubation, the fungal biomass was separated from medium by centrifugation (20 000 x g, 10 min, 0-4 °C) using Kubota high speed refrigerated centrifuge model 6500, capacity (6 x 500 mL), dimension (500 x 740 x 940 mm) followed by filtration using Whatman filter paper grade 41 before being weighed. The optimum growth temperature and pH of the lignocellulolytic fungi is defined as the condition giving the best dry weight.

Characterization of Optimum Growth pH and Temperature of the Selected Lignocelullolytic Fungi (isolates JPF 2, JPF 5, JPF 13, and JPF 14) was conducted by measuring the cellular dry weight (Fig 1,2). Cultivation 4 strains of lignocellulolytic fungi was performed on shaker incubator (72 h, 100 rpm min^{-1}) with pH variation from 4.0 to 8.0 (with interval pH 1.0) and temperature variation between 20 °C and 60 °C (with interval temperature 10 °C). Fungi isolate JPF 2 grew optimum at temperature of 30 °C with weight of biomass cell 1.3985 g and pH 5 with weight of biomass cell 1.0887 g. Then, fungi isolate JPF 5 grew optimum at temperature of 20 °C with weight of biomass cell 1.3584 g and pH 5 with weight of biomass cell 1.5375 g. Meanwhile, fungi isolate JPF 13 grew optimum at temperature of 30 °C with weight of biomass cell 1.1986 g and pH 8 with weight of biomass cell 1.9778 g. Later, fungi isolate JPF 14 grew optimum at temperature of 20 °C with weight of biomass cell 1.2978 g and pH 6 with weight of biomass cell 0.9113 g.

For Production of Lignocelullolytic Fungi Starter Inoculant for Organic Fertilizer, the experiments was conducted as follow. Fungal isolates were cultivated on PDA (potato dextrose agar) medium and incubated at room temperature for 5 d. To make starter inoculant F0, fungal isolates were transferred from PDA medium into bean sprout extract-sugar (60 g L^{-1}) medium and incubated at room temperature for 7 d. Then, the starter inoculant F0 was transferred again into sterile cassava dregs (*onggok*) to make starter inoculant F1 and incubated for 14 d. After incubation, it was dried and mixed again with sterile cassava dregs with ratio (1:20) (w/w). Then, it was incubated for another 14 d to make starter inoculant F2, which will be used to inoculate rice straw (spent champignon substrate) to produce organic fertilizer. Population density of the starter inoculant F2 was estimated by using TPC (total plate count) method (Table 2). The population density of inoculant F2 made from isolates JPF 2 and JPF 13 was 5.6 x 10^5 CFU mL^{-1}, fungi isolate JPF 5 was 4.6 x 10^5 CFU mL^{-1}, and JPF 14 was 2 x 10^5 CFU mL^{-1}. TPC method was performed by serial dilution factors 10^3, 10^4, 10^5, and 10^6.

TPC (total plate count) method indicated that the total microbial population on organic fertilizer starter inoculant F2 was between 2 x 10^5 CFU mL^{-1} and 5.6 x 10^5 CFU mL^{-1}. Microbes growing on starter inoculant F2 of organic fertilizer was dominated by fungi isolate. So, it can be assumed that there is no contamination on the production process of organic fertilizer starter inoculant.

Lignocellulolytic fungi was applied as starter to produce compost organic fertilizer from rice straw (Chang *et al*. 2012). Laccase, cellulase, and xylanase enzymes are very important in composting to produce organic fertilizer from rice straw. Laccase enzyme helps to degrade lignin from rice straw. Cellulase and xylanase enzyme have an important role main role in hydrolyzing cellulose and hemicelluloses, respectively, to become glucose and xylose (Liu *et al*. 2012). Glucose and xylose are sources of carbon and hydrogen for fungi, which are essential nutrition source to induce plant growth. In addition, there is also another important reason why fungal is used as starter for organic fertilizer, that is the fungal ability to produce endogenous auxin hormone such as indol acetic acid (IAA) hormone (Ahmad *et al*. 2005). Combination of lignocellulolytic enzyme and IAA hormone will produce organic fertilizer that is nutrition rich and containing high concentration of growth hormone regulator, which is necessary to increase the soil fertility and can accelerate the growth of roots, branches, bars, and leaves (Isikhuemhen and Mikiashvilli 2012).

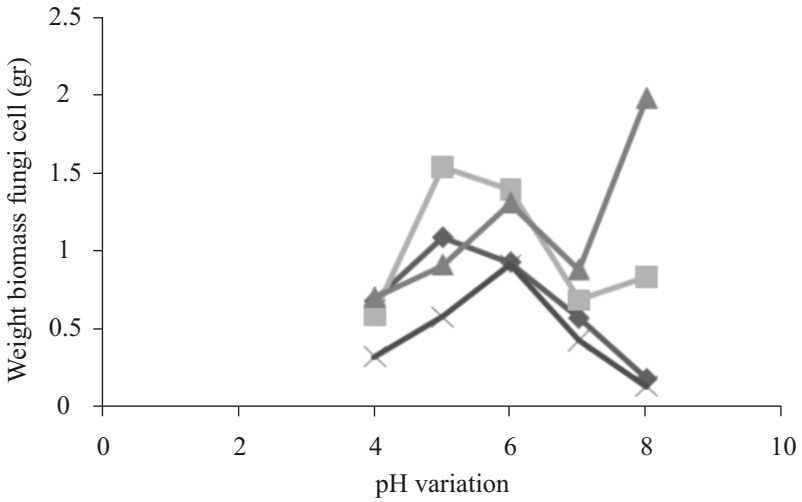

Fig 1 Characterization for optimum pH for growth of fungi isolates JPF 2 (—◆—), JPF5 (—■—), JPF 13 (—▲—), and JPF 14 (—✕—).

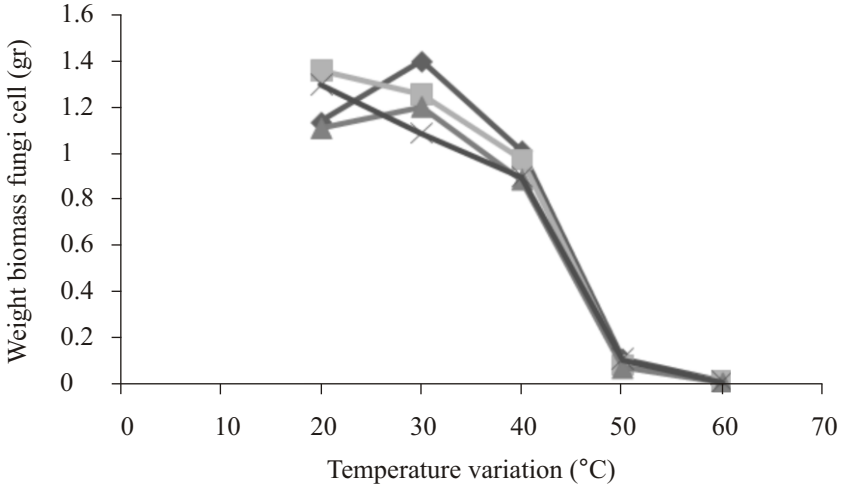

Fig 2 Characterization for optimum temperature for growth of fungi isolates JPF 2 (—◆—), JPF5 (—■—), JPF 13 (—▲—), and JPF 14 (—✕—).

Table 2 Population density of organic fertilizer starter inoculant F2 calculated based on TPC method

Fungi isolates	Results of TPC (CFU mL^{-1})			Average TPC results (CFU mL^{-1})
	1	2	3	
JPF 2	7×10^5	4×10^5	6×10^5	5.6×10^5
JPF 5	3×10^5	6×10^5	5×10^5	4.6×10^5
JPF 13	5×10^5	8×10^5	4×10^5	5.6×10^5
JPF 14	2×10^5	3×10^5	1×10^5	2×10^5

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