Characterization and pathogenicity test of indigenous cellulolytic fungi as biofertilizer candidate

F Fikrina1,3, S Susanna2, M Khalil1, R Sriwati2, S Syafruddin4 and S Sufardi3

1 Doctoral Program of Agricultural Sciences, Universitas Syiah Kuala, Banda Aceh, Indonesia.
2 Plant Protection Department, Faculty of Agricultural Sciences, Universitas Syiah Kuala, Banda Aceh, Indonesia.
3 Soil Science Department, Faculty of Agricultural Sciences, Universitas Syiah Kuala, Banda Aceh, Indonesia.
4 Agrotechnology Department, Faculty of Agricultural Sciences, Universitas Syiah Kuala, Banda Aceh, Indonesia.

E-mail: fikrina@unsyiah.ac.id

Abstract. Cellulolytic fungi have an important role in regulating plant nutrition to sustain plant productivity. This study was aimed to characterize and test the pathogenicity of cellulolytic fungi isolated from the dry land of Aceh as a candidate for biofertilizer. A total of 30 isolates was characterized by their cellulolytic and phosphatase activity semi-quantitatively. The cellulolytic activities on amorph and crystalline cellulose were determined on the Mandel medium with CMC and Avicell as carbon sources respectively while phosphatase was observed on the Pikovskaya medium. The pathogenicity test of the potential isolates on maize, rice, and soybean was determined by the Knop’s medium slants method. The results show that isolates with the ability to degrade amorph cellulose were more (93.33%) than crystalline (86.67%) and 80% isolates had activities on both cellulose types. Beside cellulolytic, most isolates (93.33 %) also had phosphatase activity. There were 23 isolates whose cellulolytic and phosphatase activities, five isolates were non-pathogenic on maize, rice, and soybean, two isolates on maize and rice, and one isolate on rice. These results indicate that the indigenous cellulolytic fungi from the dry land of Aceh were potential to be developed as biofertilizers in the cultivation of maize, rice, and/or soybean.

1. Introduction
The application of agriculturally important microorganisms is an environmental strategy to sustain plant productivity. The microorganisms called biofertilizer may sustain plant growth and yield even in an adverse environment. Biofertilizer production requires a media that functions to grow bacteria, packaging and to extend the shelf life of biological agents [1]. For this reason, the contribution of cellulolytic fungi such as Aspergillus niger Penicillium oxalicum, Paecilomyces varioti, Sporothrix carnis, Talaromyces pinophilus, and Trichoderma reesei [2-5] have been reported.

These cellulase producing microorganisms may stimulate plant growth directly and indirectly. They can directly decompose soil organic matter [6], control the availability of nutrients, such as nitrogen [7] and phosphorus [8, 9] or by the production of phytohormones [10, 11]. Indirectly,
microorganisms are capable to promote plant development as well by the suppression of pathogens [12] and involve in soil aggregation [13]. However, the capacity of indigenous microorganisms is a key point to soil restoration [14]. The term “indigenous microorganisms” refers to a group of beneficial microorganisms that are native to the area, thus those microorganisms play an important role by protecting the normal host from invasion by microorganisms with a greater potential for causing disease [15].

To the success in the special effort program of self-sufficiencies of maize, rice, and soybean termed “UPSUS PAJALE” by The Indonesian Ministry of Agriculture since 2015 [16], the adoption of this biotechnology needs special attention especially about the pathogenicity of the microorganisms applied. A diverse range of pathogens is known to cause disease on many crops which not only impede its growth but also cause loss of yield, consequently leading to starvation scenario [17, 18].

Several cellulolytic fungal species of the genera Aspergillus, Fusarium, Rhizoctonia, and Penicillium have been reported to be associated with maize, rice, and soybean diseases [19-22]. Therefore, to avoid the application of the microorganisms as biofertilizer agents, the pathogenicity test for those microorganisms is a prerequisite.

Although a lot of published reports on the role of microorganisms as biofertilizer agents are available, the information on the cellulolytic fungi and their potency as plant growth-promoting agent in Aceh are scanty. This work was aimed to characterize and test the pathogenicity of cellulolytic fungi isolated from sub-optimal dry land in Aceh on rice, maize, and soybean seeds.

2. Materials and methods

2.1. Cellulolytic fungal isolates characterization

Thirty cellulolytic fungi used in this study were obtained from the previous study. These isolates were isolated from the rhizosphere of some plants in the dry land of Ie Seuum Village, Aceh Besar. Characterization of the isolates involved cellulolytic activities on amorph and crystalline cellulose and phosphate solubilization activity.

2.1.1. Plate assay for cellulolytic activities. Based on clear zones formation on modified Mandel medium agar [22], the cellulolytic fungi were evaluated for their cellulose-hydrolyzing index values. Their activity on amorphous cellulose was assessed based on their ability to hydrolysis carboxymethyl cellulose (CMC) 1% on the medium while Avicel 1% carbon sources for crystalline cellulose. The plates were incubated for three days followed by staining with 0.1% Congo red for five minutes and destaining with washed them with 1 N NaCl solution.

Following incubation at 30 °C for five day-period, cellulase activity on the Mandel medium was recorded as the Index of Relative Enzyme Activity (IREA). The index was based on the calculation of the ratio of total diameter (colony + halo or clearing zone around colonies) and colony diameter [24]. The classification of the enzyme ratio was based on [25].

2.1.2. Plate assay for phosphate-solubilization activity. Phosphate-solubilizing fungi activity technique is based on the formation of clear halos around the colonies capable to solubilize calcium phosphate [24]. Pikovskaya medium was used to assess the phosphate-solubilizing fungal activities [27]. Their selective abilities index values were assessed based on the calculation of the ratio of total diameter (colony + halo or clearing zone around colonies) and colony diameter [26] after incubation at 30 °C for over five days. The classification of the enzyme ratio was based on Choi et al. criteria [25].

2.2. Pathogenicity test.

It is an in-vitro pathogenicity test determined by the Knop’s medium slants method of Potato Dextrose Agar (PDA) in test tubes (one seed per tube). The 23 isolates producing the activities of cellulose (amorph and crystalline) degradation and phosphate solubilization were used. The isolates for being tested were first grown on Mandel medium in petri plates and were multiplicated in 250-mL flasks
containing 50 mL of Mandel medium containing 1% w/v glucose and 0.2% w/v Tween 80 and in shake culture for 48–60 h at 28°C. The density of each isolate was 108 conidia mL⁻¹.

The pathogenicity of the isolates was tested on maize (variety Bisi 2), rice (variety Sanbei Simeulue), and soybean (variety Anjasmoro) seeds. Before being examined, every seed was sterilized in a 5% hypochlorite solution for five minutes, washed three times with sterilized distilled water, dried on soft paper and then the sterilized seeds were soaked in each inoculum culture for three minutes. The seeds were placed with the embryo facing the medium carefully. The test tubes were closed with cotton wool and then placed vertically in a slotted plastic basket and held for ten days under aseptic conditions. A three-replicate test was performed with five seeds per each replication.

2.3. Percentage of fungal infection

The percentage of fungal infection was analyzed to ascertain the most susceptibility of different seeds of different cultivars to various fungal infections. The total infection percentages of the component plating and seedlings tests were calculated by using the following equation:

\[
\text{Infection } \% = \frac{\text{No. of plants affected by a pathogen}}{\text{Total no. of plants}} \times 100
\]

3. Results and discussion

3.1. Cellulolytic fungal isolates characterization

The characteristics of the indigenous cellulolytic fungi isolates tested are presented in Table 1. Clear zones around the colonies in the medium were used as an indicator of cellulase activity [28]. The ability to hydrolysis the cellulose was tested on two cellulose types, namely amorph and crystalline cellulose. The amorph cellulose, CMC, is a water-soluble type which is easily degraded by fungi. Otherwise, the crystalline cellulose, Avicel, with a well-characterized structure and an average degree of crystallinity of 60% (measured via solid-state 13C-NMR) is a water-insoluble type that is degraded very slow [28, 29] and cannot be utilized directly by most of the microorganisms [31]. The initial degree of crystallinity of cellulose plays a major role as a rate determinant in the hydrolysis reaction [29]. Out of 30 isolates, 24 were capable of degrading both substrates, four (A 5.2, PK 1.1, PK 5.1, and S 3.1) were restricted to degrade CMC, and two (C 2.2 and C 3.1.3) were only for Avicell. These results were linear to Oetari et al [30] indicated that the amorph (CMC) was found more than the crystalline (Avicell) degraders. The microorganisms enabling degrade crystalline cellulose are important to degrade native cellulose in nature.

Based on their cellulolytic ratio index criteria [25], the isolates had a moderate to strong reaction on both cellulose types. The highest cellulolytic activity was designated by isolate K 5.2 for the amorph and isolate L 3.3. for the crystalline. These results were linear to Oetari et al [30] found members of seven genera (Anthostomella, Aspergillus, Chaetosarya, Cladosporium, Flavomyces, Penicillium, and Sarocladium) were able to use the amorph, seven genera (Anthostomella, Aspergillus, Cladosporium, Flavomyces, Penicillium, Purpureocillium, and Sarocladium) use the crystalline, and six genera (Anthostomella, Aspergillus, Cladosporium, Flavomyces, Penicillium, and Sarocladium) use both cellulose types.

The isolates exhibiting clear halo zones around the fungal growth were considered as phosphate solubilizers. Table 1 showed that most (93.33%) of the indigenous cellulolytic fungi had phosphatase activity as well. Their activity in solubilizing tricalcium phosphate (TCP) ranged from 1.11 to 2.14 and the maximum activity with a high enzyme ratio index was recorded by isolate T 3.1.1. Ceci et al [32] showed that Rhizopus stolonifer var. stolonifer, Aspergillus niger and Alternaria alternata were the best performing strains of cellulolytic fungi in terms of amounts of TCP solubilization. The phosphate solubilization also was found in the genera of Aspergillus, Fusarium, Penicillium, Talaromyces, and Trichoderma [33].
Table 1. The IREA of cellulase and phosphatase of the indigenous cellulolytic fungi from the dry land of Aceh.

| Isolates | Cellulolytic Amorph | Cellulolytic Crystalline | Phosphatase |
|----------|---------------------|-------------------------|-------------|
| A 3.1    | 1.56**              | 1.28**                  | 1.37**      |
| A 5.2    | 1.21**              | 0.00*                   | 1.25**      |
| C 2.2    | 0.00*               | 1.10**                  | 1.43**      |
| C 2.3    | 1.40**              | 1.78**                  | 1.24**      |
| C 3.1.3  | 0.00*               | 1.67**                  | 1.11**      |
| C 5.1    | 1.31**              | 1.17**                  | 1.18**      |
| K 1.2    | 1.22**              | 1.13**                  | 1.15**      |
| K 1.3    | 1.33**              | 1.13**                  | 1.27**      |
| K 2.2    | 1.55**              | 1.46**                  | 1.06**      |
| K 5.2    | 2.20***             | 1.07**                  | 1.30**      |
| L 2.2    | 1.26**              | 1.29**                  | 1.62**      |
| L 3.2    | 1.11**              | 1.09**                  | 1.23**      |
| L 3.3    | 1.16**              | 2.60***                 | 1.31**      |
| L 4.2    | 1.45**              | 1.19**                  | 1.75**      |
| L 4.3    | 1.20**              | 1.17**                  | 1.41**      |
| L 5.1    | 1.56**              | 1.22**                  | 1.67**      |
| L 5.2    | 1.32**              | 1.17**                  | 1.72**      |
| PK 1.1   | 1.44**              | 0.00*                   | 0.00*       |
| PK 5.1   | 1.30**              | 0.00*                   | 1.56**      |
| PM 2.1   | 1.17**              | 1.15**                  | 0.00*       |
| PM 4.1   | 1.20**              | 1.05**                  | 1.18**      |
| S 3.1    | 1.13**              | 0.00*                   | 1.36**      |
| T 1.2    | 1.05**              | 1.09**                  | 1.59**      |
| T 2.1.1  | 1.10**              | 1.08**                  | 1.75**      |
| T 2.2    | 1.38**              | 1.04**                  | 1.78**      |
| T 3.1.1  | 1.51**              | 1.10**                  | 2.14**      |
| T 3.1.2  | 1.10**              | 1.10**                  | 1.29**      |
| T 4.1    | 1.15**              | 1.22**                  | 1.42**      |
| T 4.2    | 1.29**              | 1.21**                  | 1.33**      |
| TR 2.1   | 1.21**              | 1.10**                  | 1.60**      |

*= no reaction; ** = moderate; *** = high

3.2. Pathogenicity test.

The pathogenicity test was conducted on 23 indigenous cellulolytic fungi owning plant growth-promoting characteristics (Table 1). The results (Table 2) showed that seven isolates (A 3.1, C 2.3, K 1.3, K 5.2, L 5.1, L 5.2, and TR 2.1) were non-pathogenic on maize, eight isolates (A 3.1, C 2.3, K 1.3, K 5.2, L 5.1, L 5.2, T 3.1.1, and TR 2.1) were on rice and five isolates (A3.1, C 2.3, K 1.3, L 5.2 and TR 2.1) were on soybean. Overall, five isolates were non-pathogenic on maize, rice, and soybean, therefore these isolates were required to be more investigated for their impact as biofertilizer agents.
Many microorganisms have a significant detrimental impact on crops. Of the plant-pathogenic microorganisms, fungi are an enormous threat to plant health. *Fusarium* attack on many important crops like maize, rice, and soybean has been reported [17]. *Rhizoctonia solani* is a major fungal pathogen for rice and soybean. Other fungal pathogens harming seeds and seedling’s life of maize, rice, and/or soybean are *Aspergillus flavus*, *A. niger*, *Curvularia* spp., *Pythium ultimum*, *Rhizopus* spp. and *Xanthomonas* sp. [17]. Therefore, those microorganisms are not suitable as biofertilizer agents even though they have plant growth characteristics.

4. Conclusions
The indigenous fungi from dry land Aceh had cellulolytic and phosphatase characteristics. The activities of indigenous cellulolytic fungi to degrade cellulose were depended on the type of the substrate. The number of amorph cellulose degraders are higher (93.33%) than the crystalline (86.67%) and only 80% of the fungi had activities on both cellulose types. In addition to the cellulolytic, 93.33% of the fungi also had phosphatase activity.

The pathogenicity of the indigenous cellulolytic fungi varied among the tested crops. There were five non-pathogenic fungi on maize, rice, and soybean, and therefore, they were potential to be technologically advanced as biofertilizers in the cultivation of maize, rice, and/or soybean.
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