

The effect of substrate composition on the activity of amylase and cellulase by *Trichoderma harzianum* strains under solid state fermentation

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Abstract

Trichoderma harzianum is a filamentous fungus that has been known to have biocontrol and plant growth-promoting ability. However, the propagation of this fungus particularly through solid state fermentation (SSF) and characterization of its enzyme activity as one the indicator of quality of fermentation process are still needed to be explored further. Rice grain and its derivative products have economically as well as nutrient composition features beneficial as substrates of fungal propagation through SSF. Therefore, the aim of this study was to investigate the effect of white rice, rice bran, and combination of white rice and rice bran on the activity of amylase and cellulase by *Trichoderma harzianum* strains under SSF. Two strains of the fungus, InaCC F116 and InaCC F89, as well as their consortium were employed as fungal inoculants. After closed fermentation in dark chamber at $30 \pm 1^\circ\text{C}$ for 7 days, the activity of amylolytic as well as cellulolytic enzyme was assayed. The result showed that the presence of rice bran as a substrate increased the activity of crude amylase and crude cellulase. In all substrates, the strain F116 has low activity of both enzymes. The fungal consortium improves the activity of crude enzymes in all substrates. Therefore, the amylase and cellulase activity by *T. harzianum* in SSF condition were strain- as well as substrate-dependent.

Keywords: amylase, cellulase, solid state fermentation, *Trichoderma*

Introduction

The utilization of *Trichoderma* genera for increasing the growth and yield production of crops and overcoming the threat of plant pathogens has been broadly investigated. *Trichoderma* spp. are filamentous fungi that presence in high abundance in soil as well plant tissues. *Trichoderma harzianum*, one of the well-known species of the genera, has been reported to have plant growth promoting fungus features such as solubilizing insoluble phosphate (Altomare *et al.* 1999), formatting plant growth hormone of indole-3-acetic acid (Napitupulu & Kanti 2019), and production of siderophore (Vinale *et al.* 2013). Moreover, the ability of *T. harzianum* as biocontrol agent has been studied for the control of various

crop diseases such as Fusarium wilt in banana (Napitupulu *et al.* 2019), rice brown spot caused by *Bipolaris oryzae* (Abdel-Fattah *et al.* 2007), and soybean charcoal rot (Khaledi & Taheri 2016). Therefore, the optimization of *T. harzianum* propagation is promising for plant growth and biocontrol agent.

Solid state fermentation (SSF) is an alternative of liquid or submerged fermentation (SmF) to produce fungal biomass, enzymes, or secondary metabolites. SSF occurs in the lack of water, thus resembles the native habitat of the fungi particularly the filamentous such as *Trichoderma harzianum* in soils (Soccol *et al.* 2017). For production of mycelial molds application in field crops, SSF is more suitable than SmF due to its viability for a stock long time. Traditionally, SSF is a suitable fermentation for investigation of enzyme activity and also has been used for enzyme production at industrial scale (Nadda 2001). Moreover, primary as well as secondary metabolite production of fungi have been commonly determined through solid state fermentation (Barrios-González & Tarragó-Castellanos 2017).

Selection of substrates is one of crucial factors for fungal solid-state fermentation. Considerations for selection of the solid substrates on SSF for production and bioassay purposes include nutrition composition, availability and price of the substrate, and the presence of inhibitors. Furthermore, enzyme activity is as one the indicator of quality of fermentation process. Rice grain and its processed products, white rice and rice bran, that rich in carbohydrate are common SSF substrates for various microorganisms. Their high abundance and relatively inexpensive cost make them versatile for various SSF purposes using wide range of microorganisms, but mostly for food-related fermentation and biofertilizer production. Therefore, the aim of this study was to evaluate the production of *Trichoderma harzianum* biomass, its activity of amylase as well as cellulase through solid state fermentation by using rice grain processed products as substrates. In this investigation, we assay two strains of *T. harzianum* collection of Indonesia Culture Collection (InaCC).

Materials and methods

Microorganisms

Two *Trichoderma harzianum* strains, InaCC F89 and InaCC F116 were obtained from Indonesian Culture Collection (InaCC). The fungi were isolated from leaf litters in Mount Salak, Sukabumi regency, Indonesia. All strains were maintained on petri dish containing potato infusion (200 g/L), dextrose (20 g/L) and agar (15 g/L), incubated at 30°C in dark condition prior to use in the experimental procedures.

Phylogenetic tree analyses

The raw ITS rDNA sequences of the InaCC strains along with selected downloaded *Trichoderma* sp. ITS rDNA on National Centre for Biotechnology Information (NCBI) were multiple aligned using ClustalW. A phylogenetic tree construction based on genetic distance with a neighbor-joining (NJ) statistical method, maximum-composite likelihood algorithm, and complete deletion gaps. The strength of phylogenetic tree was tested using bootstrap method with 1000 replications.

Preparation of fungal suspension

The mycelial of one-week fungi in solid cultures were then harvested by pouring 30 mL of sterile NaCl 0.9% (w/v) in the plate and swirl to detach the conidia. The suspension formed was then collected in a sterile bottle. The conidia were counted with a hemacytometer under the microscope. If necessary, the suspension was diluted with sterile NaCl 0.9% (w/v) to obtained 10^7 conidia/mL.

Solid state fermentation condition

Three different solid substrates, white rice, rice bran, and white rice:rice bran (1:1), obtained from traditional market in Cibinong Indonesia, were prepared for solid state fermentation. As much 200 g of the solid substrates in closed plastic bags were wet sterilized in autoclave at 121°C for 15 minutes. All substrates were supplemented with dextrose 5% (w/w). After cooled, as much of 20%, 40%, and 50% (v/w) of sterilized water then added to moisten white rice, rice bran, and white rice:rice bran (1:1), respectively. Different inoculants, namely *T. harzianum* InaCC F89, *T. harzianum* InaCC F116, and consortium of InaCC F89 and F116 (1:1) were inoculated to each group of solid substrates. As much of 5 mL of fungal suspension (10^7 conidia/mL) was added to sterile solid substrates and mixed well. A total of these 9 treatments of SSF was then incubated in dark chamber at $30 \pm 1^\circ\text{C}$ for 7 days.

Enzyme activity assay

Two enzymes, amylase and cellulase, was assayed on fermented matters. The first step was extraction of crude enzymes. For this, 1 gram of fermented matter was vigorously shaken with 10 mL of sterile tween 80 0.1% (w/w) at 180 rpm for 1 hour. The liquid part was centrifuged at 5200 rpm for 20 minutes at 4°C and the supernatant was used for enzyme activity assay.

For amylase assay, 0.5 ml of extracted crude enzyme was mixed with 0.5 ml of soluble starch 1% (w/v) in 0.1 M phosphate buffer, pH 6.0, for 30 min at 40 °C. The reaction was stopped and reducing sugar was determined with dinitro salicylic acid according to the method of Saqib & Whitney (2011). An enzyme unit (U/g) is defined as the amount of enzyme releasing one μmol of equivalent glucose from the substrate per minute at 40°C per gram of dry fermented matters.

For cellulase assay, 0.5 ml of extracted crude enzyme was mixed with 0.5 ml of carboxymethyl cellulose natrium 1% (w/v) in 0.1 M phosphate buffer, pH 6.0, for 30 min at 40 °C. The reaction was stopped and reducing sugar was determined with dinitro salicylic acid according to the method of Saqib & Whitney (2011). An enzyme unit (U/g) is defined as the amount of enzyme releasing one μmol of equivalent glucose from the substrate per minute at 40°C per gram of dry fermented matters.

Results

Trichoderma harziaum is a fast-growing filamentous fungus that relatively easy grown in various substrates. After 7 days (Fig. 1), the fermented matters were analysed for estimation of amylase as well as cellulase activities.

The enzymatic abilities of *Trichoderma harzianum* through solid state fermentation were measured. Two enzymes, amylase (Fig. 2) and cellulase (Fig. 3) of *T. harzianum* were assayed. It is obvious that both amylolytic and cellulolytic activity of *T. harzianum* was strain- as well as substrate-dependent.

Generally, the profile pattern of amylase and cellulase activity of each inoculant fungus is similar. In the observation of substrate used, white rice as fermentation substrate gave lower enzymatic activity than rice bran or combination of white rice and rice bran for all inoculants. However, the difference of enzymatic activity was obtained when rice bran and combination of white rice and rice bran were used as substrate. In those two substrates, *Trichoderma harzianum* F116 had lower amylase and cellulase activity compared to *T. harzianum* F89. Consortium of those strains in rice bran and white rice:rice bran (1:1) substrate have slightly similar enzymatic activity to single strain F89.

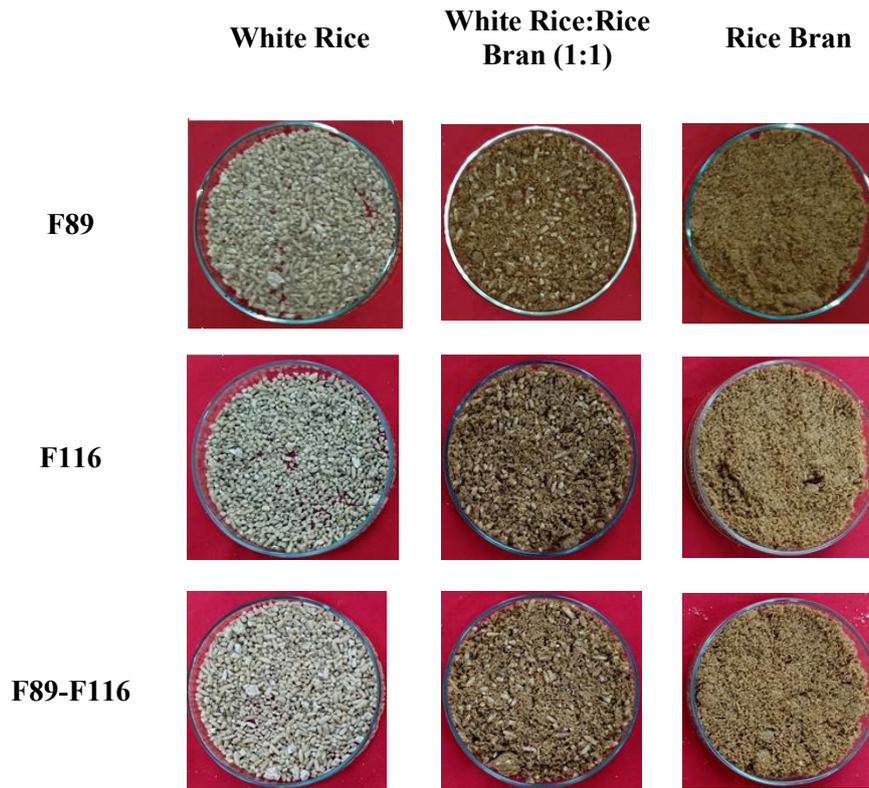


Figure 1. Visual observations of color substrates after 7 days of solid state fermentation of rice grain processed products by *Trichoderma harzianum* strains.

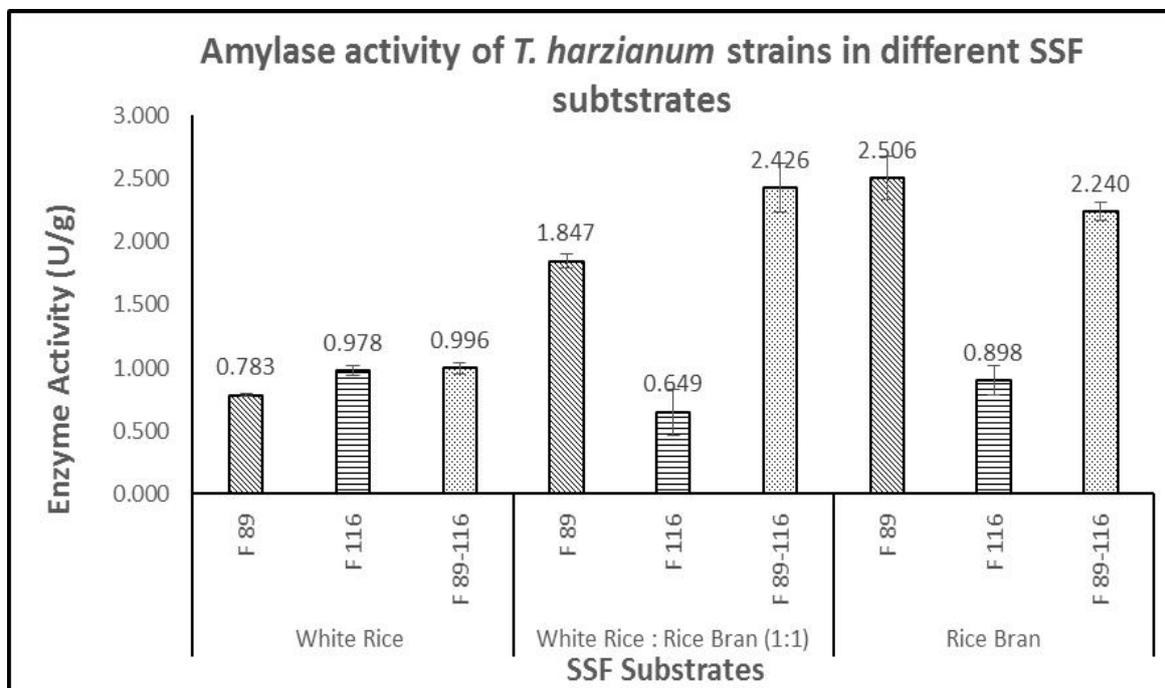


Figure 2. Comparison of amylase activity of *Trichoderma harzianum* as affected by variation of inoculant type and rice processed product substrates. The enzyme activity was determined at 7 days of SSF incubation period at 30 °C.

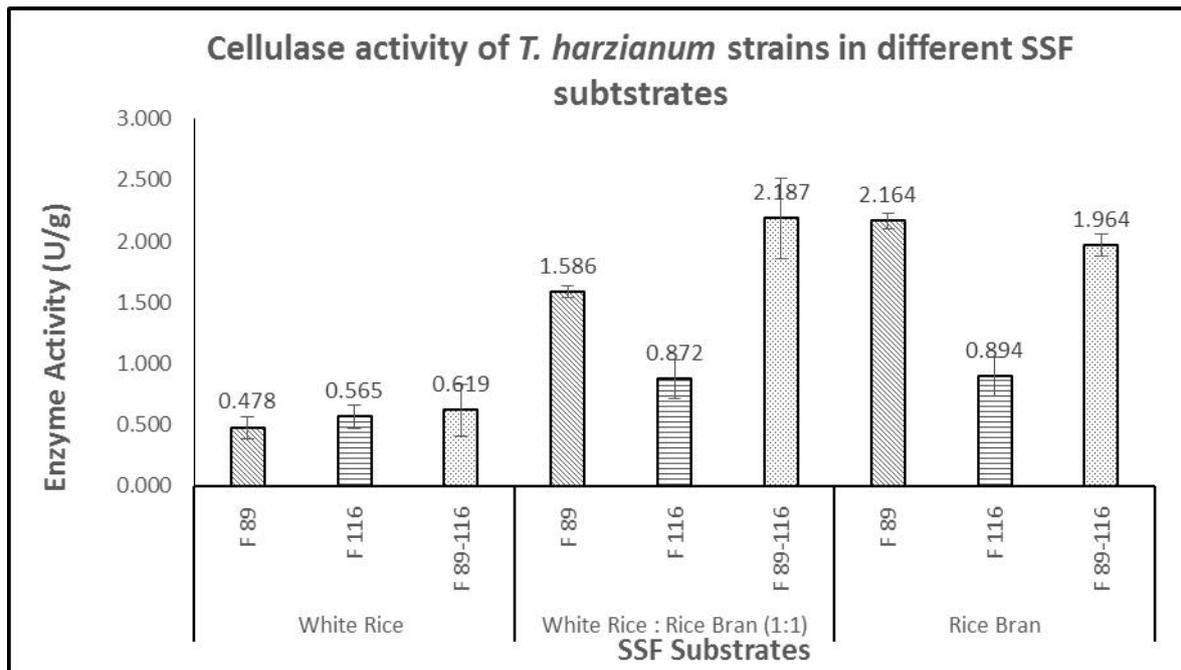


Figure 3. Comparison of amylase activity of *Trichoderma harzianum* as affected by variation of inoculant type and rice processed product substrates. The enzyme activity was determined at 7 days of SSF incubation period at 30 °C.

The relationship among strain InaCC F89 and InaCC F116 is shown in Figure 4. ITS rDNA sequences of all strains are obtained from Indonesian Culture Collection (InaCC) database. The phylogenetic analyses from ITS sequence data based on NJ method showed that all the strains have the same clade with *Trichoderma harzianum* species group.

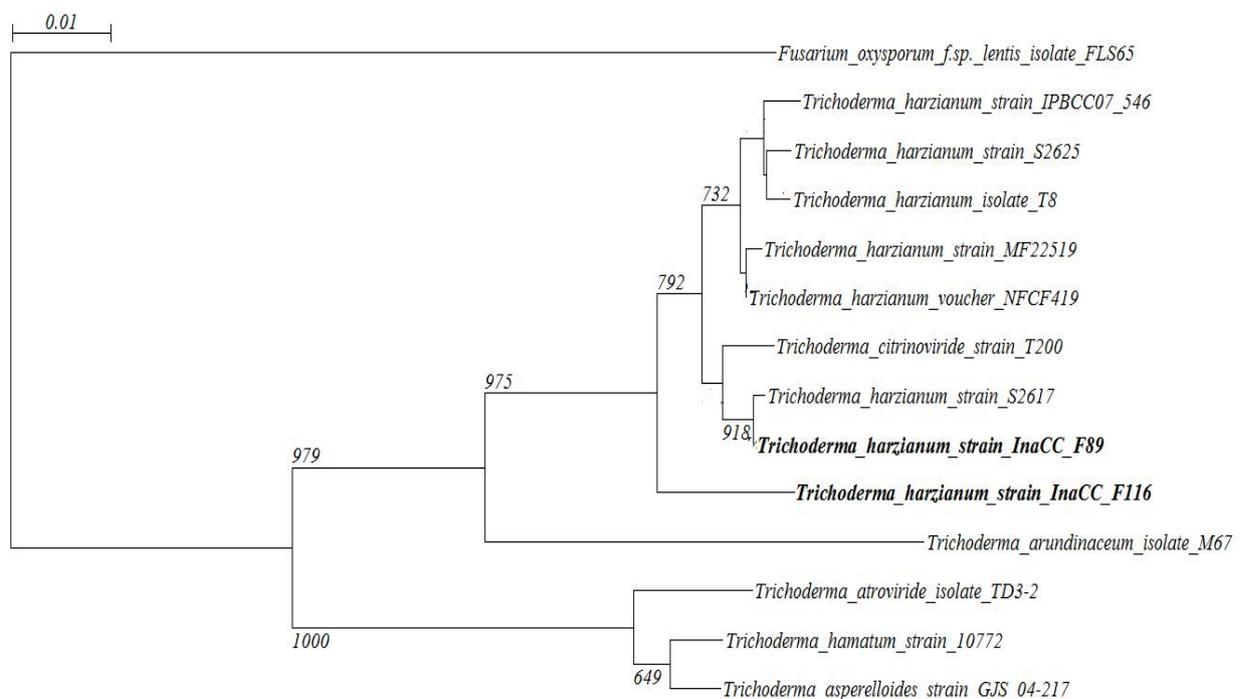


Figure 4. Phylogenetic tree of InaCC F89 and InaCC F116 strain of *Trichoderma harzianum* based on ITS rDNA sequence using neighbor-joining method and *Fusarium oxysporum* f. sp. *lentis* as outgroup.

Discussion

Trichoderma harzianum has versatile ability to grow in many substrates as well as various environmental conditions, while its physiological features were remained active. Although it has been found abundantly in soils and leaf litters, this fungus was also present as endophytic in plant tissues. Moreover, *Trichoderma* fungi also have capability to live symbiotically with wide range of plant hosts (Vargas *et al.* 2009). *T. harzianum* is also known to have a high capability to grow competitively and used nutrients as well as space effectively. The nutrient and other environmental conditions do influence the physiological characters of *T. harzianum*.

Our results indicate that the presence of substrate differences affects the enzyme activity of both amylase and cellulase. In this study, we examined rice grain derivatives products, namely white rice, rice bran, and combination of white rice and rice bran (1:1). These substrates have been applied widely for propagation of filamentous fungi together with enzyme production through a solid-state fermentation method. A proximate analysis study showed that available carbohydrate content of white rice is around 2.3 times higher than rice bran, but the crude nitrogen content of white rice is approximately 1.7 times lower than rice bran (Eggum *et al.* 1982). In contrast to the high content of carbohydrate in white rice, the activity of both amylase and cellulase were found lower than in rice bran and combination of white rice:rice bran (1:1) as fermentation substrate. As comparison, a study of alpha amylase activity of *Bacillus licheniformis* showed that increasing starch content resulting the increasing of the enzyme activity (Divakaran, Chandran, & Pratap Chandran 2011). In other works, effect of starch concentration toward alpha-amylase activity of *Bacillus cereus* showed the maximum activity at a particular starch level concentration, but then ceases above that level (Al-ZaZae *et al.* 2011). This point out that there is an optimum concentration of starch in the substrate that correlate the activity of amylase of the microorganism in such fermentation condition. Moreover, in the mean of available nutrition, the macronutrients and micronutrients status in the fermentation substrate influence the activity of amylase. A study on the amylase of *Aspergillus niger* showed that Ca^{2+} enhanced the enzymatic activity (Varalakshmi *et al.* 2009). Furthermore, the amount of phosphorus, calcium, and magnesium in rice bran are higher in the range of 15 times, 3 times, and 21 times, respectively, than in the white rice (Pedersen & Eggum 1983). Calcium itself is essential metal for stability and enzymatic activity of amylase (Bush *et al.* 1989). Similarly, cellulose activity in white rice is lower than in rice bran and combination of rice bran and white rice (1:1). Cellulolytic activity of the fungal strains was also dependent on the substrate composition. A study showed that cellulose activity of *Aspergillus niger* was higher in rice bran compare to risk husk as substrate fermentation (Mrudula & Murugammal 2011).

The amylolytic and cellulolytic activity of *Trichoderma harzianum* show variation (Fig. 2 and Fig. 3), even though both isolates are grouped in one species (Fig. 4). While the activity of both enzymes remains similar in white rice for all strains, the F116 strain showed much lower enzyme activities when the fermentation substrate was rice bran, as well as combination of rice bran and white rice compared to the F89 strain despite both isolates, have close relation based on phylogenetic tree analysis. This strain-dependent tendency of enzymatic activity of both amylase as well as cellulase was also reported in numerous microorganisms by previous other studies. As comparison, a qualitative screening of alpha-amylase production of *Aspergillus niger* isolated from soil showed diversification among the strains (Khan & Yadav 2011). Similarly, cellulose activity by six *Trichoderma harzianum* isolates showed strain variation during in vitro antagonism with *Aspergillus niger*, the causal agent of collar ROT of Peanut (Gajera & Vakharia 2012).

Consortium fungal strains, compared to single strain, did improve the amylase and cellulase activity of *Trichoderma harzianum* through solid state fermentation in mixture

substrate of white rice:rice bran (1:1). Various previous studies have been done to investigate the strain consortium toward enzymatic activity of microbes, particularly in the correlation with fermentation substrate variation. For instance, the activity level of extracellular serine proteinase of two different *Lactococcus lactis* strains was reported to be regulated by the peptide content of the medium (Meijer *et al.* 1996). In field application, the use of microbial consortium, particularly for plant growth-promoting microbes has been widely applied to enhance the growth of plants as well as the biocontrol ability against pathogens (Bradáčová *et al.* 2019).

Conclusion

The amylase and cellulase activity by *Trichoderma harzianum* through solid state fermentation are substrate- as well as strain-dependent. A condition for optimum amylase and cellulase activity was obtained by the utilized of consortium fungal strains in white rice and rice bran (1:1) as fermentation substrate.

Conflict of interest

The authors state no conflict of interest from this manuscript.

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Author contributions

All authors have reviewed the final version of the manuscript and approved it for publication. TPN and IM designed the study; TPN and NRS performed research and collected the data; TPN and NRS analysed the data; TPN, AK, and IM wrote and reviewed the paper. TPN is the main contributor of this manuscript.

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