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Abstract. Chitinase and β1-3, glucanase produced by living microbes play important role of mycoparasitism and one of known mycoparasites is *Trichoderma* species. The aim of study was to examine *Trichoderma* isolates obtained from South Sulawesi. The isolates were cultured into potato dextrose agar and then subcultured with specific media for understanding chitinase and β1-3, glucanase activities. The result shown that the diversity of enzyme activities was obtained from isolates.

1. Introduction

Cocoa development and biological control agents is not apart. Since cocoa is a better host of insect pests and pathogens [1] causing significant production losses and the death of tree [2, 3], the need for intervention for pest and disease control is necessary [4]. One of intervention methods is the use of biological control agents [5-6] and of many, *Trichoderma* species is a highly likely promising fungus. A successfulness of *Trichoderma* species in protecting health agricultural and forest ecosystems is due mainly to the role of mycoparasitism [6-9] which enzyme production e.g. chitinase and β1-3, glucanase plays important role. Chitinase and β1-3 glucanase help to lyse functional and structural cell wall of fungal pathogen during parasitism. It is known that fungal cell wall structures of crop pathogens mainly consist of chitin and glucan [10]. The effectiveness of mycoparasitism depends on amount of chitinase and glucanase resulted from *Trichoderma* species and therefore the study will examine two dominant enzyme activities generated by isolates of *Trichoderma* species obtained from South Sulawesi.

2. Methods

2.1. Culture of Trichoderma isolates

Trichoderma isolates obtained from Sulawesi were grown into solid medium, potato dextrose agar (PDA). Two volumes of PDA were 10 mL and 20 mL. Linier hyphae were counted from the central growth to the edge of petri-dish every day.

2.2. Subculture media for testing Trichoderma isolates to produce chitinase, β-1,3 glucanase and cutinise

Trichoderma isolates of interest were grown into malt extract medium (ME) with rich chitin colloid (testing chitinase), glucan flour (testing glucanase) and Yeast extract (YE) for testing cutinase. To obtain 1 Litre medium ME, reagent composition with pH 7 consisted of 1 g K_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, and for source of chitinase added 5 g chitin colloid and added 5 g glucan flour (source of glucanase) and 2 g cutin (source of cutinase). The mixture was homogenised while boiled to 100 ^oC and it subsequently was autoclaved at 121 ^oC for 15 minutes before moved to a new sterilized erlenmeyer for initial inoculum and mass production media.

2.3. Making N-acetylglucosamine solution

1 g N-acetylglucosamine was dissolved into 1000 mL water. The solution was diluted to have various concentration such as 0 ppm, 160 ppm, 200 ppm, 240 ppm, 280 ppm, 400 ppm, 440 ppm, 480 ppm and 520 ppm. Each concentration was load 1 mL to tube mixed with 3 mL dinitro-salicylate acid (DNSA).

2.4. Determining rough enzyme and its activity

Rough chitinase, glucanase and cutinase were determined in different method. Once the enzyme was collected, it was rapidly stored at -20 ^oC for several days. To measure every rough enzyme activity, chitinase activity was measured by amount of chitin colloid as carbon source [11], glucanase activity was determined by amount of glucose reductor released from laminarin [12] and glucose determination following Nelson method [13] and cutinase activity was counted by amount of p-nitrophenyl-butyrate released [14]. To examine each of enzyme activities, detail formulation was underlined in table 1.

To measure chitinase activities, the method consisted of phosphate buffer solution, DNSA solution, chitin colloid solution and acetylglucosamine solution as standard. Stock solution A and solution B were made to prepare phosphate buffer. Stock solution A consisted of 27.8 g; 0.2 M NaH_2PO_4 and stock solution B included 71.7g; 0.2 M NaH_2PO_4. $12H_2O$ and every stock solution was diluted with 1,000 mL distilled water. 81.5 mL stock solution A was mixed with 18.5 mL stock solution B pH 6.2 and final solution was diluted to reach 200 mL volume. In DNSA solution, 1 g DNSA was diluted into 20 mL 2 M NaOH and then mixed with 30 g Rochelle salt while homogenized. The stock solution was diluted to reach 500 mL volume. In chitin colloid, 100 mg chitin colloid was dissolved 100 mL purified water. 1 mL stock solution was pipetted and loaded into each concentration and subsequently mixed with 3 mL DNSA stock solution. The final solution was heated at 100 ^oC for 10 minutes and then reaction tube was placed in the ice box to cooling temperature down. Once its temperature dropped, its color absorbance at λ575 nm was measured with spectrophotometer UV/VIS. To make standard solution of N-acetylglucosamine, 1000 mg N-acetylglucosamine was dissolved with 1000 mL distilled water before pipetted to load into reaction tube with concentration of 0 ppm, 160 ppm, 200 ppm, 240 ppm, 280 ppm, 400 ppm, 440 ppm, 480 ppm and 550 ppm. Every 1 mL final solution was loaded into reaction tube with 3 mL DNSA. The reaction tube was heated at 100^oC for 5 minutes and then the tube was placed into ice box to cooling temperature down. Color absorbance at λ575 nm was measured with UV/VIS spectrophotometer. Chitin colloid is carbon source to allow chitinase production by *Trichoderma* isolates. 1.0 mL rough chitinase was loaded into reaction tube with 1.0 mL 1% (g/v) chitin colloid and phosphate buffer pH 6.2 the stock solution was heated at 37^oC for 15 minutes and it was subsequently added 4 mL DNSA stock solution before re-heated in the boiled water for 5 minutes. The final solution was measured its color absorbance at λ540 nm. To measure glucanase and chitinase activities, changing the determiner substrates from chitin colloid to laminar in which determines β1-3, glucanase.

2.5. Testing enzyme production obtained from Trichoderma isolates

Testing enzyme production by *Trichoderma* isolates were based on amount of enzyme activities released on the media. To measure amount of chitinase activity, the activity was measured once N-acetylglucosamine (NAG) performed during chitin colloid hydrolysis, glucanase activity was measured amount of glucose performed from hydrolysis of laminarin. For more detail in table 1.

Table 1. Determination of enzyme activities obtained from *Trichoderma* isolates

Objective	Formulation of enzyme activities	Marker	Source
Chitinase	$Chitin\ activity = \frac{NAG\ concentration}{mass\ molecul\ of\ NAG}\ x\ time\ incubation$	NAG	[1]
Glucanase	$\beta,1-3,glucanase\ activity = \frac{glucose\ concentration}{mass\ molecul\ of\ glucose}\ x\ time\ incubation$	Glucose	[2,3]

Note: 1 unit of enzyme activity = 1 µmol of NAG (chitinase) or glucose (glucanase) per minute

3. Results and discussion

3.1. Producing chitinase and β1-3, glucanase by Trichoderma isolates

Measuring chitinase activity, amount of NAG was examined which from culture with colloid chitin as carbon source which results to NAG. Chitin is a large polysaccharide structure synthesized from N-acetyl-D-glucosamine unit. For β1-3, glucanase, glucan is defined as polysaccharides derivative from glucose monomers which are linked by glycosidic bonds. β1-3 laminarin is one of Glucose-based polysaccharides.

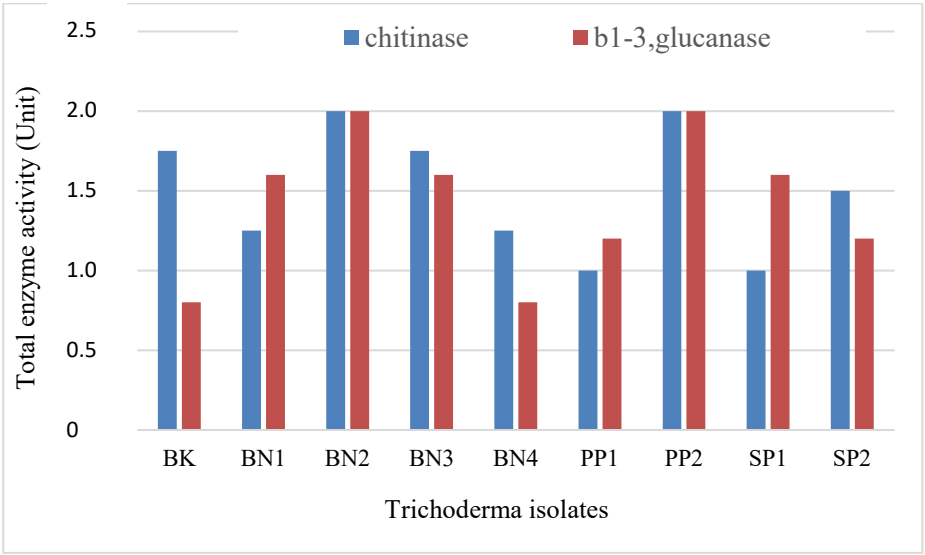

Figure 1. Diversity of enzyme activities was released by *Trichoderma* isolates.

Figure 1 depicts that all isolates of interest were able to produce important enzymes for using biocontrol services. Two isolates such as BN2 and PP2 were consistently to have much greater enzyme activities than other isolates. Overall, the enzyme activities varied depending on isolates where obtained in the nature and enzyme diversity greatly associates with genetic variation of isolates [9, 15]. In the paper, genetic variation was not discussed. In terms of biocontrol agents, two enzyme activities were focused i.e. chitinase activity and β1-3, glucanase. Those enzymes are important role to lyse the structure and function of pathogen which dominantly consists of chitin and β1-3, glucan.

3.2. General discussion

One of important roles behind successful fungal *Trichoderma* species against phytopathogenic fungi in nature is due to producing important enzymes to lyse pathogen cell wall of hyphae mainly consisting of chitin and β1-3, glucan polymers. As enzyme production, the potential of biocontrol agent against fungal plant diseases is necessary. Of many enzymes released, chitinase and β1-3, glucanase are such a 'gun' produced to combat the pathogens. There is a positive correlation between generating enzyme activity and the role of parasitism. Once chitinase or glucanase activity is considerably generated, the fungal has a robust capacity to damage cell wall of pathogen [10]. The hyphae of *Trichoderma* attach to pathogen and coils its cell wall before hydrolyses its structure and function until kills it. In the cell wall of crop pathogen, because the wall structures mainly consist of chitin and glucan polymers producing chitinase and β1-3, glucanase by *Trichoderma* species is critical to break down the polymers. The way of cell wall lysis is known as mycoparasite [15-16]. The need for *Trichoderma* isolates which produces consistantly three ezymes of interest was predominantly and the result shown that in the figure 1, only isolates of BN2 and PP2 perfomed to have relatively stable in producing three main enzymes. In this study, amount of mass mycelium obtained was not associated with enzyme production. Study of Budiarti [10] suggests to obtain the lower mass mycelium of *T. reesei* but the higher glucanase is produced. Although *Trichoderma* species is an opportunistic fungus in many occasions, it is avirulent to host-plant [15].

4. Conclusion

Two main enzymes of chitinase and β1-3, glucanase Produced by *Trichoderma* isolates addresing with mycoparasistism for biological control varied. The only two isolates (BN2 and PP2) shown the highest enzyme activities and had much greater consistent in producting amount of both enzymes.

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