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Original research article

Antibacterial Activity of Extracellular Protease Isolated From an Algicolous Fungus *Xylaria psidii* KT30 Against Gram-Positive Bacteria

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A B S T R A C T

Infectious diseases became more serious problem for public health in recent years. Although existing antibacterial drugs have been relatively effective, they do not rule out the emergence of resistance to the drug. Therefore, the intensive exploration of new bioactive compounds from natural, especially peptide compounds began in recent decades in order-handling infection. This study aimed to isolate, purify and test the potential application of *Xylaria psidii* KT30 extracellular protease as antibacterial agent against Gram-positive bacteria. *X. psidii* KT30, a marine fungus isolated from red seaweed *Kappaphycus alvarezii* showed antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus*. Antibacterial compounds of this fungus were predicted as a group of proteases. Extracellular protease exhibited an optimum activity when potato dextrose broth was used as cultivation medium. Furthermore, the highest activity of these proteases was found on fungal extract after day 15 of cultivation with value of 2.33 ± 0.19 U/mL. The partial purification of proteases using G-75 column chromatography resulted in 2 groups of fractions and showed protease activity based on zymogram assay. The extracellular proteases obtained from those fractions have 3 patterns of molecular mass based on sodium dodecyl sulfate–polyacrylamide gel electrophoresis which are 56.62, 89.12, 162.18 kDa.

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1. Introduction

Diseases caused by bacterial infections become a serious problem in public health in recent years. Antibiotic resistance has become a global pandemic and one of the largest worldwide anxieties. Resistance to antibiotics occurs because of the changing nature of bacteria that no longer can be turned off or killed. Efficacy of the drug be weakened or even lost. Bacteria that are resistant to antibiotics will not be killed by antibiotics, then multiply and spread so that they become more dangerous. Currently antimicrobial resistance among bacteria, viruses, parasites, and other disease-causing organisms is a serious threat to infectious disease. Over the past few years, there was an increase in incidence of infectious diseases caused by bacteria along with increasing human population (Nwinyi *et al.* 2009). Microorganisms such as Gram-positive and Gram-negative bacteria can cause infections in humans. Although antibacterial drug has been quite effective, it does not rule out the possibility arises of resistance to the drug. Therefore, new discoveries of antibacterial drugs are indispensable (Chopra 2007).

Exploration of new bioactive compounds from nature, especially peptide compounds, has been conducted intensively in the last decade. *Staphylococcus aureus* is a Gram-positive coccal bacterium frequently found in the human respiratory tract and on the skin. Although *S. aureus* is not always pathogenic it is a causative agent for skin infections, respiratory disease, and food poisoning. The wide use of antibiotics in the treatment of bacterial infections has led to the emergence and spread of resistant strains. *S. aureus* has successfully evolved numerous strategies for resisting the action to practically all antibiotics (Kuroda *et al.* 2001) Resistance to methicillin is now widely described in the community-acquired methicillin-resistant *S. aureus*, thus the development of new drugs or alternative therapies is urgently necessary. *Bacillus subtilis* is only known to cause disease in severely immunocompromised patients, and can conversely be used as a probiotic in healthy individuals, rarely causing food poisoning (Nakano & Zuber 1988). One of the main sources of microorganism-derived metabolites is endophytic

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fungi (Desale & Bodhankar 2013). These fungal metabolites are usually divided into 2 main groups called primary metabolites and secondary metabolites.

The biologically active proteins or peptides, which are believed to be mostly grouped into primary metabolites have attracted researchers' attention because of their potential to be developed as antibacterial compounds. Marine microorganisms, especially fungal species have recently attracted a lot of attention among researchers as a source of bioactive secondary metabolites. Until recently, marine fungi have become an important subject on research field especially in natural product isolations for diverse applications and elucidation of many new compounds with unique biological activities with significant impacts on human life quality (Holler *et al.* 2000). *Xylaria psidii* KT30 is one of the marine fungi which had been proven to show biological activity against bacteria and fungi. This marine-derived endophytic fungus potential exhibited cytotoxic activity against cancer cells (Tarman *et al.* 2011). Other study of Munandar *et al.* (2014) had convinced the importance of *X. psidii* KT30 as source of protein-based bioactive compounds applied as antibacterial agents. Moreover, Inthe *et al.* (2014) revealed that moderate cytotoxicity of *X. psidii* KT30 against HeLa cells due to protein extracted from 9th day of *X. psidii* KT30 cultural medium with LC_{50} was 104.95 ppm and IC_{50} was 69.9 ppm. Furthermore, screening test of *X. psidii* KT30 protein extract using zymogram method showed that this extract contained proteases (Budiarto *et al.* 2015). Until recently, there is no study related to the isolation and application of *X. psidii* KT30–derived protease as antibacterial agent, as well as the optimization of protease production and its protease activity against Gram-positive bacteria. The purpose of this study was to isolate and purify the extracellular protease and to examine the antibacterial activity of this protease against Gram-positive bacteria.

2. Materials and Methods

2.1. Cultivation and determination of *X. psidii* KT30 dry biomass

Marine endophytic fungus *X. psidii* KT30, was isolated from the red seaweed *Kappaphycus alvarezii* BRKA-1 from the Labuange-Village, Barru, South Sulawesi. One loop of the isolate was cultured in Erlenmeyer flask containing 25 mL of potato dextrose broth as preculture media then incubated at room temperature for 3 days on orbital shaker. After 3 days, 3 mL media containing *X. psidii* KT30 mycelium was transferred into a 300 mL PDB. Culture media was incubated at room temperature in accordance with the best results of optimization using a shaker (Tarman *et al.* 2011). After reaching the optimum incubation period, mycelium of the fungus was filtered using filter paper, then dried at 105°C for 7 hours to calculate dry biomass to create growth curve. Final pH after cultivation period was observed as well.

2.2. Optimization of protease *X. psidii* KT30 production

Culture media was optimized by distinguishing 2 types of culture media, PDB and Hagem. Media optimization was performed on each media with 3 replications and protease activity was observed at 0, 3, 6, 9, 12, 15, 18, 21 old day culture. Before protease activity test, media and mycelium were separated by filtration using filter paper. The filtrates then neutralized to pH 7.4. Protein was precipitated using ammonium sulfate according to method described by Scopes (1987) with 90% saturation (Munandar 2014). The precipitates were stored for 12–16 hours at 4°C. Precipitated media was then centrifuged ×18,000 *g* for 30 minutes at 4°C. Pellet obtained from centrifugation was dissolved in 1 mL of buffer Tris HCl 25 mM pH 7.4 and tested for the protease activity.

2.3. Protease activity testing

Reagent A contains 0.2 g of Na_2CO_3 in 10 mL of 1 N NaOH, and reagent B contains 0:05 $CuSO_4 \cdot 5H_2O$ g in 10 mL of $KNaC_4H_4O_6 \cdot 4H_2O$ 1%. Reagent C, freshly prepared, was made by mixing reagents A and B with the ratio 50:1. Reagent D was prepared by mixing folin ciocalteu with sterile distilled water (1:1) in volume. Protease activity assay was performed using the method described by Enyard (2008) with modification. The assay was done using 96 well plate. The sample wells contained 6 μL protein pellets, 6 μL phosphate buffer 10 mM and 6 μL 1% casein, while for the blank the protein pellet was replaced with aquabidest. In blank samples, wells contained 6 μL aquabidest, 6 μL phosphate buffer and 6 μL 1% casein. Tyrosine solution was used as standard ranging from 0, 25, 50, 100, 125, 250, 500 and 1000 μM. Subsequently the plate was incubated at 37°C for 30 minutes then the reaction was stopped by adding 12 μL trichloroacetic acid solution into each well sequentially followed by addition of 143 μL of reagent C and 30 μL of reagent D. The tyrosine release was monitored at 620 nm and its concentration was predicted using tyrosine standard curve already prepared.

2.4. Measurement of protein concentration

Protein concentration was measured with Pierce BCA Protein Assay Kit. Total of 10 μL sample was added to 200 μL of working reagent in a 96 well plate. The mixture was then incubated at 37°C for 30 minutes. The absorbance was measured using a wavelength of 540 nm. Standard proteins used were bovine serum albumin in the range of 0–2000 μg/mL as suggested in manual.

2.5. Gel filtration chromatography

Sephadex G-75 was equilibrated with 25 mM Tris HCl pH 7.4 and then allowed to stand overnight at 4°C. Two milliliters of extract with total protein concentration 4863 μg/mL obtained from precipitation step using 90% ammonium sulfate was then poured slowly into the column just above the surface of the gel. Column was filled with elution buffer (25 mM Tris HCl pH 7.4) to fractionate the protein content and stopped at 65th fraction. Each fraction contained ±1 mL of solution and the protein content was measured using Pierce™ (Rockford, Illinois, US) BCA Protein Assay Kit as described previously.

2.6. Antibacterial activity of protease from *X. psidii* KT30

Pathogenic bacteria used in this assay was *B. subtilis* ATCC 19659, *S. aureus* ATCC 6538, *Salmonella typhii* ATCC 25241, *Listeria monocytogenes* BTCC B693, *Pseudomonas aeruginosa* ATCC 15442. Bacteria were diluted using McFarland standard 2 (6×10^8 CFU/mL) and measured on spectrophotometer (Gene Quant; 600 nm). Bacteria were then diluted with 0.85% NaCl to reach 10^6 CFU/mL of optical density. Each well was added with 50 mL sample of pellet protein (protease activity 2.328 U/mL). Buffer Tris HCl 25 mM pH 7.4 was used as a negative control and chloramphenicol was used as positive control. Incubation was performed at 37°C for ±24 hours, then the diameter of inhibition zone (mm) was measured in each well (Lay 1994).

2.7. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Protein separation was conducted in 12% separating gel. First, sample containing loading dye, 15 μL in total volume, was incubated in a water bath (±95°C) for 15 minutes to denaturate the protein. Denaturated samples loaded into each well then run for 2 hours at 110 V. After protein separation completed, the gel was carefully removed then stained using Thermo Scientific Pierce™ Silver Stain Kit. Precision Plus Protein™ Dual Color Standards

(Biorad) was used to predict molecular mass of corresponding protein bands appeared on gel.

2.8. Zymogram assay

Zymogram used 12% separating gel containing 0.1% gelatin. The separation technique was similar with sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) except that the denaturating steps of the sample (sample mixed with non-reducing loading dye) were skipped, but the difference is the substrate used which is gelatin to detect protease activity on gel. The sample then ran for 2 hours at 110 V. After protein separation was completed, the gel was carefully removed then washed using aquadest 2 × 5 minutes, followed by renaturation step in Triton X 2.5% solution for 40 minutes to 1 hour. Gel was incubated at 37°C for 24 hours in incubation buffer. After the incubation step was completed, gel was stained in Coomassie Blue solution for 2 hours followed by destaining step until clear zone as protease activity detected (Kleiner & Stevenson 1994)

3. Results

The optimal time for fungal growth was one of the factors which would affect the fungal biomass and the bioactive compounds production. As shown in Figure 1 the difference in culture media used had different effects on dried biomass of mycelia and medium pH value. The yield of dried biomass was highest in Hagem media—cultivated *X. psidii* KT30 on day 15 of harvesting (0.1419 ± 0.01 g). Meanwhile PDB inoculated with *X. psidii* KT30 yielded 0.1233 ± 0.01 g of dried biomass at day 9 of harvesting. The pH medium of culture underwent acidification along culturing process with similar pattern. On the contrary, the pH medium of Hagem (3.17 ± 0.09—3.60 ± 0.17) is lower than PDB (5.12 ± 0.50—5.72 ± 0.07). The prominent effect of culture medium on yield of bioactive compounds was pointed out at Figure 2. The highest protein content was found in day 15 of PDB-based cultivation (2431.83 μg/mL), whereas the highest protein yield extracted from Hagem-based culturing medium was appeared on day 21 of cultivation (1884.83 μg/mL). These results indicated that variety of culturing medium used significantly affected fungal growth and medium pH, yet the dynamic of fungal growth itself as consequence of difference between medium tested did not correlate directly into extracellular protein yield.

Higher protease activity was observed in PDB-cultured *X. psidii* KT30 in comparison with Hagem medium—based culture (Figure 3). The highest activity of protease was reached at day 15 of cultivation with 2.33 ± 0.19 U/mL for PDB-based culture and 0.484 ± 0.03 U/mL for 18th day of cultivation from Hagem

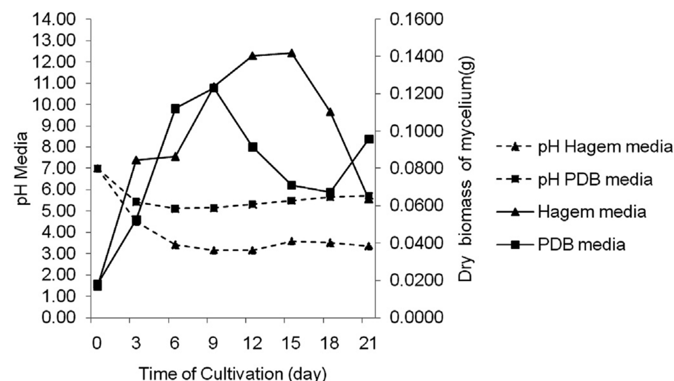

Figure 1. Effect of different culture media treatment on mycelial dried biomass and pH of end-culture medium during growth period of *Xylaria psidii* KT30.

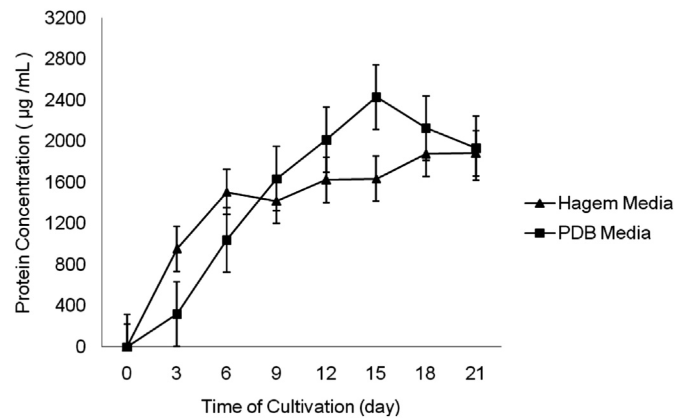

Figure 2. Effect of different culture media treatment on *Xylaria psidii* KT30 extracellular protein concentration. PDB = potato dextrose broth.

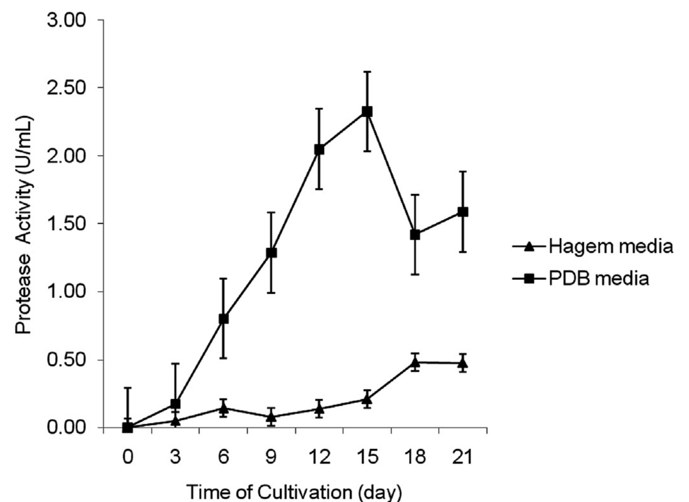

Figure 3. Effect of different culture media treatment on *Xylaria psidii* KT30 extracellular protease activity (U/mL). PDB = potato dextrose broth.

medium—based culture. Overall, the protease activity in both culturing media tended to increase along with the length of the cultivation period.

The antibacterial activity of *X. psidii* KT30 extracellular protein was evaluated using 5 pathogenic bacteria as indicated in Table. Among the 5 pathogenic bacteria tested only 2 isolates, *B. subtilis* and *S. aureus* showed inhibition zone on protein pellet—treated agar after 6th hour on *B. subtilis* and 4th hour on *S. aureus* of observation (Figure 4) with inhibition zone 8 ± 0.57 mm for *B. subtilis* and 7 ± 0.57 mm for *S. aureus*. This result indicated that

Table. Antibacterial activity of ammonium sulfate precipitated-protein pellet derived from 15th day of *Xylaria psidii* KT30 cultivated on potato dextrose broth against pathogenic bacteria

Pathogen	Gram (+/−)	Inhibition zone (mm)
Pseudomonas aeruginosa ATCC 15442	−	−
Salmonella typhii ATCC 25241	−	−
Staphylococcus aureus ATCC 6538	+	7 ± 0.57 (4th hour)*
Bacillus subtilis ATCC 19659	+	8 ± 0.57 (6th hour)*
Listeria monocytogenes BTCC B693	+	−

* Observation on antibacterial activity was done at time when clear zone was seen obviously.
− No antibacterial activity was detected.

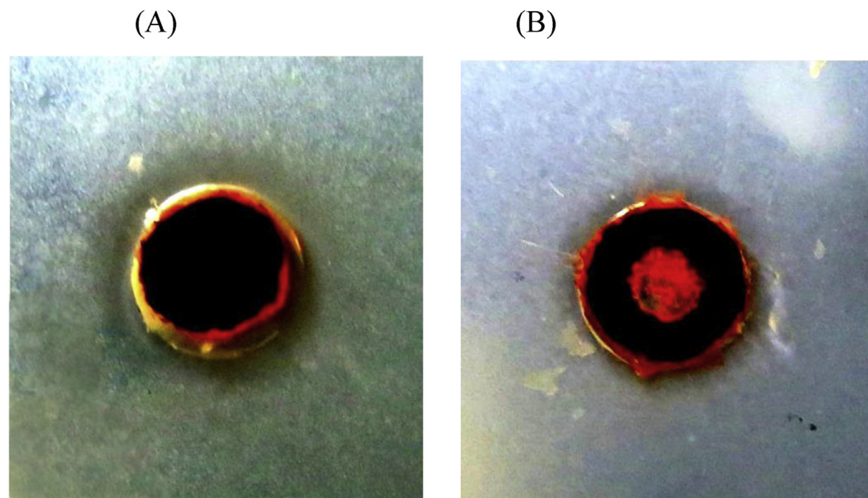

Figure 4. Inhibition zone formation due to treatment of ammonium sulfate precipitated-protein pellet of *Xylaria psidii* KT30 cultivated on potato dextrose broth on (A) *Bacillus subtilis* and (B) *Staphylococcus aureus* growth.

the protein pellet is specifically showing its antibacterial activity only to Gram-positive bacteria.

We assumed the antibacterial activity appeared in protein pellet was due to proteases activity. Before the zymogram assay was done, the protein pellet firstly was subjected to partial purification using Sephadex G-75 gel filtration (Figure 5). Sixty-five fractions were obtained from chromatography; we divided into 3 groups based on protease activity value of each fractions and we combined fractions 5–8 (0.045 U/mL), 9–12 (0.270 U/mL) and 13–15 (0.008 U/mL). The unification of these fractions based on proximity absorbance of protein concentration from each fraction that was predicted to have protease activity. Based on consideration of protease activity we chose 2 combined fractions 5–8 and 9–12 to be tested using zymogram assay. Fractions 9–15 did not use further testing because when initially screened using zymogram, no clear zone was detected. The predicted protease activity on fractions 9–15 was very low so clear zone was not detected. As shown in Figure 6, the activity of *X. psidii* KT30 extracellular proteases could be clearly detected in the zymogram result. The protease activity was appeared in all steps of purification as indicated by white clear zone band. The prominent protease activity appeared on crude extract and combined fractions 5–8 retrieved from day 15 of PDB-based cultivation. The protein bands represented the extracellular proteases on fractions 5–8 with molecular mass of 56.62, 89.12, 162.18 kDa indicating on SDS-PAGE separation. The protein with molecular mass of 162.18 kDa appeared on SDS-PAGE which was indicated by fractions 9–12 that correlated with slight protease activity based on zymogram result.

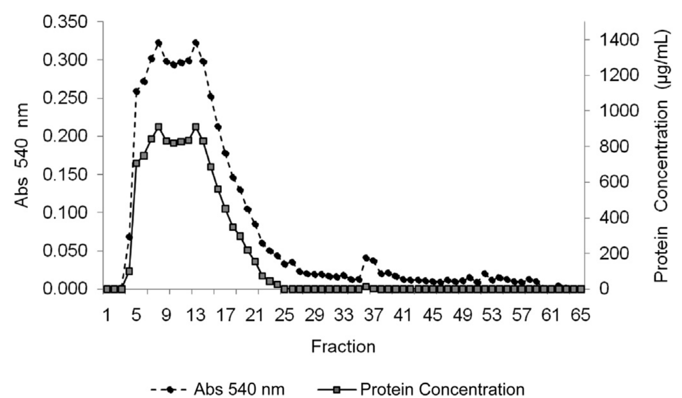

Figure 5. Size exclusion chromatography result of ammonium sulfate precipitated-protein pellet derived from 15th day of *Xylaria psidii* KT30 cultivated on potato dextrose broth eluted with Tris HCl 25 mM pH 7.4

4. Discussion

Fungi have an ability to produce various, diverse and unique primary and secondary metabolites such as biologically active pigments, polysaccharides, enzymes, toxin, etc. (Demain 1986). *X. psidii* KT30, which is fungus of marine origin, was able to produce extracellular enzymes such as proteases. The production of any metabolites by microfungi depends not only on the conditions and strains used but also on nutrient availability (carbon and nitrogen sources) and other factors. Mikiashvili *et al.* (2005) stated that the carbon source in the medium significantly influenced the enzyme activity of the fungus *Trametes versicolor*. We used 2 types of culture media which were dissimilar in carbon, nitrogen and trace-element contents where Hagem medium (Modess 1941) was modified by enriching with malt extract, yeast extract, D-glucose, NH_4Cl and some trace elements such as KH_2PO_4, $MgSO_4$ and FeCl, while PDB medium only contained potato infusion solids and dextrose. Kind of carbon or nitrogen sources and their complexity, trace-elements and their dosage used have been proven to affect the fungal growth and metabolites production, especially proteases (Nadeem *et al.* 2008; Thakur *et al.* 2009; Sarker *et al.* 2013).

Interesting finding in our study was different usage of culture media strikingly impacted not only on the dynamic of *X. psidii* KT30 growth but also on the protein yield and protease activity. Hagem culture media only prolonged the exponential phase for 3 days (from day 9 to 12 of cultivation) without significantly enhancing protease activity although its protein content was almost as high as in PDB (Figure 1). We assumed that prolonged effect observed in Hagem culture media was due to stimulation effect of yeast extract on fungal growth, while shorter exponential period observed in PDB was due to adverse effect of glucose on fungal growth (Sakamoto *et al.* 1978; Pradeep *et al.* 2013). We also noted that the protein content and especially proteases activity (from day 9 to 18 of cultivation) were much higher in PDB-cultured *X. psidii* KT30 (Figures 2 and 3) compared to Hagem media–cultured *X. psidii* KT30; we predicted that the increase of extracellular proteases

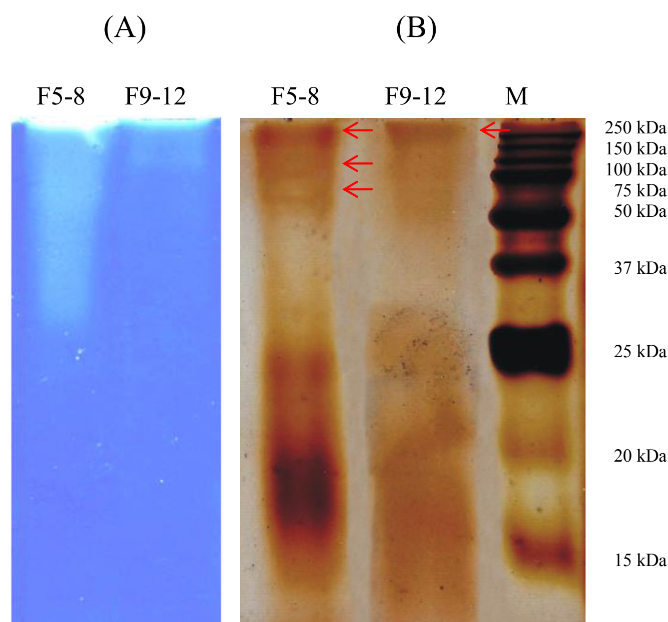

Figure 6. Zymogram activity and molecular mass of representative *Xylaria psidii* KT30 proteases extracted from day 15 of potato dextrose broth–based cultivation. (A) Extracellular protease activity using zymogram on each fractions seen as white zone band. (B) Molecular mass of representative extracellular protease on SDS-PAGE separation. M = marker; F5-8 = fractions 5–8; F9-12 = fractions 9–12.

activity during the decline phase of *X. psidii* KT30 cultivation was a result of stress response due to nutrients limitation.. For instance, these extracellular proteases help to degrade proteins into simple peptides that assemble death cells of *X. psidii* KT30 then used as nutrients to sustain the growth. Simkovic (2008) stated that the genes encoding the fungal serine proteases could be induced by some environmental stress that emerged within cultivation process. The medium acidification during the process of fungal cultivation was not solely due to glucose catabolism effect but due to other factors such as membrane ATPase activity and mineral availability also contribute to this event (Serrano 1983; Gadd 1999). In contrast with Rosling *et al.* (2004), the degree of medium acidification as observed in our result was greatly inverse with overall dry biomass of mycelium along with cultivation process. Furthermore, the degree of medium acidification was also species-specific responses to the environment (Rosling *et al.* 2004).

The presence of extracellular proteases extracted from the fungal cultivating media was well documented using zymogram assay. This method is a very powerful tool not only to detect protease activity but also to predict molecular mass of the target proteins (Kleiner & Stevenson 1994). We succeeded to determine 3 kind of *X. psidii* KT30 extracellular proteases (56.62, 89.12, and 162.18 kDa) due to stress response against unfavorable environment condition. To be highlighted, the protein bands with size of 162.18 kDa obtained from SDS-PAGE result slightly differed from the corresponding protein band which was pointed out at zymogram result. This discrepancy due to the fundamental principle of protein separation on both methods quite differs where non-reducing treatment in zymogram assay leaved protein in their native form, which retained their activity, (the protein will migrate based not only on sizes but also on N-glycosylation and protein conformations) so the protein band will appear much smaller in molecular mass, after separation on zymogram gel (Narhi *et al.* 1989; Takagi *et al.* 1995). This phenomenon has been also observed in another *X. psidii* KT30 serine-type extracellular protease as reported by Budiarto *et al.* (2015). The molecular masses of fungal proteases range from 28.1 kDa up (smaller one) to 205 kDa with diverse biological activities (Kolvenbach *et al.* 1990; Chellappan *et al.* 2011). Fungal protease (36 kDa) with keratinolytic activity from poultry soil–derived *Aspergillus parasiticus* has applications in pharmaceutical industries (Anitha & Palanivelu 2013). Serine protease (35 kDa) with function in fungal pathogenesis of *Dactylella shizishanna* has been well characterized by Wang *et al.* (2006). Furthermore, Farnell *et al.* (2012) has identified various proteases (54 kDa aminopeptidase Y, 69 kDa elastinolytic metalloproteinase, and 42 kDa alkaline serine protease) expressed by *Aspergillus fumigatus* due to different treatment of protein substrates. The diversity of extracellular proteases produced by fungi especially in this case *X. psidii* KT30 reflected the fungal adaptive response to environmental changes due to imbalance in nutrient requirement.

The biological activity of fungal proteases has been well documented and the current progress is outstanding. Some fungal proteases showed excellent anti-cancer and anti-microbial activities while others exhibited good potential in biotechnological field (Bonants *et al.* 1995; Park *et al.* 2009; Chellappan *et al.* 2011; Wang *et al.* 2006; Parente *et al.* 2010; Cavello *et al.* 2013). Our study also added the important finding related to the potential of marine-derived fungus *X. psidii* KT30 as a proteases producer to be applied as antibacterial agent against Gram-positive bacteria.

In summary, the production of *X. psidii* KT30–derived extracellular proteases has been optimized. The maximum of protein yield (2431.83 μg/mL) and extracellular proteases (2.33 ± 0.19 U/mL) was recorded at day 15 of cultivation. Optimized culture-precipitated protein exhibited inhibition zone on *B. subtilis*–poured agar (8 ± 0.57 mm) and on *S. aureus*–poured agar (7 ± 0.57 mm). Furthermore, the protein bands that represented extracellular proteases have molecular mass of 56.62, 89.12, 162.18 kDa, respectively.

Conflict of interest

We declare there is no conflict of interest.

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