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Characterization of Bacterial Isolates Producing Chitinase and Glucanase for Biocontrol of Plant Fungal Pathogens

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Abstract Cells wall degrading-bacteria as biological control agents have been intensively utilized because of their environmentally friendly control measures. The chitinolytic bacteria produced chitinase and glucanase that degrade cell walls of pathogenic microbes. This study was aimed to determine chitinase and glucanase activities of several microbial isolates both by qualitative (chitinolytic index) and quantitative analyses (spectrophotometric method). The characterization of chitinase activity of isolate PBR 3b showed that chitinolytic index was 2.19, with the highest enzyme activity of 0.0287 U/ml and specific activity of 0.8605 U/mg protein after two days incubation. Bacterial isolate PBR 3b was identified as *Pseudomonas veronii* with 99% similarity. The potential ability of bacterial isolates to hydrolyze β-glucan was shown by the clearence zone sorrounding the bacterial colony after addition of 0.2% congo red dye. Notably, *Pseudomonas aeruginosa* C 32a produced the largest clear zone with the glucanolytic index of 2.27. This study demonstrated that temperature and pH optimum for glucanase activity of *P.aeruginosa* C 32a was 40^{o}C and pH 6, respectively. The antagonistic test of *P.aeruginosa* C 32a against *Pyricularia oryzae* and *Rhizoctonia solani* showed inhibition zones of 59.11% and 37.33%, respectively. These *Pseudomonads* isolates could be promising for biocontrol with broad spectrum phytopathogens.

Keywords: chitinase, glucanase, biocontrol agent

Introduction

Serious problems that encountered in agricultural cultivation were declining of rice and estate crop yields caused by plant pathogens (bacteria, fungi and viruses). The rice blast disease caused by *Pyricularia oryzae* Cav. is a common fungal disease particularly during the rainy season. Blast spot symptoms are visually noticed on leaves with oval or elliptical with the center of the spots usually brown or brownish red. High humidity might affect the

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development of blast disease, whilst dense planting, excessive Nitrogen fertilization and soil temperatures favor blast disease development (Greer and Webster, 2001).

Aside of blast disease invading rice crops around the world, at present basal stem rot (BSR) in oil palm also treathen some producer countries. This serious plant disease is caused by fungal *Ganoderma boninense,* and become challenge in millions of hectares oil palm plantations. BSR have been observed from seedbed nursery to old plants, but new symptoms are usually seen after the seed is planted in the field where fungal develops slowly at the base of palm trunk. The disease does not only infect farmers' oil palm but also heavily damages cultivated large plantations (Breton *et al.*, 2006).

Various measures have been applied to inhibit the growth of plant fungal pathogens by culture technique and pesticides. However, the use of pesticides is considered as an inappropriate method because of its continuous application will affect for human health and unfriendly environments including the residues on agricultural products. To alternate fungicide use, microorganisms have been intensively explored for their potential antagonistic nature or competition to control plant pathogen populations (Thamthiankul *et al*., 2001) Biological control agent that showed antimicrobial compounds is believed to promisingly solve the environmental problems caused by excessive used of chemical pesticides (Compant *et al*., 2005).

Indonesia, as one of country having a large biodiversity, provides many sources of microbes with significant economic value in respect to agricultural and industrials sectors. Search of isolates that could be used in industries such as isolates capable of producing commercial enzymes need to be carried out. Many bacteria and fungi produce chitinase which converts chitin into monomers or oligomers (Moerschbacher *et al.*, 2005). The existence of chitin is abundant in nature and can serve as a constituent component of a variety of organisms. Thus, chitin plays as a substrate which is always available for soil microbes.

Chitinolytic bacteria showed ability to hydrolyze chitin to its derivatives and they were used as valuable materials in industries such as anti inflammatory, immunoajuvant, antitumour, flocculants in wastewater treatment, fungal or anti-arthropod pests agents, and fungal protoplasts technology (Patil *et al*., 2000). The β-1,3-glucanase was also involved in fungal pathogen-plant interactions that degrade callose (β-D-1,3-glucan), a component of host vascular tissue when invaded by pathogenic fungi. In fungal cell walls, specific substrates of this enzyme is found to be β-1,3-glucan and laminarin callose. They are induced in response to environmental stresses or pathogen infection. Together with chitinase, they showed a defense-related biological function

through the inhibition growth of pathogenic fungi (Fujii and Miyashita, 1993; Jach *et al*., 1995). The β-1,3-glucan might be used to improve plant defense responses of antifungal enzyme during infection (Marco and Felix, 2007). The chitinolytic bacteria produce enzymes that could degrade fungal cell walls in order to inhibit growth of plant pathogens. The aim of this study was to screen some Indonesian bacterial isolates for chitinolytic and glucanolityc activity to suppress the growth of plant pathogenic fungi.

Materials and methods

This study was conducted at the Laboratory of Microbiology and Biochemistry-ICABIOGRAD- IAARD. The bacterial isolates used in this study were provided by culture collection of ICABIOGRAD and IPB-CC (Table 1).

Table 1. List of bacterial isolates used in the study

Isolates Code	Host and isolates origin	Year collection
C.1D	Sugarcane waste, Malang-E.Java	2008
C.4	Soil, Malang-E.Java	2008
C.29B	Seawater Kenjeran, Surabaya-E. Java	2008
C.30	Pond Kenjeran, Surabaya-E.Java	2008
C.33C	Sand beach Kenjeran, Surabaya-E.Java	2008
C34A	Soil mud Sidoarjo-E. Java	2008
C34C	Soil mud Sidoarjo-E. Java	2008
PPT 3.4	Parang Tritis beach, Yogyakarta	2008
PBR 3.b	Baron beach, Yogyakarta	2008
PBR 3K	Baron beach, Yogyakarta	2008
11 UJ	Soil,Jember-E. Java	2011
5 MK	Soil,Manado-N. Sulawesi	2011
6 PJ	Soil,Jember-E.Java	2011
5J	Soil, Jember-E.Java	2011
6PM	Soil,Manado-N. Sulawesi	2011
1KM	Soil,Manado-N.Sulawesi	2011
8KM	Soil,Manado-N.Sulawesi	2011
3KM	Soil,Manado-N.Sulawesi	2011
1J	Soil,Jember-E.Java	2011
4KM	Soil,Manado-N.Sulawesi	2011
P.aeruginosa C32a	Soil-mud Sidoarjo-E.Java	2004
B. cereus I.21**	Chili pepper,Bogor-W.Java	2007
Burkholderia sp E76	Rice, Sukabumi-W.Java	2004
Ganoderma sp	Oil palm, Bogor-W.Java	2011
Pyricularia oryzae	Rice,Sukamandi-W.Java	2011
Rhizoctonia solani	Rice,Sukamandi-W.Java	2011

**=isolates was obtained from IPB CC

Determination of chitinase activity

Qualitative assay for chitinolytic activity was conducted by activation of preserved isolates onto slant agar, then transferred into 50 ml sterile tube containing chitin agar medium. After shaking and vortex, the 5 ml of culture was dropped into chitin media (125 ml colloidal chitin, $Na_2HPO_4.2H_2O$ 0.65 g, 1.5 g KH_2PO_4, 12.25 g NaCl, 0.5 g NH_4Cl, 0.12 $MgSO_4.7H_2O$ g and 0.005 g $CaCl_2$ dissolved in 1,000 ml distilled water). After 7 days incubation, the plates was soaked with a solution containing Congo red dye 0.1% (w/v) for 15 min and washed with running water. The formation of a clearence zone of the isolates was observed on petri dish.

Quantitative chitinolytic activity assay was performed using spectrophotometry. Colloidal chitin 0.3% (1.5 ml) was put into a test tube containing 0.75 ml of 10 mM phosphate buffer saline (PBS) pH 7 and 1.5 ml of crude enzyme extract, and incubated for 30 min at 37^oC in a water bath. The mixture was then centrifuged at 6,000 rpm for 4 min, and 2.5 ml of the taken supernatant was mixed with 2.5 ml of distilled water and 5 ml Schales reagent. The reaction was put into boiling water for 10 min to stop the reaction, and after cooling at room temperature, it was measured using UV-VIS spectrophotometer at a wavelength of 420 nm (Spindler, 1997).

In vitro antagonistic assay against Ganoderma sp. and P. oryzae

Antagonistic assay against *Ganoderma* sp and *P. oryzae* were done by dual culture on petri dish. The fungal mycelia were grown onto potato dextrose agar (PDA) media. The fungal pieces were placed in glucan media using cork borer. As many as 2 ml of bacterial isolates from Luria broth (LB) media was slowly dropped into a petri dish well and then incubated for 2 days at room temperature.

Bacterial isolates were cultured in 100 ml chitin medium for one day using rotary shaker, then it was centrifuged at 10,000 rpm for 10 min. The supernatant was added slowly with 60% ammonium sulphate to presipitate enzyme, and cooled at -20^oC for several hours. This mixture was centrifuged again for 10 min, and then the pellets were added with 2 ml PBS.

Characterization of PBR 3b

Isolate PBR3b was subcultured aseptically on Nutrient agar (NA) slant medium in laminar air flow. Bacterial isolates in the ampoule were diluted with 50 ml of sterile water, then streaked on NA media and incubated at room temperature. The bacteria was transfered into the tube containing Nutrient

Broth (NB) media (triptose 1%, 0.5% yeast extract, 1% NaCl), and then incubated again at room temperature under shaking conditions for 5 days. The samples were taken every day to quantify the number of bacteria which were measured using spectrophotometer at wavelength of 620 nm.

Determination for chitinase activity of PBR 3b and protein measurement

The cultures were incubated at room temperature on a shaker for 5 days and assay was performed periodically (24h, 48h, 72h, 96h, 120h, 144h, and 168h). The formation of a clear zone was observed after 5 days incubation. The chitinase activity of the supernatant was measured as descriebd above. Determination of chitinase enzyme activity by spectrophotometric method was done using N-Acetyl Glucosamine (GlcNAc) as a standard.

Protein concentration measurements were performed using the Bradford method (Bradford, 1976). The filtrate (50 ml) was added with 450 ml Bradford reagent. The homogenized solution was vortexed, incubated at room temperature for 15 min and measured using spcetrophotometer at 595 nm wavelength. Bovine serum albumine (BSA) (0.10 mg/ml) which was homogenized was used as a standar for protein determination. BSA standard series was prepared as follows: 0.00mg/ml, 0.02mg/ml, 0.04mg/ml, 0.06mg/ml, 0.08mg/ml and 0.10mg/ml.

Identification of PBR 3b

DNA isolation was done by culturing the bacteria in a test tube containing 5 ml LB media with shaking for 18 h in an orbital shaker (75 rpm) at room temperature. Cultures were centrifuged at 10,000 rpm for 5 min, and bacterial pellets were resuspended in 150 µl Buffer Solution I, 150 µl Solution II, and mixed evenly for 5-20 min at room temperature. Subsequently, 250 µl Solution III was added and centrifuged 10,000 rpm for 5 min. Supernatant was transferred into a new tube and added with 100 µl phenol chloroform-isoamyl alcohol mixture (24:1). The solution was centrifuged at10,000 rpm for 10 min, then 600 µl supernatant was retrieved and mixed with 600 µl isopropanol. After centrifugation at 10,000 rpm for10 min, the supernatant was discarded, then the pellet was air dried and resuspended in 20 µl H_2O.

Bacterial nucleotide sequence identification was based on 16S ribosomal DNA (Janda and Abbot, 2007) using primer pairs of 63F (CAGGCCTAACACATGCAAGTC) and 1387R (GGGCGGA/TGTGTACAAGGC). The composition of 20 µl PCR reactions consisted of 2 µl 10X PCR buffer, 1.5 µl 50 mM $MgCl_2$, 0.5 mM dNTP 4 µl, 1 µl of ecah forward and reverse primers (20 mM), 0.5 µl 5U Taq polymerase,

and 13,5 µl of sterile distilled water. PCR Thermal Cycler (Biometra, USA) was run with the following programs: initial denaturation of DNA at 94^{o}C for 10 min, denaturation 94^{o}C for 1 min, annealing 50 ℃ for 45 sec, and 72^{o}C extension for 1 min 30 sec with 35 cycles, and final extension at 72^{o}C for 10 min. PCR products were electrophoresed on gel agarose using 90 volts for 30 min in TAE buffer. Subsequently, PCR products were purified using ethanol precipitation method. The pellet was then washed twice with 70% ehanol. DNA sequencing was performed with ABI PRISM 3070-DNA Sequencer. The nucleotide sequences of 16S ribosomal DNA was analyzed using Blastn of NCBI (www.ncbi.nlm.nih.gov). Phylogenetic analysis was performed using PHYLIP program version 3.6 (University of Washington).

Screening of glucanase activity, characterization of glucanase isolate C 32a for biocontrol plant pathogen

A total of 5 ml bacterial culture was grown on agar medium containing 0.2% glucan (Wood and Weisz, 1984), and then incubated at room temperature for 2-3 days. Formation of clear zone was observed after staining with 0.2% Congo red solution and rinsing with 1 M NaCl. The clearence zone formed by the hydrolysis of glucan was calculated based on glucanolytic index.

Quantitatively glucanase activity assay was determined by modified dinitrosalicylic acid (DNS) method (Miller, 1959). A 1 ml crude enzyme extract obtained was reacted with l ml glucan substrate and incubated at 37^{o}C for 60 min. The reaction was stopped by adding l ml DNS reagent. The samples was heated for 15 min, cooled and the absorbance was measured at λ = 540 nm. One unit of glucanase activity was defined as the amount of enzyme that produces 1 µmol of glucose in one minute. Standard curve was done by measuring various concentrations of glucose reacted with l ml of DNS reagent.

The inhibition assay of the selected bacterial isolate *P.aeruginosa* C 32a to fungal pathogens (*P. oryzae* and *R. solani*) were performed based on in vitro test. PDA medium on petri dishes (diameter of 9 cm) was made in hole/well using 0.5 cm diameter cork borer. About 50 µl crude extract of glucanase produced from selected bacterial isolates was put on the well and then pieces of pathogenic fungi cultured was placed with a distance of 3 cm. A negative control was prepared using sterile distilled water. The inhibition zone was observed after 7 days incubation at room temperature (± 30^{o}C), and then calculated using the formula of Fokemma (1973) as follows: Inhibition zone (IZ) = r1-r2/r1 x100%; where: r1 = radius of the control; r2 = radius of the treatment.

Results

Chitinase activity

The chitinolytic index was calculated by comparing the diameter of the clear zone with the diameter of the colony formed. The chitinolytic index was ranged from 1.353 ± 0.15 to 3.861 ± 0.44. The largest chitinolytic index was observed on isolate 11 UJ with the chitinolytic index of 3.861 ± 0.44 (Table 2).

The largest clear zone of isolate 11 UJ indicated that the bacteria produced chitinase in large quantities. Other isolates also formed chitinase, but the clear zone was varied depending on enzyme production of bacterial isolates. The results showed that chitinolytic index did not vary much, however; some isolates (C 34A, C 34C and PBR 3K) were not detected for its chitinase activity. This might occur because of several factors affecting the rate of enzyme reaction. Isolates with the highest activity (0.00277 U/ml) was C 33C suggested that the number of units of the enzyme could produce GlcNAc in a certain incubation time of the isolate (Table 3).

Table 2. Qualitative chitinase activity assay of isolates

Isolates	Chitinolytic index ± SD
PBR 3b	1.725 ± 0.22
C29B	2.339 ± 0.33
6PM	1.353 ± 0.15
C30	1.780 ± 0.22
C33C	2.175 ± 0.13
C34C	1.408 ± 0.17
11UJ	3.861 ± 0.44
3KM	1.625 ± 0.17

Note: chitinolytic index (CI) < 1=weak reaction; CI 1-1.5= medium reaction, CI >1.5=strong reaction

Table 3. Chitinase activity of isolates

Isolates code	Chitinase activity (U/ml)
C1D	0.00187
C4	0.00198
C29B	0.00095
C30	0.00198
C33C	0.00277
PPT 3.4	0.00198
PBR 3b	0.00053
11 UJ	0.00075
5 KM	0.00143

6 PJ	0.00118
5 J	0.00111
6 PM	0.00055
1 KM	0.00111
8 KM	0.00064
3 KM	0.00140
1 J	0.00122
4 KM	0.00221
C34A	-
C34C	-
PBR 3K	-

-= not detected

In vitro antagonistic assay to fungal pathogens (Ganoderma sp and *P. oryzae)*

Based on the antagonistic assay to fungal pathogens *Ganoderma* sp and *P. oryzae*, antifungal bacteria showed the inhibitory growth effects against the pathogenic fungal isolates. Fungal pathogens and bacterial isolates were grown close to the distance of about 1-1.5 cm. After incubation for 1-2 days, the fungal growth was observed compared with control. Of this assay, the strong inhibitions zone were observed on isolates E 76, C 33C, C 1D, PBR 3b, whilst only E 76 and C 33C could inhibit growth of *P. oryzae* (Table 4). Antagonistic assay against fungal pathogen *P. oryzae* was only performed to five isolates of bacteria that previously showed higher chitinolytic index. Isolates E 76 and C 33C which be able to inhibit the growth of fungi could be promising a biocontrol agent. Further observation showed that isolates C 33C and C 1D produced inhibitor compounds (proteins or metabolites). This result, however, can not prove as antibiotic compound, but the isolate inhibited the growth of pathogenic fungi. Thus, this isolate needed further investigation for its potency.

Table 4. Antagonistic test against *Ganoderma* sp and *P. oryzae*

Isolates	Reactions to Ganoderma	Reactions to *P. oryzae*
C30	-	-
11 UJ	-	-
E76	++	++
C29B	-	-
C33C	++	++
PBR 3b	++	ND
C34A	-	ND
PPT 3.4	-	ND
C1D	++	ND
C4	-	ND

++= inhibits; -= no inhibition, ND=not determine

Characterization of PBR 3b isolate producing chitinase

Determination of PBR 3b growth was done in NB medium based on the incubation time of bacteria versus Optical Density (OD). Optical reading of bacterial suspension correlated with the number of cells contained in the media that can be measured at a wavelength of 620 nm. The higher the OD value was obtained, the more the number of bacterial cells was observed. The exponential phase of bacteria occurred after 3 days, indicating that the bacteria were able to produce optimum energy to perform cell division and increased the number of cells. Isolate PBR 3b had the highest growth with a OD value of 2.087.

Chitinase activity and Protein concentration

Positive chitinase activity assay of isolate PBR 3b was tested in solid medium containing colloidal chitin. The result was characterized by the formation of a clear zone sorroundings the colony (Fig. 1). Transparent color medium was caused by the degradation of chitin in the growing medium. Based on the results of chitinolytic index, the highest chitinolytic index of PBR 3b was 2.19.

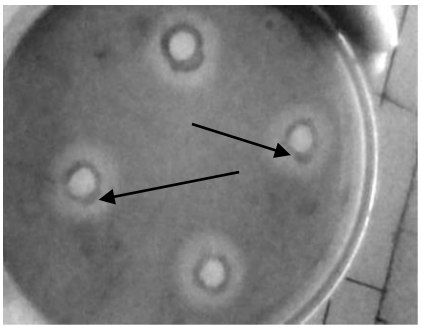

Fig.1. Formation of a clearence zone sorroundings the colony of PBR 3b on coloidal chitin agar (arrow).

PBR 3b was grown on the media during the optimum growth period, in which an enzyme was generated the highest activity. Chitinase activity was measured at the specified time for 7 days incubation. PBR 3b showed the highest chitinolytic activity on the second day with the value of enzyme activity of 0.0287 U/ml (Fig.2). Chitinase purity was determined based on the specific activity which expressed as mg of protein (number of enzyme units (U) per milligram of protein) that necessary to determine protein content in the chitinolytic bacterial isolates. Based on standard curve equation (y = 1.442 x

+0.002 with R^2 is 0.995), the PBR 3b protein content was 0.0333 mg/ml, and the known specific activity of PBR 3b was 0.8605 U/mg protein.

Identification of isolate PBR 3b

Sequencing result was obtained based on nucleotide sequences of 16S rRNA with 1294 bp in length. Based on the comparison of 16S rRNA bacterial sequence with other bacteria in the GeneBank database through Blastn analysis showed that bacteria have a 99% level of similarity with *Pseudomonas veronii* AIX/3A, AIX/4B and AIY/3A (Fig. 3).

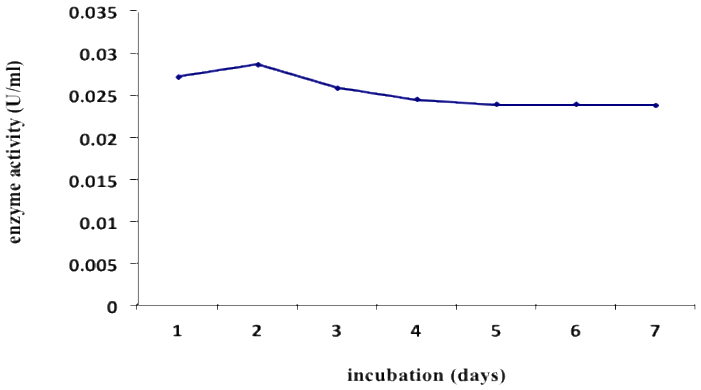

Fig. 2. Rate of chitinase activity of PBR 3b isolate at different incubation time

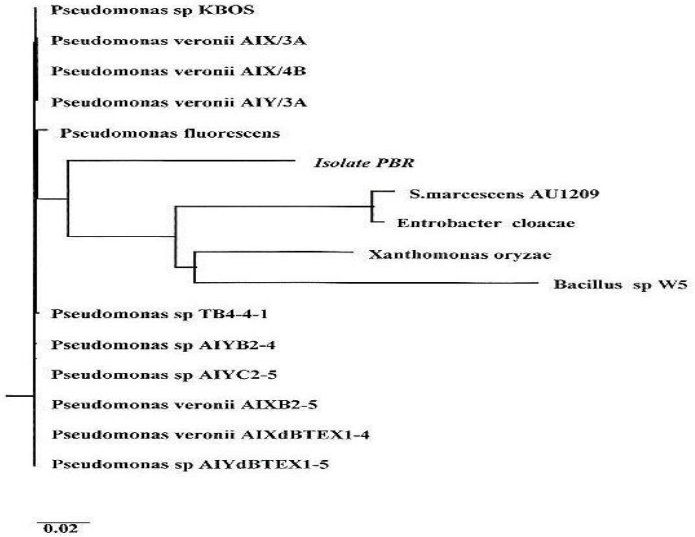

Fig. 3. Phylogenetic tree of PBR 3b with other 16S rDNA sequences

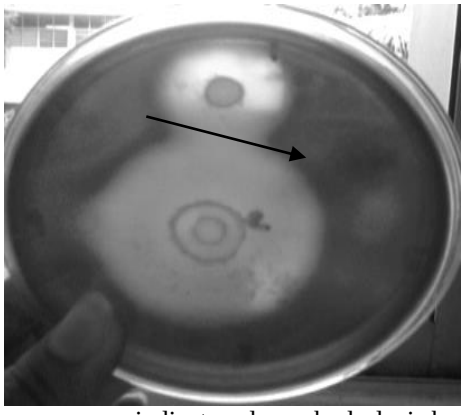

Fig. 4. Clearence zone indicates glucan hydrolysis by glucanase.

Screening of glucanase activity, characterization of glucanase isolate C 32a for biocontrol of rice fungal pathogen (P.oryzae and R. solani)

Screening of quantitative glucanase activity was carried out in solid medium in a petri dish containing glucan substrates. The glucan substrates derived from oat flour which was extracted with 20% Na_2CO_3 adjusted to the alkaline pH (pH 10). The isolate was dropped on petri plates containing glucan media, and after incubation at room temperature for 1-2 days, the clear zone will appear sorroundings the colony (Fig.4). From the results of this test, it could be seen that the isolates 11 UJ, E76, *B.cereus* 1.21, and C 1D showed biger clearance zone. This suggests that these isolates have a high glucanase activity. The qualitative examination resulted the leading bacterial isolates showing glucanase activity with the highest glucanolytic index was *Pseudomonas aeruginosa* C 32a (Table 5).

Characterization of pH indicated that the crude enzyme extract of bacterial isolates *P. aeruginosa* C 32a had an optimum glucanase activity of of 0.029 U/ml at pH 6 (Fig.5). Glucanase activity was also detected to be sensitive to temperatures. The highest glucanase activity of crude extract was shown optimum at temperature of 40^{o}C (Fig.5).

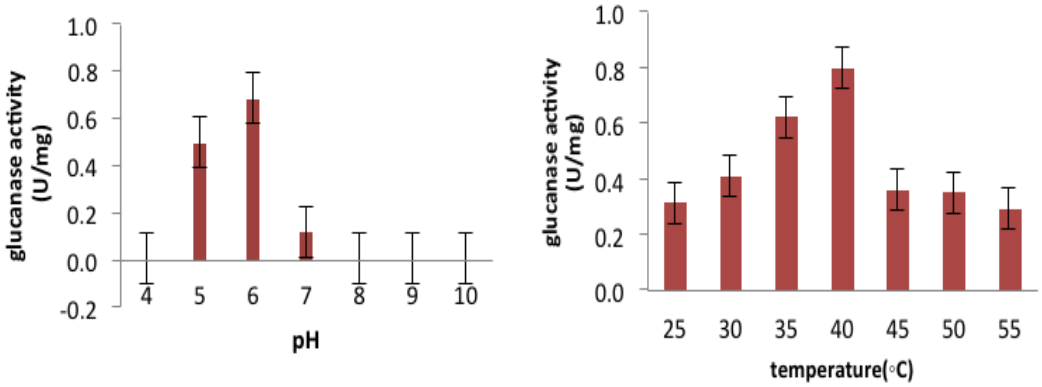

Fig. 5. Glucanase activity of *P. aeruginosa* C32a at different pH and temperature.

The crude enzyme extract of *P. aeruginosa* C 32a was selected for its antagonistic acivity against fungal pathogen *P. oryzae* and *R. solani* (Table 6). It was showed that the bacteria could inhibit the growth of both fungal mycelia with inhibition zones of 59.11% and 37.33%, respectively.

Table 5. Glucanase assay to the isolates

Isolates	Glucanolytic index
C30	0.9
11 UJ	1.7
E76	1.6
C29B	1.2
C33C	1
PBR 3b	1
C34A	-
PPT 3.4	-
C1D	1.7
C4	1.1
C33C	1.1
P. aeruginosa C32a	2.27
B. cereus I.21	1.92

-=not detected

Table 6. Antagonistic test of *Pseudomonas aeruginosa* C32a against fungal pathogens

Bacterial Isolates	Fungal pathogens	Inhibition zone (%)
P. aeruginosa C32a	*P. oryzae*	59.11 ±5,02
P. aeruginosa C32a	*R. solani*	37.33 ±14,86
Control (-)	-	0.00 ± 0.00

Discussion

Prior to chitinase assay, the bacterial isolates were firstly rejuvenated in liquid media containing colloidal chitin to increase chitinase activity. Sampling was done after 18 h under shanking to determine the optimum growth. Chitinase activity was assayed qualitatively by observing the formation of clear zone. A clear zone was virtually the result of chitin hydrolysis in the specific period. The larger the zone was formed, the greater the activity also owned. Not all isolates gave good results, only a few isolates showed a clear zone. Isolates with no clear zone might be due to the rate of reaction that did not make chitin hydrolysed well.

Enzymes were known as proteins that play an important process of biological activity, and they could loose its activity due to heat, acids or strong alkaline, and protein denaturation. The enzymes is very specific and only works on certain surface of molecules substrates. The factors that affect the speed of reaction of the enzyme are temperature, pH, enzyme concentration, substrate concentration, the influence of activators, inhibitors, coenzyme and electrolyte concentration (Folders *et al*., 2001).

The chitinase activity by both assays revealed that not all isolates produced the same results. It is likely due to different performance of the tested isolates on the test medium or the isolates did not hydrolyze well due to the reaction of enzyme-substrate. Isolates producing high chitinase or glucanase could inhibit fungal growth such as *P. oryzae* and *Ganoderma sp*. There were four isolates inhibited fungal growth i.e; E 76, C 33C, PBR 3b, and C 1D isolates. Isolates C 33C and C 1D were also excellent in inhibiting the fungal isolates, however compounds that inhibit fungal growth have not yet determined. Thus, it was possible that these bacteria could be used to protect plants from fungal infection. Moreover, *Ganoderma* sp was saprophyte and would become pathogen when met with the roots of palm trees growing nearby.

Chitinase produced by chitinolytic bacteria could degrade chitin into monomers and supposedly capable of inhibiting the growth of fungal pathogens on rice plants. These bacterial isolates were obtained from various sources such as the rhizosphere, phylosphere, soil or water environments such as ocean, lake or shrimp waste and so on. In addition to environmental mesophyll, chitinolytic microorganisms could also be isolated from thermophilic environments such as hot springs, geothermal areas and others. Several genus of bacteria that were able to produce chitinase included *Aeromonas, Enterobacter, Pseudomonas, Serratia, Vibrio, Chromabacterium*, and *Pseudoalteromonas*. In addition to bacteria, fungi were also known as a group of organisms that showed good chitinase activity, including groups of Deuteromycetes, Myxomycetes and Ascomycetes such as *Aspergillus, Trichoderma, Verticillium, Thielavia,*

Penicillium, Humicola, Colletotrichum, and *Physarium polichepalum* (Cohen-Cupick *et al*.,1999; Gimenez-Pecci *et al*., 2002).

Chitinase was an extracellular enzyme that might play an important role in the hydrolysis of chitin.The enzyme was produced in bacterial cells, but secreted into the growth medium. Based on chitinolytic index observations, the promising isolate was directly proportional to its ability to degrade chitin. Formation of a clear zone around the colony, caused the termination of the β-1,4 N-acetylglucosamine bond by chitinase into monomer GlcNAc. The greater the number of monomer GlcNAc was produced, the larger clear zone was formed around the colonies. To further clarify the invisibility of clear zone, Congo red dye 0.1% (w / v) was added into the medium. Congo red dye which has a positive charge can detect nonpolar hydrogen bonding in carbohydrates to give orange-red color, whereas the clear zone formed from the chitin reduction remains clear.

The increment of chitinase activity of PBR 3b was almost similar at early stage then declined slowly. This generally occurs because the enzyme has a specific reaction rate. Several factors could affect the rate of enzyme reaction including pH, temperature, and substrate concentration (Fukamizo, 2000). The phylogenetic tree analysis, the bacteria was in the same group with *P. veronii*. This suggests that the bacterial isolate of PBR 3b could be ascertained as *P. veronii* as shown by closely genetic relation.

The glucanase produced from *P. aeruginosa* C 32a showed activity in the pH range of 5-7. At pH above 7 no glucanase activity was observed. This phenomenon occured because glucanase produced by every organism has a specificity towards certain types of β-glucan. This result is in good agreement with previous reports. Celestino *et al*. (2006) pointed out that the glucanase produced by *Rhizopus microporus* var. *microporus* showed specificity against glucan from oats (β-1,3-1,4-glucan) and not specific to laminarin (β-1,3-glucan) and carboxy-methylcellulose (β-1,4-glucan), with glucanase activity is at pH 3-7. β-1,3-glucan is an acidic protein, and similar results were also reported in the glucanase group produced by fungi such as *Trichoderma harzianum* and *Trichoderma asperellum* where optimum activity was occured at acidic pH (Marco and Felix, 2007).

The glucanase of *P. aeruginosa* C 32a was more suitable used in pH to be neutral or acid tolerant to obtain optimum results. Similar results have also been reported that the glucanase produced by *Pseudomonas* sp. was the optimum at pH 6 conditions. In addition, *Pseudomonas* sp. showed β-1, 4 glucanase activity at the optimum temperature of 45^{o}C (Cheng and Chang, 2011). According to Ogawa *et al*. (2002), Gram-negative bacteria such as

Burkholderia cepacia produced not only chitinase but also β-1,4-glucanase under the optimal temperature of 50^{o}C.

The optimum temperature for enzyme is very important because it affects the geometry of the enzyme. The high temperature would denature enzymes, while too low temperature also would lead to inactivation of the enzyme. *P. aeruginosa* C 32a produced glucanase at the optimum temperature of 40^{o}C with activity 0.034 U/mg; which was comparable with other reports. Optimum temperature of glucanase produced by *Moniliopthora perniciosa* was at 40^{o}C, whilst *Streptococcus salivarius* subsp. *thermophilus* produced glucanase at a higher temperature of 70 ℃ (Asan and Ozcan, 2007). Apiraksakron (2006) pointed out that glucanase produced from the bacterium *Bacillus subtilis* was in the temperature range of 40^{o}C-60^{o}C.

The glucanase production of *P. aeruginosa* C 32a was obtained from 24 h-old bacterial cultures. Glucanase production with similar incubation period was also produced by following microorganisms, such as *Bacillus* sp., *Talaromyces emersonii* and *Trichoderma* sp. (Bhat and Wood, 1989). *Pseudomonas aeruginosa* C 32a produced the largest glucanase with glucanolytic index of 2.27. The isolate could inhibit the mycelium growth of *P. oryzae* and *R. solani* with the inhibition zone of 59.11% and 37.33%, respectively.

It was shown from this study that the potential benefit of chitinolytic bacteria to degrade chitin and glucan was the highest, hence these bacteria can be used as a biological control agent. This biological agent should be applied directly into agriculture in the form of biological fungicide products that would reduce the use of chemicals. In addition, further characterization should be done based on different environmental conditions and broad spectrum phytopathogens.

Conclusion

It may concluded from this study that chitinase activity of isolate PBR 3b showed chitinolytic index of 2.19, with the highest enzyme activity of 0.0287 U/ml and specific activity of 0.8605 U/mg protein. Bacterial isolate PBR 3b was identified as *Pseudomonas veronii* with 99% similarity. Isolate *Pseudomonas aeruginosa* C 32a produced the largest clearence zone with the glucanolytic index of 2.27, while temperature and pH optimum was 40^{o}C and pH 6, respectively. The antagonistic test of *P.aeruginosa* C 32a against *Pyricularia oryzae* and *Rhizoctonia solani* showed inhibition zones of 59.11% and 37.33%, respectively.

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