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Potential antifungal of Chitinolytic Bacteria *Pseudomonas sp* TNH54 from Mud Field

Nuniek Herdyastuti [1]*, Sari Edi Cahyaningrum [1], Tri Joko Raharjo [2], Mudasir [2] and Sabirin Matsjeh [2]

[1] Department of Chemistry, Surabaya State University, Ketintang Surabaya, Indonesia
[2] Department of Chemistry, Gadjah Mada University, Sekip Utara Yogyakarta, Indonesia

ABSTRACT

Bacteria isolated from mud field in the Ketintang Surabaya area showed chitinolytic activity in media containing 0,4 % colloidal chitin. Morphological and phisiological characteristic showed Gram negative, rod-shape kokoid, can produce acid from mannitol, sucrose, sorbitol, innositol also able to oxidize glucose. Based on the nucleotide sequence of 16S-rRNA gene showed 98 % genetic relationship to *Pseudomonas sp*. Molecular weight chitinase determination by SDS-PAGE and zymogram have size 26.1 and 29.0 kDa. Extraselluler chitinase can showed inhibition of growth of *Aspergillus parasiticus* fungi that are pathogenic on rice and potatoes.

Keywords: Anti-fungal, Chitinolytic Bacteria, Mud field, *Pseudomonas sp*.

Corresponding author

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INTRODUCTION

Chitinolytic bacteria are bacteria that can degraded chitin using chitinase enzyme. Tese bacteria can be obtained from water environments such as oceans, lakes, ponds, shrimp waste and so on. In addition on mesophyll enviroment, chitinolytic bacteria also have been isolated from termophyllic environments such as hot springs, geothermal areas and others. Chitinase producing bacteria is still not widely known either about the number, diversity and function of chitinase produce, although chitin which a polymer abundance in nature. Some microorganism of chitinolytik from various sources have been isolated and characterized. Chitinolytik bacteria from Jeziorak on the surface of the lake water and bottom sediment layer such as Pseudomonas sp, Alkaligenes denitrificans, Agrobacterium sp, Aeromonas hydrophila that chitin degraded and utilized N-acetyl-glucosamine as a carbon source [5]. Exploration of soil chitinase has also been carried out to obtain the types of microorganism [1, 4, 18, 19]

Chitinase enzymes can be utilized in plants as a defense against pathogenic organisms attack (in this fungus) that contain chitin. As it is known that nearly 3 – 60 % yeast cell wall composed of chitin. Chitinase enzyme has the ability to degrade cell walls and or prevention of hyphal growth in fungi. The use of biological agent made from raw biofungisida be an appropriate alternative for the control of microbial pathogens. This is because the control of disease with synthetic fungicide and bactericide has been ineffective, because it raises the issue of harm to human life directly or indirectly. Bactericide or fungicide can cause residue attached to the plant so that it will harm the health of consumers, environmental pollution and kill other non-target organisms [17]. That phenomenon led researchers to develop products biofungisida friendly to the environment such as chitinase enzymes [7, 9, 16].

MATERIAL AND METHODS

Materials used are: bacto agar (Difco), bacto-tripton, yeast extract, Chitin isolated from shrimp shells. Pa purity chemicals: 3,5-dinitrosalisilat acid (SIGMA), N-acetyl-glucosamine (SIGMA), chitin are biosynthesized of glycol chitosan (SIGMA).

Organism and Cultural Condition

Pseudomonas sp TNH 54 was isolated from field mud in Ketintang area Surabaya by using a minimal medium containing colloidal chitin. The strain was maintained at medium contained : 0.4 % colloidal chitin, 0.7 % K_2HPO_4, 0.3 % KH_2PO_4, 0.5 % $MgSO_4.5H_2O$, 0.01 % $FeSO_4.7H_2O$, 0.001 % $MnCl_2$ and 0.5 % peptone, incubated at room temperature for 40 h on a rotary shaker (150 rpm). The supernatants were collected for measurements of chitinase activity.

Preparation of colloidal chitin

Colloidal chitin was prepared according to the method of Hsu and Lockwood (1975). Chitin powder was dissolved in 400 mL of concentrated HCl by stirring for 30 to 50 min. The chitin was precipitated as a colloidal suspension by adding it slowly to 2 liters of water at 5 to 10° C. The suspension was collected by filtration with suction on a coarse filter paper and then washed by suspending it in about 5 liters of tap water and refiltering. The washing was repeated at least three times or until the pH of the suspension was about 3.5.

Crude enzyme production

After incubation, the culture cells were centrifuged (10000 x g, 4 °C for 20 min). The supernatant was brought to 0 – 50 % saturation with ammonium sulphate (4 °C, for 30 min) stirring by magnetic stirer. The precipitate was recovered by centrifugation (4 °C 6000 x g for 30 min) and pellet formed was solubilized in 0,1 M phosphate buffer pH 7.0.

Chitinase Assay

Chitinase activity was measured by colorimetric medhod based on released N-acetyl-glucosamine (Monreal and Reese, 1969). The colloidal chitin solution 2.0 mL of 1.25% (w/v) dissolved in 200 mM potassium phosphate buffer was added to 0,5 mL enzim solution and incubated for 2 h at room temperature. After two hours, place the vials in to a boiling water for 5 minutes and cool to room temperature by placing the vials in a cold water bath. The suspension was centrifugated (4 °C, 6000 x g, for 10 min). Place the vials 1.0 mL supernatant was added 2.0 mL deionized water and 1.5 mL color reagent solution which containing 5.3 M sodium potassium tartrate and 3,5-Dinitrosalicyclic acid 96 mM. Place the mixed solution in to a boiling water for 5 min and remove and allow the containers to cool at room temperature. Transfer the solution to suitable cuvettes and record the absorbance at 540 nm. One unit activity will liberate 1.0 mg N-acetyl D-glucosamine from chitin per hour.

Protein Determination

Protein was determined by the Bradford method at 595 nm using Coomassie Briliant Blue G-250 with bovine serum albumine as a standart [2].

Electrophoresis and Zymogram

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970), using 12 % gel. After electrophoresis, gels were stained either with Coomassie brilliant blue R-250. The chitinolytic activities were detected on gels by zymogram using 0.1 % glycol chitin from acetylation of glycol chitosan (Trudel and Asselin, 1990). After electrophoresis, the gels were incubated for 2 h at 37 °C in 100 mM sodium acetate buffer, pH 5.0 containing 1 % (v/v) Triton X-100. The gels were then stained with 0.01 %

Calcofluor white M2R in 0.5 M Tris-HCl, pH 8.9 for 7 min, and destained with aquadesh. The lytic zones were photographed under the UV-transilluminator.

Antifungal assay

Antifungal activity was tested by using a modification of the bioassay described by [9]. Paper dish (6 mm in diameter) with the Aspergillus parasiticus which was growing state previously cultured on PDA (Potato dextrose agar), was placed in the center of a petri dish. 20 µL of chitinase enzyme was added the a paper dish. The growth of A.parasiticus was inhibited by addition of the enzyme as compared to posphate buffer without enzyme. The plates were incubated for 72 h at room temperature and then photographed.

Identification of bactery

Fenetik classical systems based on shared phenotypic characteristics including morphological characteristics (shape, colony size and staining reaction), physiological characteristics (need for CO_2, O_2, vitamins, biochemical assays), ecological characteristics and serology. The results were then compared with the Bergey's Manual of Systematic Bacteriology to determine the species of microorganism. Physiological stages of identification using Identification kits Microbact^{TM} GNB (Gram Negative Bacilli) 12ª/B/E, 24E – Oxidase Positive (Oxoid). 16S rRNA gene-identification is done by stages of DNA isolation, DNA amplification by PCR, electrophoresis results of PCR, PCR purification and sequencing results consisting of cycle sequencing, purification of the sequencing, capillary electrophoresis with sequencing and analysis of results.

RESULT AND DISCUSSION

Microorganisms that have been isolated from mud fields in the screening media containing 0.4% colloidal chitin showed chitinase activity after incubation for 40 hours. The results of morphological and physiological tests one of the isolates showed chitinase activity are shown in Table 1.

Based on morphology and physiology of bacteria test results show the isolation of the mud fields are Gram negative, rod – kokoid cell shape, having colony of yellow, round, convex elevation, and the average margin of colony diameter 3 – 5 mm, could produce acid from mannitol, sucrose , sorbitol, innositol also able to oxidize glucose. The analysis is based on the nucleotide sequence of 16S-rRNA gene showed 98% genetic relationship of the Pseudomonas sp (Fig. 2). Chitinase from the same genus produced by Pseudomonas sp TKU008 obtained from soil in Taiwan [20] also Pseudomonas sp LB-1 from red algae in the waters of Haenam, Korea [14].

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Table 1. Characteristic of morphology and Phisiology chitinolityc microorganism from mud field

Characteristic	Result
Morphology	
Shape	Rod– kokoid, 3-5 mm
Staining gram	Negative
Spore formation	Negative
Motility	Positive
Phisiology of characteristic	
Lysin dekarboksilase	Positive
Arginin dehydrolase	Positive
Ornithine decarboxylase	Positive
Tryptophan deamynase	Negative
Voges proskauer test	Positive
Gelatin liquefaction	Negative
Hidroyisis Esculin	Negative
Hidrolysis Urea	Positive
Indol formation	Negative
Production of H_2S	Negative
Use of Sitrate	Positive
Inhibition of Malonate	Positive
Acid from :	
Glucose	Positive
Sorbitol	Positive
Lactose	Negative
Raffinose	Positive
Mannitol	Positive
Rhamnose	Negative
Arabinose	Positive
Salysin	Positive
Xylose	Positive
Innositol	Positive
Sucrose	Positive
Adonitol	Negative

Production of chitinase from Pseudomonas sp TNH54 precipitated with ammonium sulphate 0-50% with a purity of 1.3 times compared to prior fractionation. Estimation of molecular weight by SDS-PAGE and zimogram indicate the size of 26.1 and 29.0 kDa (Fig. 3). Both bands produced an allegedly because both isoenzymes can hydrolyze chitin into simpler compounds. Chitinase enzymes from bacteria have a molecular weight size varies from 20-110 kDa. Some of the bacterial chitinase reported to have molecular weights of different sizes vary. Chitinase enzymes reported to consist of three types, namely chitinase Chia, ChiB and chic as found in Serratia marcescens [21]. As reported by the combination of Chia and Chia and ChiB and Chic showed synergism in both hydrolyze insoluble substrates (Brurberg et al., 2000). Pseudomonas aeruginosa K-187 has a size 30 and 32 kDa [19]. Chitinase produced by Streptomyces sp size of 20 kDa [12]; Bacillus cereus 28-9 produce chitinase to the size of 37 kDa

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[11]Pseudomonas fluorescens produce chitinase with sizes 33, 37, 55, 56, 65 and 69 [15]; chitinase from Bacillus sp DAU101 with a molecular weight of 66 kDa [13]. Chitinase enzymes from bacteria have a molecular weight size varies from 20-110 kDa. Some of the bacterial chitinase reported to have molecular weights of different sizes vary.

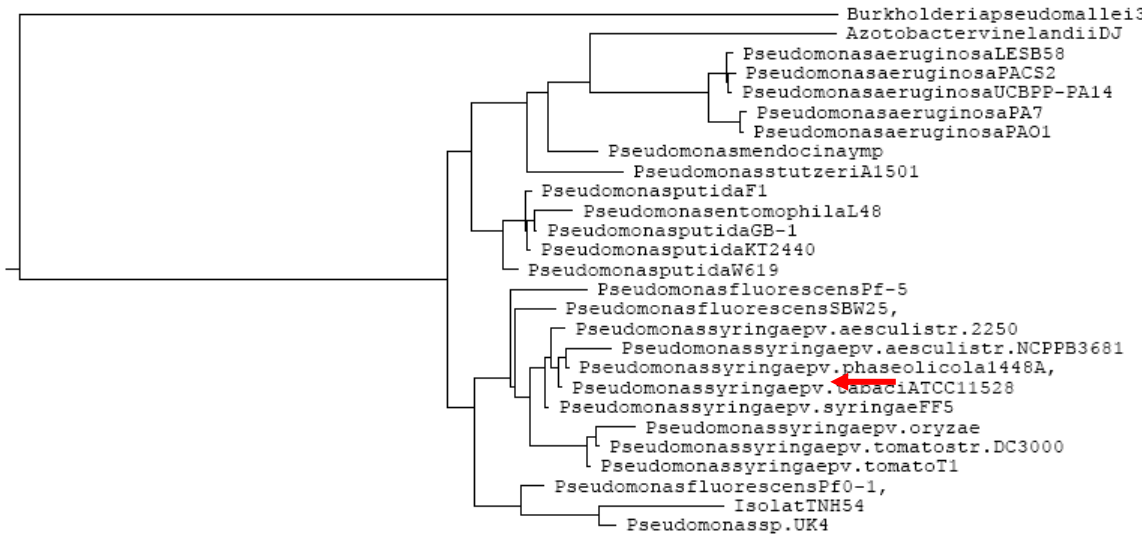

Fig 2. Phylogenetic tree of bacteria isolated from the mud of the paddy fields (TNH54 isolates) showed kinship with Pseudomonas sp UK4 to 98% nucleotide sequence similarity

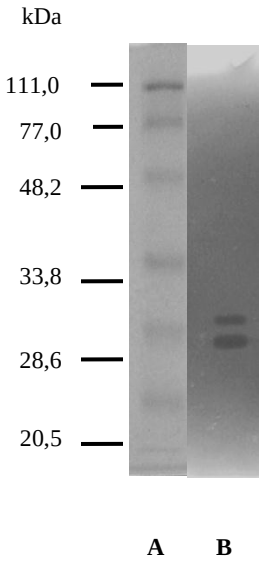

Fig 3. SDS-PAGE chitinase of Pseudomonas sp stained with Comassie Blue R-250; A : marker, B : crude extract enzyme, analysis with 0.1% glycol

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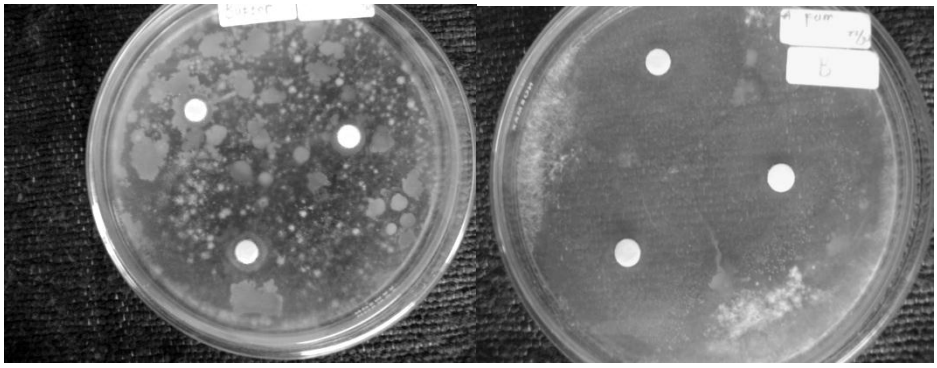
Inhibition of fungal growth by purified chitinase

The test results chitinase activity against fungal pathogens A. parasiticus showed significant inhibition compared with controls (Fig. 4). Fungus of A. parasiticus are pathogenic on potato and rice crops. The use of bacteria as biocontrol fitopatogenik kitinolitik to fungi has been carried out. Lysis process mainly lies in the production of lytic enzymes such as chitinase and glucanase that can lead to degradation of fungal cell walls. This activity is based on the ability of extracellular chitinase enzyme to degrade the cell wall and or prevention of growth of the hyphae [6]. A number of biocontrol agents Trichoderma sp kitinolitik microorganisms and Pseudomonas sp has been successfully used as a biocontrol on several pathogenic fungi[6, 7, 9, 15].



A B

Fig 4. The test results as an anti-fungal activity of chitinase in A.parasiticus (A) were incubated for 4 days and (B) is control.

CONCLUSION

Based on the analysis of bacterial 16S rRNA kitinolitik-fields of mud has a relationship with the Pseudomonas sp. Chitinase with a size of 26.1 and 29.0 kDa are thought as isozyme to act synergistically to inhibit the growth of fungi and could A.parasiticus.

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