MOLEKUL
eISSN: 2503-0310

Articles
https://doi.org/10.20884/1.jm.2024.19.1.9422

Chitinase Enzyme-Producing Endophytic Bacterias From the Roots of the Plant Gembolo (*Dioscorea bulbifera*): Isolation, Characterization and its Potential as an Antifungal Agent

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Received September 06, 2023; **Accepted** January 09, 2024; **Available online** March 20, 2024

ABSTRACT. Chitinase is an enzyme of the chitinolytic group that has many roles in agriculture, especially as an antifungal, because chitin is one of the constituent components of the fungal cell wall. This study aimed to isolate and identify endophytic bacteria from gembolo (*Dioscorea bulbifera)* roots and to characterize the chitinase enzyme from these endophytic bacteria to be used as an antifungal. Isolation and identification of gembolo plant root endophytic bacteria using PCR method of 16S rRNA gene amplification and sequencing. Characterization of the chitinase enzyme produced includes determining of optimum pH, temperature, and substrate UV-Vis spectrophotometer. Antagonistic test of the chitinase enzyme and endophytic bacterial isolates (isolate K4) *Fusarium oxysporum*. The results showed that bacterial isolate K4 had with chitinolytic index of 2.45 mm. Electrophoresis results with PCR 16s rRNA gene; the length of the amplified fragment is the position of 1300 bp. By doing the BLAST process in GenBank, the bacterial isolate has 97.93% similarity with *Enterobacter cloacae*. Then, this endophytic bacteria is called *Enterobacter cloacae* K4-G. This bacterium produced chitinase enzyme reaching maximum chitinase activity at the 38 hours with an activity of 0.0312 U/mL. The chitinase characterization results of *E. cloacae* K4-G showed that the optimum conditions were reached at at pH 6, temperature 45 ^{o}C, and 2.5% substrate with a chitinase activity value of 0.2467 U/mL. Chitinase enzyme and bacteria *Enterobacter cloacae* K4-G can inhibit the growth of the fungus *Fusarium oxysporum*. Therefore, Chitinase from *Enterobacter cloacae* K4-G can be used as an antifungal pathogen in plants.

Keywords: Chitinase, endophytic bacteria, *Enterobacter cloacae*

INTRODUCTION

Maintaining crop productivity and minimizing losses due to fungal pathogens is a significant challenge as the human population grows and the demand for agricultural products increases. In order to cope with these challenges, the use of chemical pesticides has become a common practice in modern agriculture. However, the adverse effects of chemical pesticide use on human health have become a significant concern. Cancer, disability, and infertility are among the known risks of exposure to chemicals (Kapka-Skrzypczak et al., 2018). Therefore, environmentally friendly and sustainable solutions are needed to control plant diseases. One promising approach is the use of endophytic bacteria, microorganisms that live symbiotically in plant tissues, as biological control agents.

Recent studies have shown that endophytic bacteria have great potential for enhancing plant disease resistance. They not only stimulate plant growth but also act as bioprotectants against pest and disease attacks (Pradana et al., 2022). Several studies, including research by (Chaouachi et al., 2021), have found endophytic bacteria in tomato plants that produce volatile organic compounds (VOCs) with significant antifungal activity. The success of these endophytic bacteria in controlling plant diseases can be primarily attributed to the production of chitinase enzymes.

Chitinase (EC 3.2.1.14) is an enzyme with the ability to hydrolyze chitin, a major component of fungal cell walls containing chitin compounds. In the context of plant disease control, chitinase acts as an antifungal agent that attacks pathogenic fungi by degrading chitin. Chitinase enzymes are found in a wide variety of organisms, including bacteria, fungi, insects, plants, and animals. The presence of chitinases in plants is part of a natural defense mechanism, where these enzymes trigger the production of secondary metabolites and activate plant defense responses against pathogen attack (Gasmi et al., 2019; Gohel et al., 2006; Nguyen et al., 2015).

The gembolo (*Dioscorea bulbifera*), a monocotyledonous plant of the genus Dioscorea, is a plant of interest for this study. The tuber of gembolo contains carbohydrates and glucomannan as its main components. In addition, gembolo has potential as an analgesic, aphrodisiac, and diuretic. The compound dihydrorodioscorine extracted from this plant has been shown to have antifungal properties (Natori et al., 2022). Under conditions of nutrient deficiency, gembolo plants produce secondary metabolites in response to self-defense, which can also inhibit the growth of pathogenic microorganisms in plants (Kuete et al., 2012).

Based on this background, this study aims to isolate, identify, and characterize chitinase enzymes from endophytic bacteria from the roots of gembolo (*Dioscorea bulbifera*) plants. This research will also explore the potential application of this chitinase enzyme as an antifungal agent in plant disease control. It is hoped that this research will provide new contributions to the development of more effective, sustainable, and environmentally friendly methods of plant disease control.

In a broader context, this research is expected to not only provide solutions to the use of chemical pesticides in agriculture but also support sustainable agriculture that is not only productive but also environmentally friendly. By understanding and harnessing the potential of endophytic bacteria and chitinase enzymes in agriculture, we can create a healthier agricultural system for both humans and the environment.

EXPERIMENTAL SECTION

Materials and Instruments

Gembolo root (*Dioscorea bulbifera*) as a source of chitinolytic microorganisms, colloidal chitin as an enzyme substrate, yeast extract, bacto agar, bacto peptone, peptone, Potato Dextro Agar (PDA), Aqua Pro Injection, NaOH, HCl, $(NH_4)_2SO4$, NaH_2PO_4, Na_2CO_3, K_2HPO_4, NaCl, $CuSO_4.5H_2O$, $K_3[Fe(CN)_6]$, $MgSO_4.7H_2O$, N-acetyl-glucosamine, reagen schales. Laminar Air Flows Cabinets (LAFC), Analytical balance (Ohaus), autoclave (model 8000-DSE Napco), shaker water bath (Memmert), spectrophotometer UV-Vis Genesys 20, Polymerase chain reaction (PCR) machine Bio-Rad.

Isolation of Endophytic Bacteria from Gembolo Roots

Isolation of endophytic bacteria was carried out using gembolo plant roots washed with distilled water. Root surface sterilization was carried out in LAFC. Gembolo plant roots were then crushed with the addition of 3 mL of aqua pro injection. Next, pipetted as much as 1 mL into *Luria Broth* (LB) medium, then shaken for 24 hours. The LB media suspension was then diluted 5 times. Each dilution was then spread on the surface of the Luria Agar (LA) medium and incubated at 37°C for 4 days. Colonies that grew well were then purified by re-culturing on LA media and then incubated at 37°C. After obtaining a single isolate, it was then scratched again in the chitinolytic selective medium of chitinase-producing bacteria and incubated for 3 days at the optimum temperature (Bahmani et al., 2021).

Isolation of Total DNA from Bacteria

Isolation of total DNA from bacterial isolates was carried out as follows: selected symbiont bacterial isolates with known morphology were cultured in LB (Luria Bertani) medium for 14-16 hours at 37°C with a centrifugation speed of 170 rpm. A total of 10 mL of bacterial suspension was centrifuged at 6000 rpm for 2 minutes at 4°C. The supernatant (upper liquid) was discarded, and the bacterial pellet was washed by adding 1 mL of TE 1x buffer solution and centrifuged at 6000 rpm for two minutes at 4°C. Next, the pellet was taken and 760 µL of 1x TE buffer solution, then 10 µL of lysosime was added. For optimal results, it was incubated at 37°C for 130 minutes, and then 40 µL of 10% SDS and 10 mg/mL proteinase K were added. The tube was closed, gently inverted, and incubated at 37°C for one hour. Next, 100 µL of a mixture of 10% CTAB in 5 M NaCl that had been heated at 65°C was added and incubated for 20 minutes. After that, 500 µL of the mixture (phenol:chloroform: isoamyl alcohol = 25:24:1) was added and vortexed, then centrifuged at 10,000 rpm for 10 minutes. The supernatant was pipetted and placed in a new micro-tube. Then 600 µL of cold propanol was added and incubated at 20°C for 20 minutes and centrifuged at 10,000 rpm for two minutes. The supernatant was discarded, and the pellet (DNA) was dried. Then, 50 µL of ion-free water was added and stored at -2°C (Tamura et al., 2011).

16S rRNA Gene Duplication and Analysis

This DNA amplification uses the Polymerase Chain Reaction (PCR) method, usually known as chain polymerization. Purified template DNA was as much as 0.4-0.5 µl. (±200 ng) was duplicated with Ready-to-Go PCR Beads Kit (Pharmacia, Biotech) using bacterial universal primers viz: Forward (5'-CCA GCA GCC GTA ATA CG-3) and reverse (5'-ATC GGC TAC CTT GTT ACG ACT TC-3) mixture of 18.5 µL ddH2O; 2.5 µL buffer, 1.0 µL primer 63f; 1.0 µL 1387r; 0.5 µL dNTP mix 1.0 µL DNA polymerase; 0.5 µL template DNA was put in an eppendorf tube, then put into PCR (Gene Amp PCR System) under the conditions: Pre-PCR (94 °C, 2 min), denaturation (94 °C, 30 s), anneling or primer attachment (42 °C, 30 s), elongation or primer extension (72 °C, 1 min) for 30 cycles, and post-PCR (75 °C, 5 min) (Tamura et al., 2011).

Production of Extracellular Chitinase Enzyme

Production of the chitinase enzyme was started by preparing an inoculum medium (yeast extract 0.5%, peptone 0.1%, $CaCl_2$ 0.01%, KH_2PO_4 0.01%, NaCl 0.1%, and $MgSO_4.7H_2O$ 0.01%) for starter containing 0.5% colloidal chitin, then bacteria with the highest

chitinolytic index were inoculated into the starter medium and incubated on an incubator shaker at 37 ºC at 180 rpm for 24 hours. Starter medium as much as 10% (v/v) was inoculated into the production medium (yeast extract 0.5%, bacto peptone 0.1%, NaCl 0.1%, KH_2PO_4 0.01%, $MgSO_4.7H_2O$ 0.01%, $CaCl_2$ 0.01%, colloidal chitin 0.5%) and then incubated for 4 days at the same temperature and speed for the starter medium and every 12 hours sampling was carried out to measure optical density at a wavelength of 660 nm. The fermentation product was cold centrifuged at 4 ºC at 4500 rpm for 15 minutes. The supernatant was obtained, followed by measuring the chitinase activity and determining its protein content using the Lowry method (Natsir et al., 2013).

The Optimization (pH, Temperature, and Substrate)

A mixture of 200 µL of chitinase enzyme supernatants, 200 µL of the substrate, and 200 µL of 0.1 M citrate buffer and 0.1 M phosphate buffer at various pH ranges (4.0, 5.0, 6.0, 7.0, and 8.0), for various temperatures (40, 45, 50 and 55 °C) and various colloidal chitin concentration (2%, 2.25%, 2.5%, 2.55% and 2.6%) was homogenized and then incubated for 30 minutes at 45ºC. The enzymatic reaction was stopped by placing it in a 100°C water bath for 15 minutes. Then 400 μL was taken into a test tube, 1600 μL of distilled water, and 2 mL of Schales reagent, then covered with aluminum foil and heated until boiling, centrifuged at 3500 rpm for 10 minutes at 4°C, and measured the absorbance at the wavelength 419 nm (Natsir et al., 2010).

Enterobacter cloacae K4-G Antagonist Test against *Fusarium oxysporum*

Antagonism test of *Enterobacter cloacae* bacteria in vitro was carried out on PDA media (Potato Dextrose Agar). *E. cloacae* K4-G bacteria that have been cultured for 48 hours, then placed pure culture of the fungus *Fusarium oxysporum* using a preparation needle between *E. cloacae* K4-G bacteria. Furthermore, it was incubated for 7 days. The same method was carried out using bacterial isolate that did not have chitinase enzyme activity as negative control. (Diarta et al., 2016)

Chitinase Enzyme Antagonist Test against *Fusarium oxysporum*

Chitinase enzyme activity test against bacteria was tested in vitro PDA media. The antagonist activity on chitinase crude extract using agar diffusion method. Chitinase crude extract was put into wells as much as 20 μL at a distance of at least 3 cm from the place of pathogenic fungi on PDA media. Incubation was carried out for 10 days at room temperature (±25°C). The same method was used using sterile distilled water as a negative control (Astika., 2022).

RESULTS AND DISCUSSION

Isolation and Identification of Endophytic Bacteria from the roots of Gembolo Plant (*Dioscorea bulbifera*)

Endophytic bacteria isolated from plant roots originate from the rhizosphere because the bacteria can approach the root surface as a producer of exudate, namely plant roots. Bacterial isolates have varied shapes based on their morphology. The results of isolation from the roots of the gembolo plant (*Dioscorea bulbifera*) were obtained, namely K4 isolates, which were selected in terms of macroscopic observations and their inhibition zone with a chitinolytic index of 2.45 mm. The wide inhibition zone around the colony indicates a high chitinolytic index. The inhibition zone shows the breaking of the β-1,4 bond of the N-acetylglucosamine homopolymer in chitin by chitinase to become N-acetylglucosamine monomer (Setia and Suharjono., 2015). The results (Azizah et al., 2017) showed that the chitinolytic index obtained was 2.3 mm from *Serratia marcescens* KAHN.15.12. Differences in the chitinolytic index are due to the degree of adaptation of bacteria to the media or environment and are caused by different types of bacteria.

Identification of endophytic by PCR and DNA sequencing. PCR results produced amplicons measuring 1300 bp. These results can be seen in **Figure 1.**

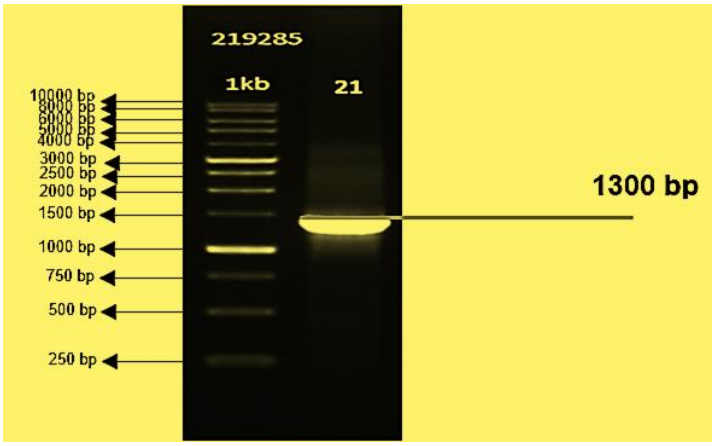

Figure 1. PCR results from K4 bacterial isolates using the 16S rRNA gene fragment (M = 1 kb plus ladder)

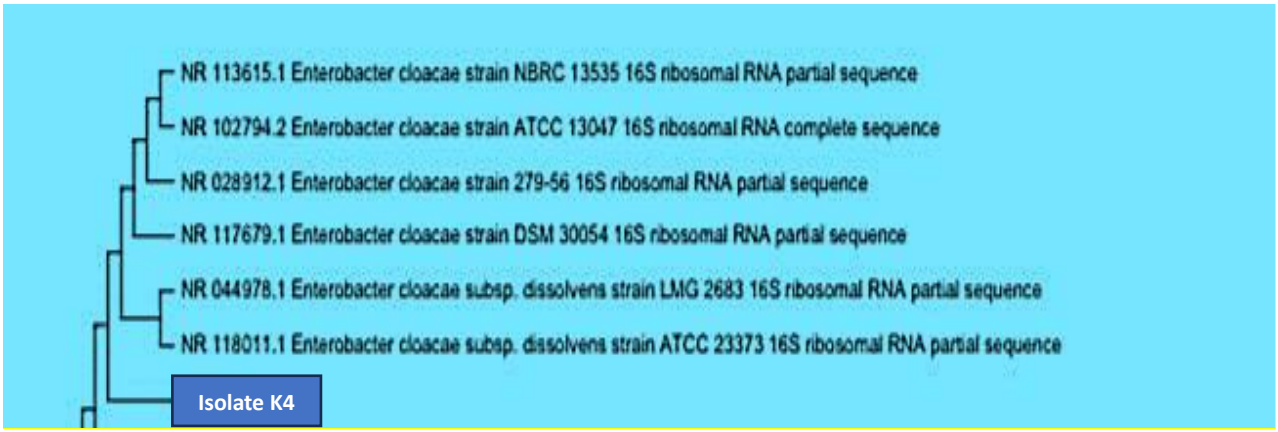

Figure 2. The phylogenetic tree of isolate *Enterobacter cloacae* K4-G obtained from the NCBI database based on the 16S rRNA gene sequence

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Enterobacter cloacae strain ATCC 1304 16S ribosomal RNA, complete sequence	Enterobacter cloacae	254	254	12%	1.00E-66	97.93	1543	NR_102794.2
Enterobacter mori strain YIM Hb-3 16S ribosomal RNA, partial sequence	Enterobacter mori	254	254	12%	1.00E-66	97.93	1522	NR_146667.2
Enterobacter wuhouensis strain WCHEs120002 16S ribosomal RNA, partial sequence	Enterobacter wuhouensis	254	254	12%	1.00E-66	97.93	1536	NR_180450.1
Scandinavium goeteborgense strain CCUG 66741 16S ribosomal RNA, complete sequence	Scandinavium goeteborgense	254	254	12%	1.00E-66	97.93	1542	NR_180446.1
Enterobacter huaxiensis strain 090008 16S ribosomal RNA, partial sequence	Enterobacter huaxiensis	254	254	12%	1.00E-66	97.93	1370	NR_180236.1
Enterobacter sichuanensis strain WCHECL1597 16S ribosomal RNA, partial sequence	Enterobacter sichuanensis	254	254	12%	1.00E-66	97.93	1528	NR_179946.1
Enterobacter asburiae strain JM-458 16S ribosomal RNA, partial sequence	Enterobacter asburiae	254	254	12%	1.00E-66	97.93	1422	NR_145647.1

Figure 3. Results of BLAST analysis at NCBI

The sequencing results were used to obtain sequences with high similarity in the Gen Bank using the BLAST *nucleotide through database* (NCBI) method to identify possible genera isolates based on homology. The identification results showed that isolate K4 had a 97.93% similarity with *Enterobacter cloacae* called *Enterobacter cloacae* KG-4 can be seen in **Figure 2** and **Figure 3.**

E. cloacae bacteria is a gram-negative proteobacterium in the *Enterobacteriacea* family (Yuliar et al., 2019). *E. cloacae* strain is used as an effective antagonist for biological control of plant pathogens (Takikawa et al., 2002). *E. cloacae* are involved in degrading chitin, of which there are four chitins and two N-acetyl-glucosamines. In addition, *Enterobacter cloacae* have a gene that codes for one monooxygenase lytic polysaccharide and one polysaccharide deacetylase, which have a role in chitin defamation (Mallakuntla et al., 2017). *E. cloacae* has a catalytic domain in producing chitinase (Sivaramakrishna et al., 2020). Based on research (Yuliar et al., 2019), the *E. cloacae* strain, with the addition of copper, can suppress plant diseases, and the addition of manganese reduces plant diseases due to *R. solani* by 85%. In addition, based on the research of (Liu et al., 2007), *Enterobacter cloacae* can inhibit the growth of the fungi *Fusarium* moniliforme and *Fusarium oxysporum* so that it is considered a plant growth-promoting *Rhizobakter* (PGPR).

The Production of Chitinase Enzyme and Protein Level by Lowry Method

Production of the chitinase enzyme was carried out by *E. cloacae* K4-G bacteria, which has a chitinolytic index of 2.45. Sampling was carried out on the production medium for 12 hours with chitinase activity values from the culture supernatant at 0 to 24 hours, undergoing an adaptation phase. For some time, the bacteria *E. cloacae* K4-G began to adapt to their environment. The adaptation phase time for bacteria varies depending on the composition of the media, pH, temperature, aeration, the number of cells in the inoculum, and the physiological characteristics of the microorganisms in the medium (Abna & Fauzi, 2023). The exponential phase occurred at 36 hours and was optimal at 48 hours with a chitinase activity value of 0.0312 U/mL, which was marked by a period of rapid growth. The effect of incubation on protein and chitinase enzyme activity can be seen in **Figure 4**.

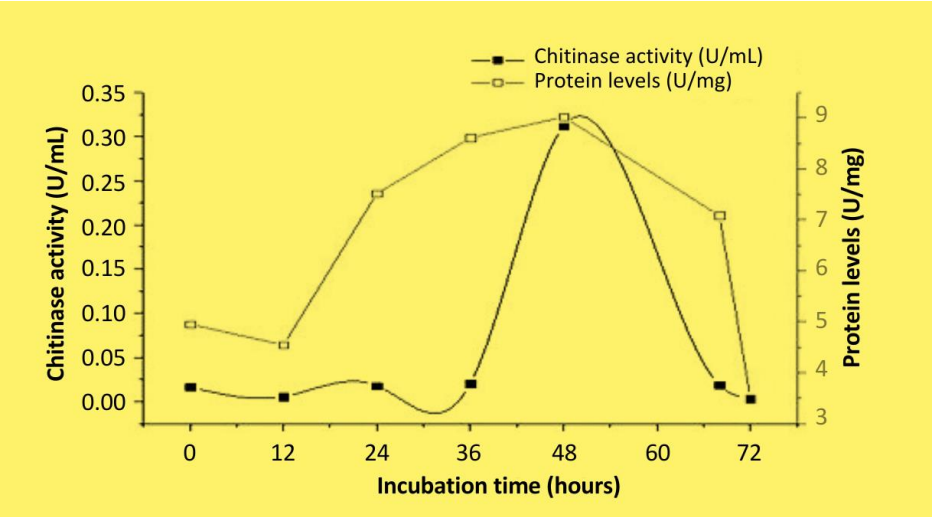

Figure 4. Effect of incubation time on protein content and chitinase enzyme activity of *Enterobacter cloacae* K4-G

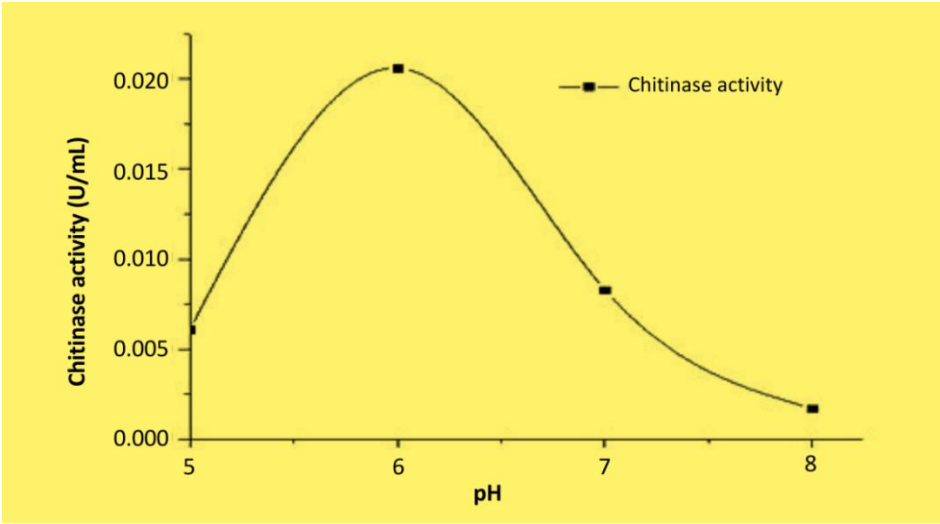

Figure 5. Effect of pH on chitinase activity at conditions: temperature 37°C, 1% substrate concentration

The highest protein content was obtained at the 48th hour of production with a value of 9.0184 mg/mL with a specific chitinase activity of 0.0034 U/mg. Meanwhile, based on research (Natsir et al., 2010), it was found that the protein content of isolate ST-3.2b, which was produced at the 40th hour, was 0.1021 mg/mL. Research (Yadi et al., 2020) found that the protein content obtained from *Bacillus cereus* 11 UJ isolate was 21390 mg with a specific activity of 0.00020 U/mg. To characterize the effect of pH on enzyme activity, phosphate buffer and acetate buffer were used with a pH of 5.0, 6.0, 7.0, and 8.0 at 45°C. Enzyme activity is influenced by various factors, one of which is the degree of acidity (pH). The effect of pH on chitinase enzyme activity can be seen in **Figure 5**.

The activity of the chitinase enzyme *Enterobacter cloacae* K4-G reached an optimum pH of 6.0 with an activity of 0.0206 U/mL. It decreased at pH 7.0 with an activity value of 0.0083 U/mL and a pH of 8.0 of 0017 U/mL. This result was similar to the study of (Noviendri et al., 2008). The maximum activity of isolate T5a1 was achieved at pH 6.0 phosphate buffer of 0.696 U/mL. Different from research (Soeka, 2009), the chitinase activity of isolate BB2.6 was highest in enzymatic reactions at pH 8 and 8.5, with each chitinase activity value of 3.8 x 10^{-2} U/mL and 3.06 x 10^{-2} U/mL. This difference is based on several factors, namely the environment, growth media, and nutrition. The activity of the chitinase enzyme decreased after the optimum pH due to changes in the state of the enzymes and substrates, which resulted in the denaturation of the enzymes accompanied by a loss of activity of the enzymes. The optimum pH aims to determine the use of enzymes that are in accordance with their characterization with the effect of pH on enzymes affecting the isoelectric point of enzymes. pH values that are too high or low will cause instability in the conformation of the enzymes, causing decreased enzyme activity (Yadi et al., 2013).

Enzyme characterization against the effect of temperature was carried out at a temperature range of 40°C -55°C. Chitinase activity values of *Enterobacter cloacae* K4-G from the results of temperature variations can be seen in **Figure 6.** The activity of the chitinase enzyme increased at 45°C with an activity value of 0.0199 U/mL and decreased to 55°C at 0.0083 U/mL. Based on the growth temperature, microbes are classified into five groups, namely psychrophiles growing at 5-20°C, mesophylls at 20-45 °C, thermophiles 45-65°C, extreme thermophiles 65-85°C and hyperthermophiles 85-100°C, so *Enterobacter cloacae* bacteria are classified as thermophiles. Based on (Soeka, 2009), the bacterial isolate BB2.6 belongs to the thermophilic group because the chitinase activity is 3.8×10^{-2} U/mL at 50°C. This result was also similar to (Selvia et al., 2013), whose highest chitinase activity was at an optimum temperature of 40.5°C with a value of 57.77 U/mL. Changes in temperature affect hydrogen bonds or hydrophobic interactions, which play a role in maintaining the conformation of the enzyme molecule because it affects the active site of an enzyme (Selvia et al., 2013). In addition, increasing the temperature until it reaches the optimum point causes an increase in the rate of enzyme reactions because it increases the energy of enzymes and substrates. However, if the enzyme is above the optimum temperature, the activity will decrease. This is because enzymes include proteins that will experience denaturation at high temperatures.

Enzyme characterization on the effect of substrate concentration was carried out at 45°C using colloidal chitin as a substrate. Based on the results of the study, the activity of the chitinase enzyme with varying concentrations of 2, 2.25, 2.5, 2.55, and 2.6% can be seen in **Figure 7.** Based on research by (Hardi et al., 2017), the optimum substrate concentration of the thermophilic bacterial isolate B1211 was produced at a concentration of 2.5% with chitinase activity of 0.2467 U/mL. One of the factors that influence enzyme activity is the substrate concentration. The higher the substrate concentration used, the higher the enzyme activity or the higher the reaction rate catalyzed by the enzyme (Noviendri et al., 2008).

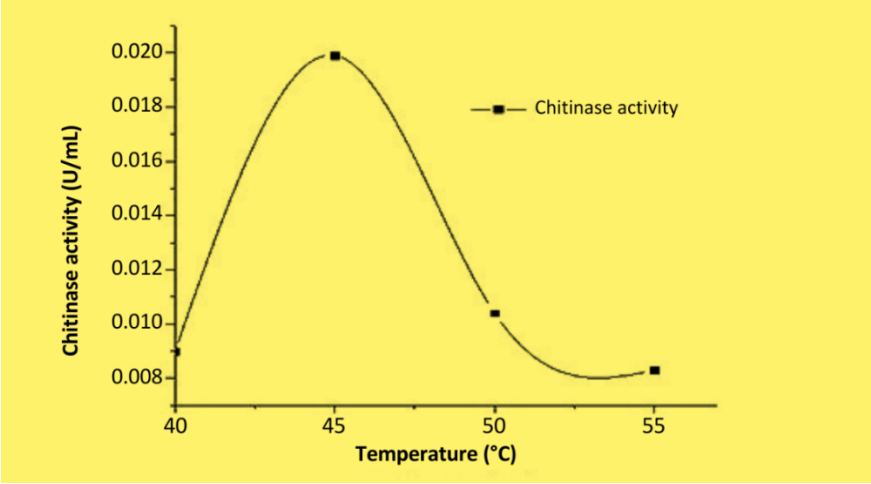

Figure 6. Effect of temperature on chitinase activity at conditions: pH 6, 1% substrate concentration

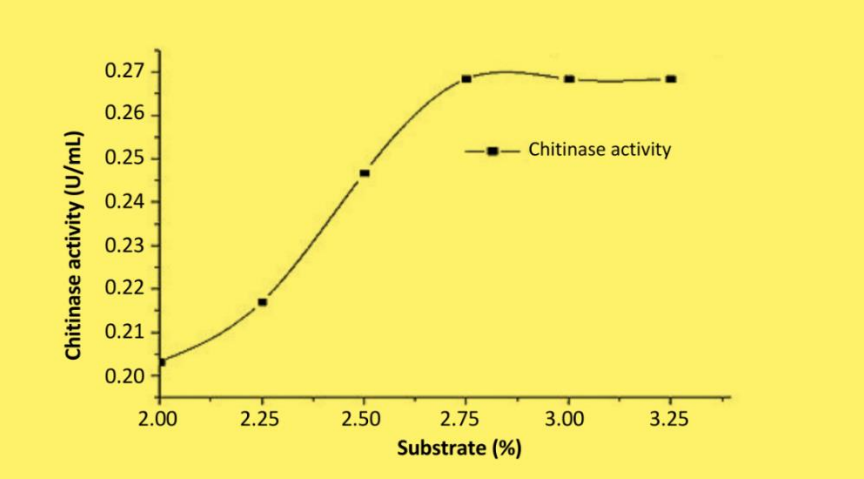

Figure 7. Effect of colloidal chitin substrate concentration on chitinase activity at pH 6 at 37°C

The results of the antagonist test showed that *Enterobacter cloacae* K4-G was able to inhibit the growth of *Fusarium oxysporum*. In contrast, the mycelium of the fungus *Fusarium oxysporum* tested with *Enterobacter cloacae* K4-G had an inhibition zone formed at the confluence of the two fungal colonies. *Fusarium oxysporum* tends to grow away from *Enterobacter cloacae* K4-G, which is found in the middle of the mushroom colonies. Mycelium growth, which tends to stay away from *Enterobacter cloacae* K4-G, is a Self-defense mechanism to avoid chitinolytic bacteria is also carried out to look for oxygen in the air (Ferniah et al., 2012). This result was compared to other bacterial isolates that have a low chitinolytic index value. The two fungal colonies tend to cover the bacterial isolates in the middle of the colony. The observation results can be seen in **Figure 8**.

Antagonistic mechanisms of *Enterobacter cloacae* K4-G in inhibiting the growth of pathogenic fungi include competition, parasitism, antibiosis, and lysis. Competition inhibition mechanisms occur if there is competition for nutrients between *Enterobacter cloacae* K4-G and *Fusarium oxysporum* fungi in the same media. Antibiosis and lysis mechanisms involve the production of toxic metabolites or extracellular enzymes produced by *Enterobacter cloacae* (Karim et al., 2020).

Chitinolytic bacteria can degrade chitin because they produce the enzyme chitinase. The product of chitin degradation in the form of the compound N-acetyl D glucosamine is then used as a source of nutrition for bacteria so that the bacteria grow to block the growth of the fungus *Fusarium oxysporum*. Bacteria produce bioactive compounds that are structural components of fungi (Ferniah et al., 2012). Based on research by Yurnaliza et al. (2011), the chitinase enzyme from *Streptomyces* RKt5 is able to inhibit the pathogen *Fusarium oxysporum*. In addition, according to (Wulandari & Zakiatulyaqin, 2012), endophytic bacteria from pepper plants are able to inhibit the growth of velvet blight pathogens.

The ability of the chitinase enzyme as an antifungal in different types of fungi. The fungus *Fusarium oxysporum* is more resistant to chitinase because its cell wall composition is different from that of *R. solani* or *Ganoderma*. The composition of the cell wall of the fungus *Fusarium oxysporum* on the outer layer contains glycoprotein compounds that protect the surface of the mycelium. While chitin and glucan are found in the inner layer, the glycoprotein content in the cell wall of *Fusarium oxysporum* is as much as 50-60% of the total cell wall mass (Yurnaliza et al., 2011). The results of the chitinase enzyme antagonist test for *Fusarium oxysporum* can be seen in **Figure 9.**

The description of degrading chitin involves the chitinase enzyme, which produces diacetyl chitobiose $(GlcNac)_2$. Furthermore, the enzyme 1,4-β-N-acetyl-glucosaminidase hydrolyzes the 1,4-β-glycoside bond to produce N-acetyl glucosamine (GlcNac), then further hydrolyzed by deacetylase to become glucosamine, as shown in **Figures 10** and **11**.

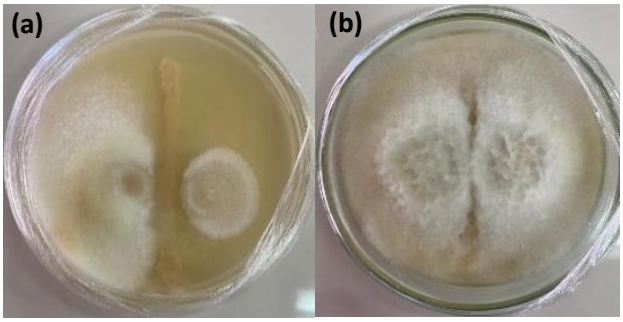

Figure 8. Antagonistic test results (a) *E. cloacae* K4-G with *Fusarium oxysporum* (b) isolates of low chitinolytic index bacteria with *Fusarium oxysporum*

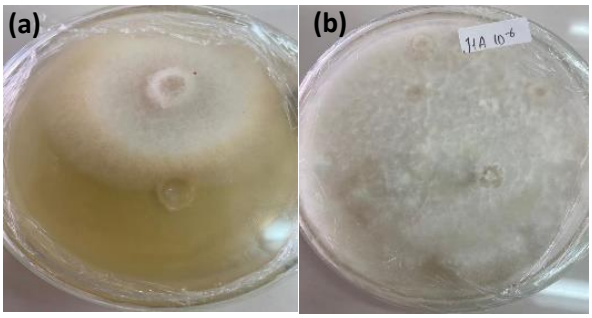

Figure 9. Chitinase enzyme antagonist test against *Fusarium oxysporum* (**a**) chitinase enzyme with pathogens (**b**) sterile distilled water as a control with pathogens

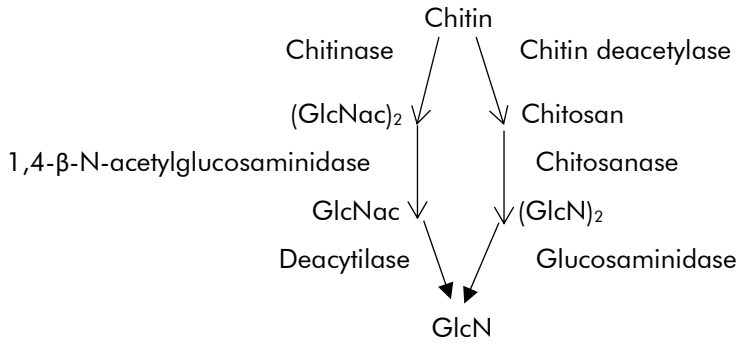

Figure 10. Chitin degradation pathway by microbes (Gooday, 1990)

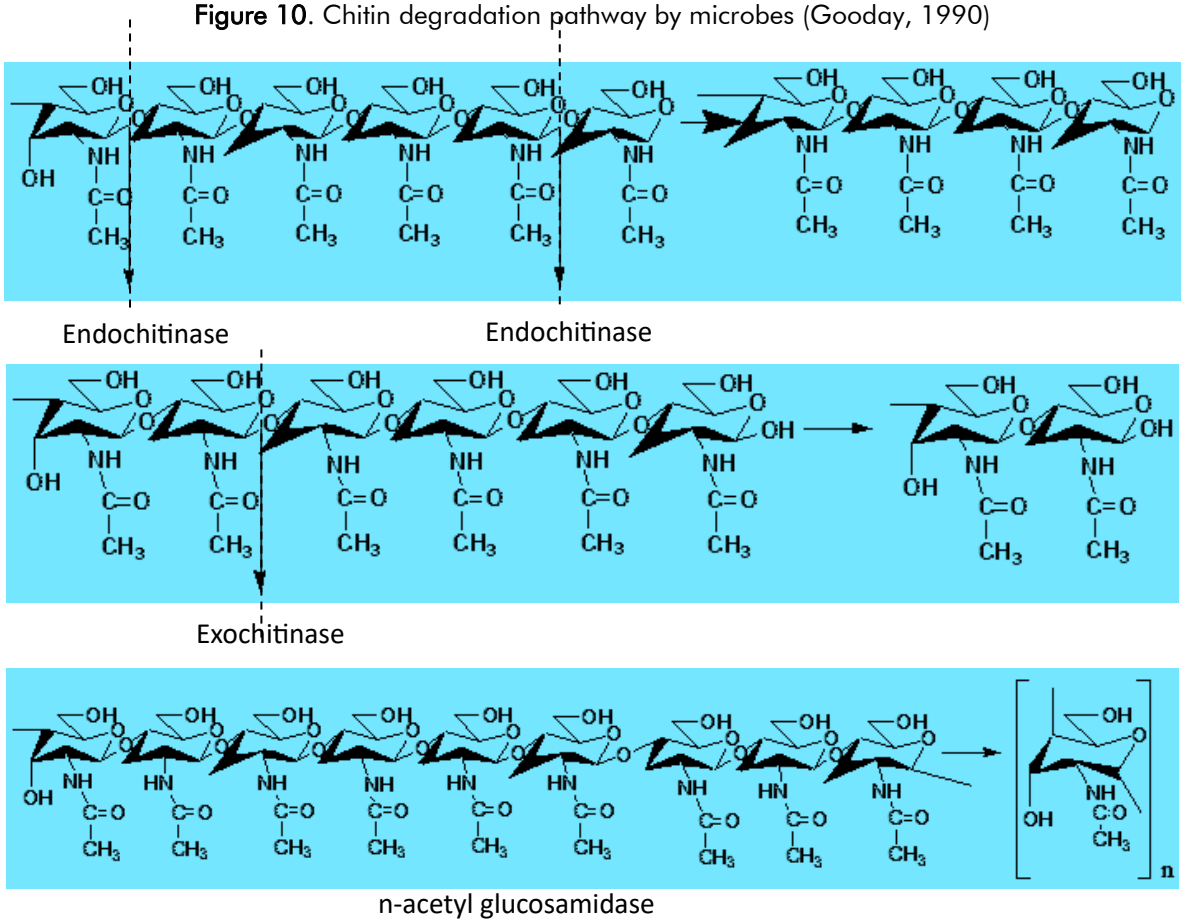

Figure 11. Illustration of hydrolysis process on chitin polymer by chitinolytic enzymes

Petri dishes containing the chitinase enzyme derived from *E. cloacae* K4-G were less able to inhibit the growth of *F. oxysporum* because the fungus *F. oxysporum* was able to grow around the chitinase enzyme during 10 days of incubation. Meanwhile, a petri dish containing the negative control contained the *F. oxysporum* fungus, which grew to fill the entire petri dish on day 10. This result was in line with the study of Yurnaliza et al. (2011) that the mycelium of the fungus *F. oxysporum* is difficult to penetrate by chitinase, and the activity of this enzyme will be effective if the mycelium mushrooms tested in the form of pieces. In addition, based on the research of Khairah et al. (2023), *P. aeruginosa* ABS 4.1.2 and its chitinase enzyme have the potential to be biocontrollers against phytopathogens, especially *F. proliferum*.

CONCLUSIONS

Based on the results of the research obtained, endophytic bacteria from the roots of gembolo plants (*Dioscorea bulbifera*) were isolated K4 with 97.93% similarity to the RNA of Enterobacter cloacae bacteria, called Enterobacter cloacae K4-G (*E. cloacae* K4-G). It has chitinase activity at the 48th hour, which is 0.0312 U/mL, with a protein content of 9.0184 mg/mL. Characterization of chitinase obtained optimum at pH 6, optimum temperature at 45°C, and optimum substrate concentration at 2.5% concentration with chitinase activity of 0.247 U/mL. *E. cloacae* K4-G bacteria was able to inhibit the growth of the pathogenic fungus *F. oxysporum.* At the same time, the chitinase enzyme was able to inhibit the growth of the pathogenic fungus *F. oxysporum*.

ACKNOWLEDGMENTS

The author would like to thank the staff of the Biochemistry Laboratory, Faculty of Science and Technology, Alauddin State Islamic University Makassar, Biochemistry Laboratory, Faculty of Mathematics and Natural Sciences, Hasanuddin University, and the Pests and Diseases Laboratory. Faculty of Agriculture, Hasanuddin University, for their support for this research.

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