



Production, purification and characterization of chitinase from *Micromonospora* sp. AR17

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ABSTRACT N-acetylglucosamine (NAG) is the monomer product of chitin, which has been widely used as a bioactive compound in applications such as anti-tumor, anti-microbial, and antioxidant activities. In production, biological processes using enzymes are preferable to chemicals due to environmental issues. This study aims to determine the activity, purity level, and molecular weight of purified chitinase from *Micromonospora* sp. AR17 determines the concentration of NAG produced by purified chitinase that has been characterized. Chitinase was produced by fermentation in colloidal chitin broth at 40 °C, pH 7, for 7 days, while chitinase activity was checked every 24 h. The optimal fermentation time was used to produce chitinase for a further purification step. Enzyme purification was carried out by ultrafiltration, ammonium sulfate precipitation, ion exchange chromatography (Q Sepharose Fast Flow), and gel filtration (Sephacryl S-300). The purified enzyme was then characterized for optimum time, pH, and temperature to produce NAG. The results suggested that the fourth day was the optimal time for chitinase production, with chitinase activity of 0.0040 U/mL and a NAG concentration of 7.62 µg/mL. The purification step successfully increased the purity by 6.82 times with chitinase-specific activity at 1.4648 U/mg. Production of NAG with purified chitinase produced a NAG concentration of 32.472 µg/mL with an incubation time of 30 min at 40 °C and pH 7.

KEYWORDS Chitinase; *Micromonospora* sp. AR17; N-acetylglucosamine; Purification

1. Introduction

Chitin, a linear polymeric polysaccharide composed of β-1,4 N-acetyl-D-glucosamine (NAG) monomers, is widely distributed in the Earth's biosphere, such as in crustacean shells (shrimp, crab, and lobster), structural components of the exoskeleton of insects, jellyfish, cell walls of fungi (22-40%), nematodes, plants, and algae (Qin and Zhao 2019). Chitin and its derivatives have high economic value because of their biological activity, which is widely used in industry and biomedical fields (Brzezinska et al. 2014). However, chitin has weaknesses, one of which is its relatively low solubility due to the large size of the chitin molecule (Roy et al. 2017). Therefore, chitin derivatives such as oligosaccharides or its monomer (NAG or GlcNAc) are preferred due to more soluble in water and have greater biological activity (Therien et al. 2019; Yang et al. 2019).

N-acetylglucosamine has been widely used as a bioactive compound, such as an anti-tumor (Zeng et al. 2014), antibiotic and antioxidant (Wyllie et al. 2022; Azam et al. 2014). The potential development of N-

acetylglucosamine causes its demand to increase. Production of N-acetylglucosamine can be carried out using biological methods that can minimize the danger of chemical residues and environmental damage in chemical production methods (Kim et al. 2018). The biological method can be carried out with the chitinase enzyme produced by chitinolytic microorganisms. Several chitinolytic microorganisms from various sources have been isolated, purified, and characterized in the last decade. Most of them are members of the genera *Vibrio* (Revathi et al. 2012), *Streptomyces* (Karthik et al. 2015), *Pseudomonas* (Suganthi et al. 2017), *Serratia* (Li et al. 2020), *Bacillus* (Akeed et al. 2020), and *Micromonospora* (Patantis et al. 2019).

Actinomycetes are important enzyme producers. More than 90% of Actinomycetes are chitinolytic in nature (Ansari et al. 2020). One member of the Actinomycetes group, namely *Micromonospora* sp. which until now has not been widely explored in Indonesia and has the potential to be investigated as a chitinase producer. Several studies have shown that these bacteria are capable of producing chitinase. *Micromonospora chalcae* had a chitinase activity of 0.6 U/mL after three days of incubation at pH

7 and 30 °C (O’Riordan et al. 1989). *Micromonospora* sp. T5a1 also had a chitosanase activity of 0.010 U/mL after purification (Patantis et al. 2019). *Micromonospora* sp. AR17 is the newest strain isolated from the wastewater from the shrimp freezing industry at PT Toxindo Prima (Cilacap, Central Java, Indonesia). This bacteria can potentially be studied as a chitinolytic bacteria mainly derived from industrial waste. Therefore, this study aims to determine the specific activity, purity level, and molecular weight of the purified chitinase from *Micromonospora* sp. AR17. Moreover, to determine the concentration of N-acetylglucosamine produced using purified enzymes that have been characterized.

2. Materials and Methods

2.1. Bacterial strain and culture condition

Micromonospora sp. AR17 is a collection of bacteria at the Fishery Product Quality and Safety Laboratory, Universitas Gadjah Mada, which was isolated from the liquid waste of the shrimp freezing industry at PT Toxindo Prima (Cilacap, Central Java, Indonesia). Bacterial isolates from glycerol stock were grown by scratching on colloidal chitin agar (CCA) media and incubated at 37 °C for 7 d until a clear zone was formed. The composition of CCA media refers to the Hsu and Lockwood (1975) method by dissolving several ingredients, namely K_2HPO_4 0.07% (w/v), KH_2PO_4 0.03% (w/v), $MgSO_4 \cdot 5H_2O$ 0.05% (w/v), $MnCl_2$ 0.0001% (w/v), $ZnSO_4$ 0.0001% (w/v), bacto agar 2% (w/v) and colloidal chitin 2% (w/v). Furthermore, the inoculum was prepared by transferring a colony of bacteria from colloidal chitin agar into the nutrient broth (NB) and then incubated at 37 °C for 36 hours.

2.2. Chitinolytic index

20 μ L of bacterial culture from NB was inoculated on sterile paper discs (d = 0.8 mm, Whatman No.1) and placed on the surface of colloidal chitin media using sterile tweezers. Then it was incubated at 37 °C for 7 d to calculate the chitinolytic index. The chitinolytic index was obtained by comparing the diameter of the clear zone formed with the diameter of the colony (Ahmed et al. 2014).

2.3. Preparation of colloidal chitin

Colloidal chitin was prepared from a chitin commercial (CV. Bio Chitosan Indonesia) and shrimp shell chitin (Sigma-Aldrich) according to the method of Arnold and Solomon (1986). Briefly, 20 grams of commercial powdered chitin was dissolved in 150 mL of 37% HCl, and stirred without heat for 1 hour. Then the mixture was filtered with glass wool. The filter results were accommodated in 800 mL of cold distilled water (4 °C) and incubated at 4 °C for 24 hours. The precipitate will be formed as colloidal chitin. Then rinse with water continuously until the pH is neutral. The chitin pellets were collected by centrifugation.

2.4. Chitinase production

Chitinase production was carried out by growing isolates on colloidal chitin broth media for three replications. Chitin colloidal broth media has the composition K_2HPO_4 0.1% (w/v), $MgSO_4 \cdot 7H_2O$ 0.01% (w/v), NaCl 0.1% (w/v), $(NH_4)_2SO_4$ 0.7% (w/v), yeast extract 0.05% (w/v), and 2% (w/v) colloidal chitin. A total of 10 mL of NB inoculum was put into 500 mL of colloidal chitin broth in a 1,000 mL Erlenmeyer flask and incubated in a water bath shaker for 7 d at 40 °C and pH 7 with 100 rpm agitation. Observations were made every 24 h to test chitinase activity and measure the concentration of N-acetylglucosamine by Reissig et al. (1955).

2.5. Purification of chitinase

After cultivation, undegraded chitin and bacterial cells were removed by centrifugation at 4,000 rpm for 15 min (4 °C). The supernatant was continued for the purification process.

2.5.1 Ultrafiltration

The first stage of purification used the ultrafiltration method with Regenerated Cellulose (RS) membrane with a Molecular Weight Cut Off (MWCO) 30 kDa (Merck). The membrane was mounted on the Amicon® Stirred Cells, which have a capacity of 50 mL. Ultrafiltration was carried out in stages at a stirrer rotation speed of 400 rpm and a pressure of 50 psi until it reached a concentration of 10 times. The enzymes collected in the ultrafiltration tube filter were then measured for specific chitinase activity.

2.5.2 Ammonium sulfate precipitation and dialysis

The solution collected after ultrafiltration was purified using the ammonium sulfate precipitation method in stages of 20%, 40%, 60%, 80%, and 100%. The supernatant was added with ammonium sulfate with a saturation level of 20% and stirred until dissolved at 4 °C for 1 hour. The samples were centrifuged at 3,500 rpm at 4 °C for 15 min. The protein fraction of 20% ammonium sulfate precipitate was added with 5 mL of pH 7 phosphate buffer. Meanwhile, the supernatant obtained was added with ammonium sulfate at a saturation level of 20% and treated the same to obtain a 40% ammonium sulfate protein fraction. The process was repeated until the fractions of 60%, 80%, and 100% ammonium sulfate were obtained. The fraction with the highest specific activity value was selected for dialysis. The dissolved precipitate was put into a dialysis tubing cellulose membrane (14,000 MWCO), then immersed in phosphate buffer pH 7 with a ratio of 10:1 with the volume of solution in a dialysis tube overnight at a temperature of \pm 4 °C. The dialysate collected was pooled for further purification.

2.5.3 Ion-exchange chromatography

A total of 20 mL of Q Sepharose Fast Flow (GE Healthcare) resin equilibrated with 0.05 M Tris-HCl buffer so-

lution (pH 7.4) was added to the column chromatography (15 × 300 mm). Furthermore, the enzymes resulting from precipitation-dialysis purification were inserted into the column and flowed into the column for 30 min. Samples were collected in a microtube. The matrix was rinsed using a linear gradient of NaCl solution (0.1 M; 0.5 M; 1 M) in 0.05 M phosphate buffer solution pH 7.0 at a flow rate of 1.0 mL/min, and every 5 ml of elution was collected. Total 4 fractions of 5 ml each were collected and stored at 4 °C until further use. The fraction that showed the highest specific activity was referred to as the active fraction.

2.5.4 Gel filtration chromatography

The active fraction was further purified using gel filtration chromatography. Gel filtration chromatography used HiPrep Sephacryl S-300 (GE Healthcare) as the stationary phase, and the mobile phase was 0.05 M Tris-HCl buffer pH 7.4. The enzymes were rinsed using the same buffer solution at a 1.0 mL/min flow rate, and every 5 mL of elution was collected. Total of 4 fractions of 5 mL each were collected and stored at 4 °C until further use. The fraction that showed the highest specific activity and was confirmed by bands on SDS-PAGE was called pure chitinase.

2.6. Protein estimation

The protein was estimated by the Bradford method (Bradford 1976). An amount of 500 µL of enzyme solution was added to 1000 µL of Bradford reagent. Different concentrations of BSA (Bovine Serum Albumin) were used for a standard curve. Absorbance was measured at 595 nm on UV Vis Spectrophotometer (Genesys 20, Thermo Scientific, USA)

2.7. Enzymatic activity assay

The cell-free supernatant was separated by centrifugation at 6,000 rpm 4 °C for 10 min. A total of 2 tubes of 500 µL supernatant were used to test the chitinase activity. One tube without treatment was incubated for 3 min in boiling water. Another tube was reacted with 1 mL of 1.3% colloidal chitin (in 50 mM phosphate buffer pH 7.4) and incubated for 30 min in a water bath shaker at 37 °C. The enzymatic reaction was boiled in boiling water for 3 min to stop the reaction. Then it was centrifuged at 10,000 rpm for 5 min (Wang et al. 2011). The supernatant from the reaction was taken as much as 0.25 mL and added 0.05 mL of potassium tetraborate pH 9.1, and the solution was immersed in boiling water for 3 min. A total of 1.25 mL of p-dimethylaminobenzaldehyde (DMAB) was added, then incubated at 37 °C for 30 min in a water bath. The absorbance of the sample was then measured at 585 nm with a UV-Vis spectrophotometer. The absorbance values of the samples and controls were then compared with the absorbance values of the standard NAG solution. One unit of chitinase activity was defined as the amount of NAG (µmol) released by the enzyme per min at the condition mentioned. The specific activity of the enzyme is the ratio of the enzyme activity to the protein content in the sample

with units of U/mg (Reissig et al. 1955).

2.8. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Gallagher (2012). SDS PAGE 10% gel was used to analyze the purified protein. The electrophoresis process was carried out with an electric current of 150 V, 30 mA for 40 min and washed with ddH₂O for 20 min. The marker used was a precision plus protein dual color standards (Bio-rad, California, United States) with a range of 10-250 kDa. Determination of molecular weight is based on calculations using a line equation plot between the R_f value and the log molecular weight

2.9. Characterization of purified chitinase at various time incubation, pH and temperature conditions

The purified chitinase was characterized to observe chitinase activity and NAG concentration at various incubation times (30, 60, 90, and 120 min), various pH (4-9), and various temperatures (30, 37, 40, 45, and 50 °C). Chitinase activity testing at various pH was carried out using 0.1 M citrate buffer (pH 4-5), 0.2 M phosphate buffer (pH 6-7), and 0.2 M borate buffer (pH 8-9). The analysis for enzyme activity and concentration of NAG followed the method of Reissig et al. (1955) and Wang et al. (2011).

2.10. Production of NAG using purified chitinase

Enzymatic NAG production was carried out using purified chitinase following Wang et al. (2011). 1,000 µL colloidal chitin 1.3% was added to 500 µL purified chitinase and were incubated in a water bath shaker. Incubation time, pH, and optimum temperature were determined from the results of the characterization of the purification enzymes. After the incubation, the enzymatic reaction was stopped by immersion in boiling in water for 5 min. The sample was then centrifuged at 10,000 rpm for 5 min at room temperature. The supernatant was taken to determine the concentration of NAG and the yield obtained.

$$Yield (\%) = \frac{NAG \text{ hydrolyzed } (\mu\text{g/mL})}{reacted \text{ chitin } (\mu\text{g/mL})} \times 100\% \quad (1)$$

2.11. Data analysis

Statistical analysis using the IBM SPSS Statistics 26 program was conducted to analyze specific activity data obtained from each purification stage. The statistical test used Analyze of Variance (ANOVA) at a 95% confidence level if it showed a significant difference, a Duncan Multiple Range Test (DMRT) was performed to determine the effect of each purification stage.

3. Results and Discussion

3.1. Bacterial strain, chitinolytic index and culture condition

The growth of *Micromonospora* sp. AR17 on colloidal chitin agar media is presented in Figure 1. Colonies were

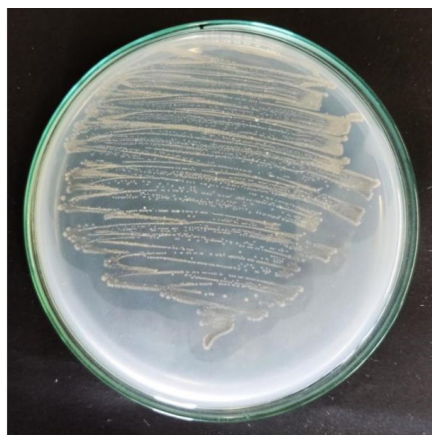


FIGURE 1 Colonies of *Micromonospora* sp. AR17 on colloidal chitin agar media.

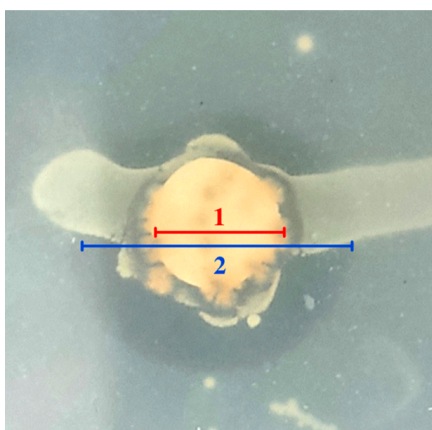


FIGURE 2 Chitinolytic index of *Micromonospora* sp. AR17 on colloidal chitin agar media. 1: Red line indicate diameter of colony = 8 mm; 2: Blue line indicate diameter of clear zone = 17 mm.

visible at day 3 (incubation temperature 37 °C and started to form a clear zone on colloidal chitin after day 6 of incubation. The formation of a clear zone around the colony of microorganisms indicated the production of an extracellular enzyme, namely chitinase, which was able to degrade

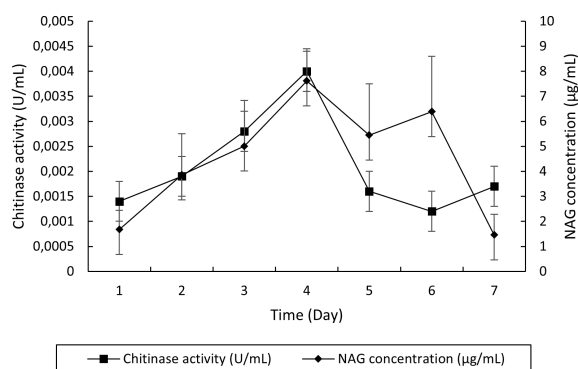


FIGURE 3 Chitinase activity and NAG concentration of *Micromonospora* sp. AR17 on colloidal chitin broth fermentation. The mean values were significantly different in ANOVA with $p < 0.05$. The standard deviation is calculated from 3 repetitions.

the colloidal chitin substrate contained in the chitin agar into monomeric units of GlcNAc (Herdyastuti et al. 2021). Macroscopic observations of *Micromonospora* sp. AR17 showed colony characteristics as in *Micromonospora* sp. research by Amin et al. (2018), which looks brownish yellow with a leathery texture, flat surface, and round colonies with flat edges (Figure 1). Colonies were ready to be transferred to NB medium as pre-culture. Macroscopic observations of *Micromonospora* sp. AR17 also exhibited a chitinolytic index of 2.125 (Figure 2). The chitinolytic index considers a high value if it is more than 2, while it is declared low if it is less than 2 (Haliza and Suhartono 2012).

3.2. Chitinase production

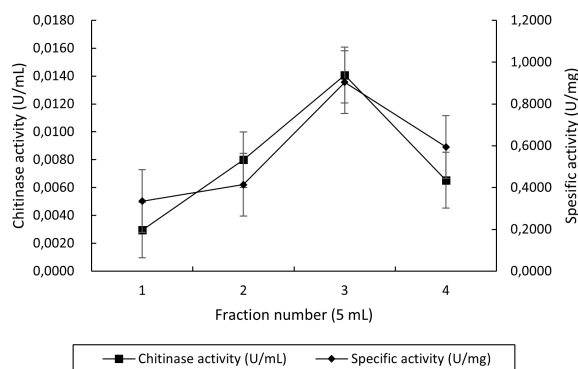
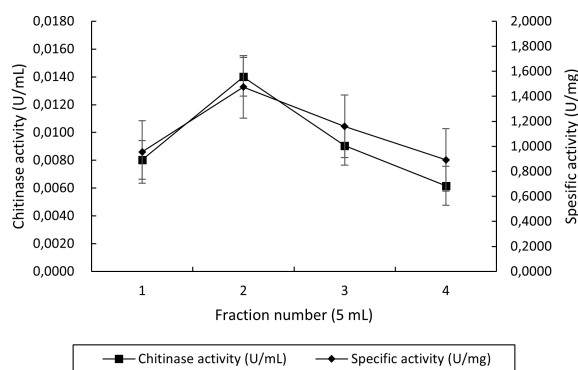
Chitinase production in the colloidal chitin broth fermentation medium was optimum on the fourth day. It showed chitinase activity of 0.0035 U/mL, specific activity of 0.2148 U/mg, and NAG concentration of 6.59 µg/mL. The complete production optimization results can be seen in Figure 3. The activity of this bacterium is lower than that of other Actinomycetes group bacteria such as *Microbispora*, *Micromonospora chalcae*, and *Streptomyces*. *Microbispora* sp. V2 has a specific chitinase activity of 1.16 U/mg after 6 d of incubation at pH 7 and a temperature of 40 °C (Nawani et al. 2002). *Streptomyces* sp. has a specific chitinase activity of 12.97 U/mg after 60 h of incubation at pH 8 and a temperature of 30 °C (Karthik et al. 2015). *Micromonospora chalcae* had a chitinase activity of 0.6 U/mL after three days of incubation at pH 7 and 30 °C (O'Riordan et al. 1989). The time required to produce the chitinase enzyme is in the exponential phase, which varies depending on each microbe (Horak et al. 2019). According to Luo et al. (2017), bacteria will produce extracellular enzymes for nutrient uptake. Extracellular enzymes are generally released by cells into the environment to hydrolyze polymer molecules in their environment. Enzyme performance is also influenced by the appropriate substrate concentration, inhibitor, temperature, and pH. The effect of pH and temperature on chitinase production will affect the formation of NAG. Chitinase secreted by microorganisms into liquid chitin media causes the chitin polymer in the medium to be degraded into monomeric units of GlcNAc or NAG, which bacteria will use as carbon and nitrogen sources for their metabolism (Keffeler et al. 2021). Furthermore, the culture was centrifuged, and a crude enzyme (supernatant) was further purified.

3.3. Purification of chitinase

The purity level is determined by comparing the specific activity of each enzyme purification step with the specific activity of the crude extract so that the specific activity will increase along with the purification step. Ultrafiltration can be used as a separation and purification technique for proteins and other macromolecules with high retention (Liu et al. 2020). Ultrafiltration results from 480 mL of crude enzyme obtained 45 mL of ultrafiltration enzymes. The ultrafiltration enzyme had a specific activity of 0.3353

TABLE 1 Purification of chitinase from *Micromonospora* sp. AR17.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme (supernatant)	16.589	77.213	0.2148	100	1.00
Ultrafiltration	0.5344	15.936	0.3353	32.2	1.56
40% ammonium sulfate precipitation	0.5756	14.591	0.3945	44.6	1.84
Dialysis	0.4997	0.7840	0.6374	30.1	2.97
Ion-exchange	0.0703	0.0778	0.9046	4.2	4.21
Gel-filtration	0.0700	0.0478	14.648	4.2	6.82

**FIGURE 4** Chromatogram of purified chitinase enzyme from *Micromonospora* sp. AR17 using Q sepharose FF matrix, elution rate of 1 mL/min at room temperature (Fraction 1: No salt; Fraction 2: NaCl 0.1 M; Fraction 3: NaCl 0.5 M; Fraction 4: NaCl 1 M). The mean values were significantly different in ANOVA with $p < 0.05$. Standard deviation is calculated from 3 repetitions.**FIGURE 5** Chromatogram of purified chitinase enzyme from *Micromonospora* sp. AR17 used sephacryl S-300 HR matrix, elution flow rate was 1 mL/min at room temperature. The mean values were significantly different in ANOVA with $p < 0.05$. The standard deviation is calculated from 3 repetitions.

U/mg and a purity level of 1.56 times that of a crude enzyme (Table 1).

In the ammonium sulfate precipitation stage, the 40% fraction had the highest specific activity of 0.3945 U/mg with a purity level of 1.84 times (Table 1). This shows that most of the chitinase enzymes are salting out at a percentage of 40% ammonium sulfate. This concentration has the best ability to precipitate protein and reduce its solubility

compared to crude extract of enzymes and other fractions because of the highest specific activity. Ammonium sulfate is used because it has high ionic strength advantages and does not cause conformational changes in the enzyme structure (Su'i and Suprihana 2013). DMRT statistical test results also showed that the 40% fraction differed significantly from all fractions. Therefore, the 40% fraction will be continued with the dialysis process.

In the dialysis stage, the enzyme has a specific activity of 0.6374 U/mg and a purity level of 2.97 times that of the crude enzyme (Table 1). Further purification using Q sepharose FF matrix resulted in 4 fractions with fraction number 3 possessed highest chitinase activity at 0.0141 U/mL. The fraction with the highest specific activity was also found in fraction number 3, with a specific activity of 0.9046 U/mg (Figure 4) and a purity level of 4.21 times that of the crude enzyme (Table 1). The chitinase enzyme fraction 3 was then proceeded to the purification step by gel filtration column chromatography using the sephacryl matrix S-300 HR.

Purification using the sephacryl matrix S-300 HR resulted in 4 fractions and the highest chitinase activity of 0.0140 U/mL and highest specific activity of 1.4648 U/mg shown by fraction 2 (Figure 5). The purity level increased 6.82 times that of the crude enzyme (Table 1). Chitinase enzyme fraction 2 resulted from gel filtration column chromatography was pure chitinase enzyme. Comparison between *Micromonospora* sp. AR17 in this study with other Actinomycetes, seen from the purification method, specific activity, and purification fold have been described in Table 2. Although the yield is low for commercial production of the enzyme, this method gives sufficient pure enzyme for initial characterization studies. The purification scheme demonstrated in this study can be performed in any laboratory with ease, and does not require any sophisticated systems.

3.4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Chitinase enzyme samples from the crude enzyme, up to ultrafiltration, 40% ammonium sulfate precipitation, dialysis, ion-exchange chromatography, and gel filtration chromatography were analyzed for molecular weights using the SDS-PAGE technique. Through the SDS PAGE electrophoresis method, it can be known whether the iso-

TABLE 2 Comparison of methods and purification results from different Actinomycetes sources.

Organism name	Purification method	Specific activity (U/mg)	Purification fold	Reference
<i>Micromonospora</i> sp. AR17	Ultrafiltration, ammonium sulphate, Q sepharose, Sephacryl S-300	14.648	6.82	Present study
<i>Microbispora</i> sp. V2	Ammonium sulphate, Sephadex G-100	4.6	4.0	Nawani et al. (2002)
<i>Streptomyces sporovirgulus</i>	Ammonium sulphate, Chitin affinity chromatography	24.8	2.3	Swiontek Brzezinska et al. (2013)
<i>Streptomyces violaceusniger</i>	Ammonium sulphate, Chitin affinity chromatography, Q Sepharose	45.86	7.45	Nagpure and Gupta (2013)
<i>Saccharothrix yanglingensis</i> Hhs.015	Ammonium sulphate, HisTrap HP	5.8	6.4	Lu et al. (2018)

lated or purified protein is a protein with the desired molecular weight and whether there are still non-target protein molecules (Gallagher 2012). The molecular weight of chitinase in bacteria ranges from 60-110 kDa, while the molecular weight of chitinase in the Actinomycetes ranges from 30 kDa or lower (Tran et al. 2019). It is also explained that bacterial chitinase has a molecular weight range of 20-60 kDa, smaller than insect chitinase (40-85 kDa) or plant chitinase (40-85 kDa) (Hamid et al. 2013).

The double bands on SDS-PAGE revealed that the purified chitinase still had two types of enzymes which differ in their molecular weights, with estimated molecular weights of 12.60 kDa and 17.32 kDa (Figure 6). This result is slightly different from the molecular weight of chitinase *Micromonospora* sp. L5 is about 27 kDa (Agheshlouie et al. 2015). Molecular size of chitosanase *Micromonospora* sp. T5a1 is about 20 kDa (Patantis et al. 2019). This reveals that probably the isolate AR17 contains novel chitinases not previously reported with MW less than equal to 12.60 kDa and 17.32 kDa. *Micromonospora* can contain chitinase types chiA, chiC and others, which means that the molecular weights of chitinase from various strains of *Micromonospora* may have differences (Agheshlouie et al. 2015). Whether the strain produces any endochitinase or exochitinase, needs to be evaluated.

The possibility of differences in molecular weight of the same bacteria was also found in other studies. Suryadi et al. (2016) found that the chitinase *Beauveria bassiana* isolate BB200109 had a molecular weight of 60.25 kDa, while the molecular weight reported by Bhagwat et al. (2021) from chitinase isolates of *Beauveria bassiana* AR-SEF 2860 at 45.06 kDa. Then several other studies also explained the possible causes of differences in the molecular weight of chitinase from the same type of bacteria depending on how many types of chitinase were produced. Stoykov et al. (2015) also stated that many organisms synthesize chitinases with different molecular weights because they are encoded by different genes or often as a result of post-translational modifications. Molecular weight (MW) can affect the antibacterial properties of chitinase. Chitinase with low molecular weight can be applied more widely because of its low viscosity and higher solubility

in neutral solutions. It will also expand its application as both an antioxidant and anti-tumor agent (Liaqat and Eltem 2018). It would be of interest to identify and determine the internal peptide sequences or N-terminal amino acid (AA) sequences of 12.60 kDa and 17.32 kDa chitinases. So that it will help find similarities between AR17 isolates and other strains.

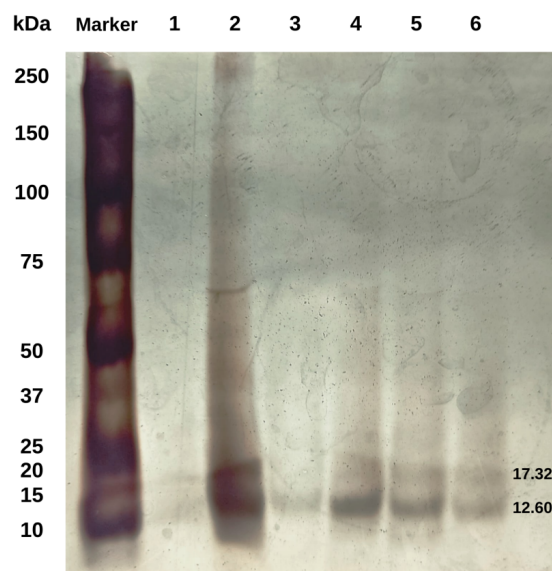


FIGURE 6 Molecular weight of chitinase from SDS-PAGE. Crude enzyme (Lane 1), protein of ultrafiltration (Lane 2), protein of 40% ammonium sulfate precipitation (Lane 3), protein of dialysis (Lane 4), protein of ion exchange chromatography (Lane 5), protein of and gel filtration chromatography or protein target (Lane 6), which after purification gave the double bands at 12.60 kDa and 17.32 kDa.

3.5. Characterization of purified chitinase at various incubation time, pH and temperature conditions

The purified chitinase performance was optimal at an incubation time of 30 minutes with a chitinase activity of 0.0107 U/mL and a NAG concentration of 12.12 µg/mL (Figure 7). Although there was an increase in the incubation time of 60 to 90 min, the results were not significantly different ($p < 0.05$). At 120 minutes, there was a signifi-

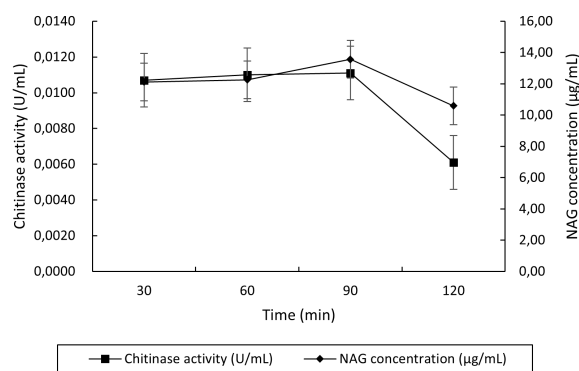


FIGURE 7 The effect of time incubation on enzymatic activity and NAG concentration of the purified chitinase. The mean values were significantly different in ANOVA with $p < 0.05$. Standard deviation is calculated from 3 repetitions.

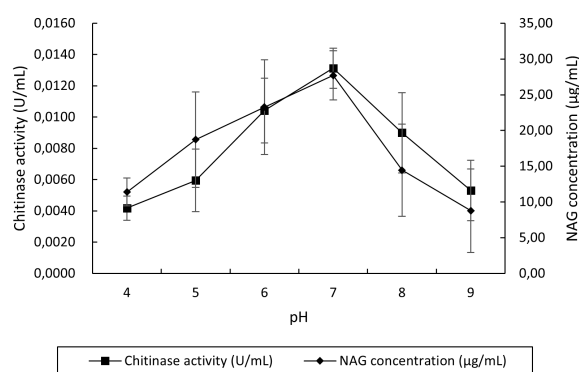


FIGURE 8 The effect of pH on enzymatic activity and NAG concentration of the purified chitinase. The mean values were significantly different in ANOVA with $p < 0.05$. Standard deviation is calculated from 3 repetitions.

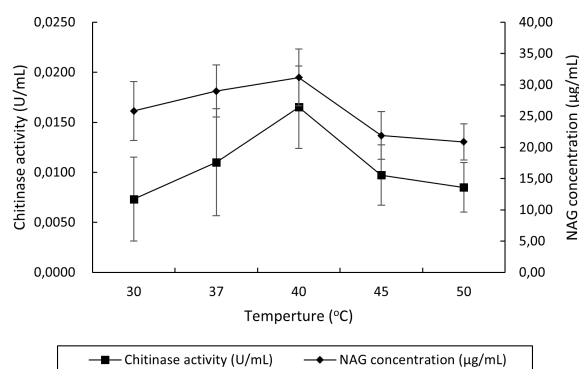


FIGURE 9 The effect of temperature on enzymatic and NAG concentration activity of the purified chitinase. The mean values were significantly different in ANOVA with $p < 0.05$. Standard deviation is calculated from 3 repetitions.

cant decrease in chitinase activity. This decrease can occur because the chitinase enzyme is denatured during the reaction, or inhibition occurs during the formation of N-acetylglucosamine (Cardozo et al. 2019).

The purified chitinase performance was optimal at pH 7.0 with chitinase activity of 0.0131 U/mL and NAG con-

centration of 27.70 µg/mL (Figure 8). A change in pH will affect changes in the ionization of the amino acid functional group of the enzyme or substrate. Changes in pH affect changes in the conformation of the enzyme structure in the solution (substrate) due to the formation or breaking of ionic interactions, especially in the part of the enzyme that contains R-NH³⁺ and R-COO⁻ ions and can result in a decrease in enzyme activity (Secundo 2013; Bisswanger 2014).

Another important factor influencing the activity of the chitinase enzyme is temperature. The purified chitinase performance was optimal at 40 °C with chitinase activity of 0.019 U/mL and NAG concentration of 31.17 µg/mL (Figure 9). Temperature affects the energy required by enzymes to carry out reactions. Based on the results of the study, it is known that an increase in temperature causes chitinase activity to reach the optimum temperature. The kinetic energy increases facilitate the collision process between the enzyme and the substrate to bind. If it passes the optimum temperature, the enzyme slowly undergoes denaturation, which begins with the partial formation of the enzyme molecule's secondary, tertiary, and quaternary structures as a result of breaking the physical and chemical bonds in the enzyme molecule. It is characterized by decreased chitinase activity (Bisswanger 2014).

3.6. Production of NAG using purified chitinase

The production of NAG from chitin using chitinase is influenced by several factors, including incubation time, temperature, pH, and substrate concentration (Cardozo et al. 2019). This production process uses environmental parameters resulting from the characterization of the chitinase enzyme. The results of NAG production from three replications showed that the concentration of NAG from the purified enzyme was shown significantly increased compared to the crude enzyme, with an average increase of 4.93 times and a yield of 0.25% (Table 3). These results indicate that the purified enzyme in this study has a higher purity than the crude enzyme because of the ability to produce greater NAG. The purified enzyme was also proven to be effective in producing NAG because the production time was shorter, which was only 30 min, and the amount was 32.47 µg/mL. This result is undoubtedly better than crude enzyme, which takes four days to produce only 6.59 µg/mL of NAG. However, this result is still much smaller than Herdyastuti and Cahyaningrum (2017) research, chitinase from *Pseudomonas* sp. TNH54 produced N-acetylglucosamine with a concentration of 1,360 µg/mL, a fermentation time of 8 h, and an enzyme concentration of 0.1 U/ml.

Enzymatic production of NAG can produce a purer product because, at the time of testing, there was only a reaction between colloidal chitin and the chitinase enzyme. Meanwhile, at the fermentation stage, the supernatant and the resulting hydrolyzate product still contained several other compounds besides NAG. However, the enzymatic NAG production needs to be optimized. The goal is that the resulting NAG product is not only purer but can be pro-

TABLE 3 Comparison of the concentration of NAG produced from crude enzyme and purified chitinase from *Micromonospora* sp. AR17.

Replication	Concentration of NAG ($\mu\text{g/mL}$)		Fold enhancement	Yield (%)
	Crude enzyme	Purified chitinase		
1	6.30	33.85	5.37	0.26
2	6.74	37.45	5.56	0.29
3	6.74	26.11	3.87	0.20
Average	6.59	32.47	4.93	0.25

duced in larger quantities. So that NAG production can be carried out effectively and efficiently and can produce optimal products both in quality and quantity.

4. Conclusions

Purification of chitinase from *Micromonospora* sp. AR17, which includes ultrafiltration, ammonium sulfate precipitation, dialysis, ion-exchange chromatography, and gel filtration chromatography, can increase the specific activity of the enzyme from 0.2148 U/mg to 1.4648 U/mg or a purification fold of 6.82. *Micromonospora* sp. AR17 had two types of enzymes which differ in their molecular weights, with estimated molecular weights of 12.60 kDa and 17.32 kDa.

The characterization of the purified chitinase enzyme showed the characteristics of the optimum incubation time of 30 minutes, optimum pH of 7, and optimum temperature of 40 °C. The concentration of NAG resulting from enzymatic hydrolysis with purified chitinase enzyme was 32.47 $\mu\text{g/mL}$.

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Authors' contributions

YH, U, and MMPP designed the research and wrote the manuscript. YH performed the field, laboratory works, and analyzed the data. All authors read and approved the final version of the manuscript

Competing interests

The authors declare no competing interest.

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