



Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Production, purification, and characterization of inulinase from dahlia rhizosphere-isolated *Aspergillus clavatus*

Saryono^{a*}, Chainulfiffah A. M.^a, Aulia Ardhi^a and Nova Wahyu Pratiwi^b

^aDepartment of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Riau

^bDepartment of Biology, Faculty of Mathematics and Natural Sciences, Universitas Riau

ABSTRACT

This study undertook exploration of Indonesian Dahliaplant (*Dahlia variabilis*), that can potentially be developed as the source of inulin. *Aspergillus clavatus* Gmn11.3 was an endophytic fungi isolated from Brastagi's Dahlia rhizosphere which had been previously reported to show high inulinase activity by producing wide halo zone around the colonies. This research aimed to optimize the production of inulinase by *A.clavatus* Gmn11.3. Inulinase production was carried out using the best inducer; 1% inulin in a medium containing 0.05% $MgSO_4 \cdot 7H_2O$; 0.015% $FeSO_4$; 0.2% NaCl, 0.05% KCl, and 1.0% yeast extract at 37°C, pH 4.5, and 5 days incubation (120 hours). The crude extract of extracellular enzyme was purified by ammonium sulphate precipitation, followed by Sephadex G75 and G50 gel filtration, DEAE cellulose and DEAE-Toyopearl anion exchanger-column chromatography. The purity of extracellular enzyme was monitored using SDS PAGE. Inulinase purity increased 5.91 fold after ammonium sulphate-fractionation, and 71.66 fold after purification using gel filtration-column chromatography and ion exchange-column chromatography. Inulinase from *A. clavatus* Gmn11.3 showed higher affinity than to its substrates with K_m 0.078mM and 0.039mM, and V_{max} were 0.098mM/h and 0.041mM/h respectively at optimum pH 5.0; temperature 50°C at four hours-incubation time; and its molecular weight were estimated to be 65.2KDa and 62.4KDa. While inulinase from *A. niger* Gmn11.1, as a comparison, had a K_m and V_{max} value 0.021mM and 0.022mM/h respectively at optimum pH 4.6; temperature 45°C with 15 hours incubation time; and its molecular weight was estimated to be 63KDa.

Keywords: *Aspergillus clavatus*, Dahlia, fungi, inulinase, purity rhizosphere.

INTRODUCTION

Inulinase is an enzyme that hydrolyses inulin into fructose or fructooligosaccharide. Exo-inulinase (β -D-fructanfructohydrolase, EC 3.2.1.80) splits off the terminal fructose units from non-reducing end of the inulin molecule; it also hydrolyzed sucrose and raffinose. Endoinulinase (2,1- β -D-fructanfructanohydrolase, EC 3.2.1.7) targets the β -2,1 linkage of inulin (a polyfructan consisting of linear β -2,1-linked fructose) and hydrolyzes it into fructose [1,2]. This polymer is a recognized source for the production of ultra-high fructose syrups, which after exo-enzymatic hydrolysis yielding over 95% D-fructose content, while the production of oligofructose syrup is carried out by endo-enzymatic hydrolysis, or for ethanol production using fermentation process [2].

Inulin can be hydrolyzed by a chemical approach, but there are many disadvantages [1,3]. On the other hand, complete hydrolysis of inulin by inulinase yields 95% pure fructose and fructo-oligosaccharides. Inulinase can be produced by many microorganisms including *Staphylococcus*, *Xanthomonas*, *Pseudomonas*, and *Arthrobacter urea faciens*; yeasts such as *Kluyveromyces fragilis*, *K. marxianus*, *Candida kefir*, and *Debaryomyces cantarelli*; and fungi including *Penicillium* and *Aspergillus* species [1,2,4,5]. The enzyme convert inulin into an oligosaccharide, DFA III (di-D-fructofuranose 1,2':2,3'dianhydride), and a small amount of other oligosaccharides. This enzyme was designated as inulin fructotransferase (DFA III-producing, EC 2.4.1.93). There have since been several reports on

the inulin fructotransferase (DFA III-producing) from *Arthrobacter* species [6,7,8,9]. Another enzyme had been purified from *Bacillus sp* by Kang *et al.* [10].

In foreign countries, Chicory root plants (*Cichoriumintybus*) have been commercially used as a possible candidate for alternative crop to sugar beet contains inulin [11]. However, people from some countries including Indonesia have never used to take the benefits from Chicory. Dahlia tubers are known to contain compounds which have high bioactivity. In some regions in Indonesia, especially in Sumatera with the mountainous topography, Dahlia can naturally grow well. For instance, in the province of Sumatera Barat, such as in Solok, Padang Panjang, Payakumbuh, and BatuSangkar; Brastagi (Sumatera Utara), Curup (Bengkulu), and LubukLinggau (Sumatera Selatan) Dahlia thrives in an environment of settlements. While in Kalimantan, Dahlia is relatively difficult to find due to environmental factors affecting the growth of Dahlia [12]. In this study, exploration of *Dahlia variabilis* endophytic fungi that can potentially be developed as the source of inulin was carried out. Soil samples and Dahlia tuber crops which have become rotten are sources of inulinase-producing microbes [13]. Our previous studies resulted that a number of fungi isolated from some regions in Sumatera showed medium to high inulinase activity and *Aspergillus clavatus* was found to be the highest [14]. Since there was lack of reports on inulinase from this fungi, this research aimed to produce, purify, and characterize the inulinase from *Aspergillus clavatus* strain Gmn11.3.

EXPERIMENTAL SECTION

Materials

The strain, *Aspergillus clavatus* Gmn11.3, used in the present study was isolated from the rhizosphere of Dahlia (*Dahlia variabilis*) which was grown in Brastagi, Province of Sumatera Utara. This strain had produced the widest clear zone in the previous study [15] and it maintained in PDA slants and taken for further studies. The fungal culture was inoculated into 25 ml PDA medium with pH 7.0 then incubated in a shaking incubator at 100 rpm speed for 3 days at 30°C [14]. The culture was used for the subsequent fermentation experiments. A total of 2.5 mL fermentation culture was inserted into the flask containing 25 mL of medium pH 5 consisting of 1% inulin, 0.5% (NH₄)₂HPO₄, 0.05% MgSO₄·7H₂O, and 0.015% FeSO₄ [16]. The mixture was incubated for 5 days in an incubator shaking at 100 rpm and 30°C.

Inulinase Production by *Aspergillus clavatus* Gmn11.3

Determination of the length of incubation

Determination of the length of incubation in inulinase production was carried out by varying the incubation time from 0 to 12 days. Inulinase activity was determined by measuring the reducing sugars formed by the method of ortho-toluidine and biomass was determined gravimetrically.

Determination of the effect of inducer concentration

A series of inulin concentration used were 0.1%; 0.25%; 0.5%; 0.75%; 1.0%, and 1.5%. The medium carbon source change was made with glycerol, lactose, cellulose, starch and sucrose to see whether inulinase which had been produced was inductive or constitutive. Experiment was carried out with three replications. This mixture was incubated at a temperature of 30°C and pH 7.0. Harvesting is done every 12 hours with an initial hour as control. All samples were examined of fructose level formed, the number of biomass produced, and inulinase activity.

Determination of the influence of the medium composition

Experiment was conducted in a variety of medium composition to determine the effect of various composition of the medium on enzyme activity as showed in Table 1. Determination of fructose, reducing sugars, and biomass that formed with three replications was undertaken in 10 fermented medium. The most appropriate medium in this experiment was used in subsequent experiments.

Table 1. Various compositions used to measure enzyme activity

No. medium	Composition
1	1% inulin
2	1% inulin + 0,5% (NH ₄) ₂ HPO ₄
3	1% inulin + 0,5% (NH ₄) ₂ HPO ₄ + 0.05% MgSO ₄ ·7H ₂ O
4	1% inulin + 0,5% (NH ₄) ₂ HPO ₄ + 0.05% MgSO ₄ ·7H ₂ O + 0,015% FeSO ₄
5	1% inulin + 0,5% (NH ₄) ₂ HPO ₄ + 0.05% MgSO ₄ ·7H ₂ O + 0,015% FeSO ₄ + 0,2% NaCl
6	1% inulin + 0,5% (NH ₄) ₂ HPO ₄ + 0.05% MgSO ₄ ·7H ₂ O + 0,015% FeSO ₄ + 0,2% NaCl + 0,0-5% KCl
7	1% inulin + 0,5% (NH ₄) ₂ HPO ₄ + 0.05% MgSO ₄ ·7H ₂ O + 0,015% FeSO ₄ + 0,2% NaCl + 0,0-5% KCl + 1% yeast extract
8	1% inulin + 0,05% MgSO ₄ ·7H ₂ O + 0,015% FeSO ₄ + 0,2% NaCl + 0,0-5% KCl + 1% yeast extract
9	1% inulin + 1% yeast extract
10	1% inulin + (NH ₄) ₂ HPO ₄ + 0.05% MgSO ₄ ·7H ₂ O + 0,015% FeSO ₄ + 0,2% NaCl + 0,0-5% KCl + 1% yeast extract + 1% sucrose

Effect of fermentation temperature

Experiment was conducted with 25ml of medium pH 5 and agitation 100rpm with various temperature used 30°C; 35°C; 37°C; 40°C, and 50°C, and then determined the relationship between biomass growth and enzyme activity against temperature.

Effect of pH fermentation medium

A series of pH value 3.5; 4.0; 4.5; 5.0; 5.5; and 6.0 was used. Subsequently, the relationship curve between biomass growth and enzyme production with pH treatment at the optimum temperature was made.

Biomass concentration measurement

Cells produced during the fermentation process were separated from the liquid medium by centrifugation at 5000 rpm for 15 minutes and washed with distilled water and filtered with filter paper that had been known the weight quantitatively. Subsequently, the samples were dried in an oven for 3 hours at a temperature of 60-70°C and after cooling in a desiccator for 15 minutes the samples were weighed. Samples were reheated for 20 minutes and cooled in a desiccator for 15 minutes and then weighed again until reaching constant weight.

Determination of inulinase activity

Inulinase activity was determined by adding 1ml of enzyme solution with 9 ml of 1% inulin. Incubation was carried out at a temperature of 40°C for 8 hours, and then the enzyme activity was stopped by immersing the system in boiling water for 10 minutes [14]. In addition, fructose formed was determined using ortho-toluidine method.

Inulinase Purification**Inulinase Fractionation**

Inulinase production was carried out in a 600 ml erlenmeyer flask with 300 ml medium at 37°C, pH 4.5, and agitation of 100 rpm for 5 days incubation. After incubation, extracellular enzymes were separated by centrifugation at 5000 rpm, for 20 minutes at a temperature of 40°C. A crude enzyme extract fractionated by precipitation using ammonium sulphate with degree of saturation of 60-100%. The result of fractionation was dialyzed in 0,02 M phosphate buffer pH 7, and then inserted into the gel filtration chromatography column. Purification by column chromatography Sephadex G75 gel filtration was using a 1.5 x 60 cm column with a flow rate of 1 ml/h and 0.01 M acetic acid as elution media. Purification followed by Sephadex G50 column chromatography sized 1,2 x 28cm. The active fraction resulted from Sephadex G50 column chromatography was purified further by anion exchange column chromatography with DEAE-Cellulose and DEAE-Toyopearl.

Determination of protein content

Protein content was determined by the Lowry method [17] using bovine serum albumin as a standard.

Determination of kinetic parameters

The Michaelis-Menten kinetic parameters K_m and V_{max} were determined from Lineweaver-Burk plots using inulin at concentrations ranging from 0.5 to 2.0 mM and 20 to 200 mM, respectively, as substrate.

RESULTS AND DISCUSSION

The rising potential of inulinase applications, such as production of high fructose syrups, fructooligosaccharides, ethanol, and inulo-oligosaccharides which are extensively used in pharmaceutical and food industries, has been promoting to search for new sources of inulinase. Hence, microorganisms are the best sources for commercial production of inulinase because of their easy cultivation and high yields of the enzyme. The attempt of this research was made to optimize and identify inulinase production by *Aspergillus niger* isolated from the rhizosphere of Dahlia.

Saryono *et al.* [15] had isolated 33 inulinase-producing fungi isolated from Dahlia's rhizospheres which were grown in various places on the island of Sumatra and Java and there were 15 species that had never been reported as a producer of inulinase, namely: *Cunningha mellaelegan*, *Rhizopus stolonifer*, *Aspergillus clavatus*, *Fusarium culmorum*, *F. solani*, *F. culmorum*, *Cylindro cephalumaureum*, *Humicola grisea*, *Oidio dendrongriseum*, *Geotricum sp.*, *G. candidum*, *Penicillium melinii Thom*, *P. citreoningrum*, and *Cephalosporium sp.* The isolates were tested using submerged fermentation which inulin was used as the only source of carbon with the addition of some minerals. Further more, *A. clavatus* Gmn11.3 from Brastagi showed a very high inulinase activity with reducing sugar of 2.94 mg/ml.

Optimization for inulinase production by *Aspergillus clavatus* Gmn11.3

Various treatments such as the length of incubation, inducer concentration, carbon sources, and medium composition were prepared as described in materials and methods as an optimization for inulinase production by *A. clavatus* Gmn11.3 grown in the basal medium for 24 h.

The length of incubation for inulinase production

The incubation time of inulinase production by *A. clavatus* Gmn11.3 varied from 0 to 12 days with pH 5, temperature 30°C and agitated at 100 rpm. Inulinase activity produced by *A. clavatus* Gmn11.3 increased during the growth phase until the stationary phase. This result was consistent with the research of Vandamme and Dirk [13] who found that the production of extracellular inulinase enzyme produced by *Kluyveromyces fragilis* increased even during stationary phase. The length of incubation for inulinase production by *A. clavatus* Gmn11.3 was five days with the highest activity of 2.51 mg/ml. The highest biomass produced on 8th day was as much as 0.0197 g, and the highest level of reducing sugars in the medium was found on 4th day of 3.97 mg / mL, as shown in Fig 1.

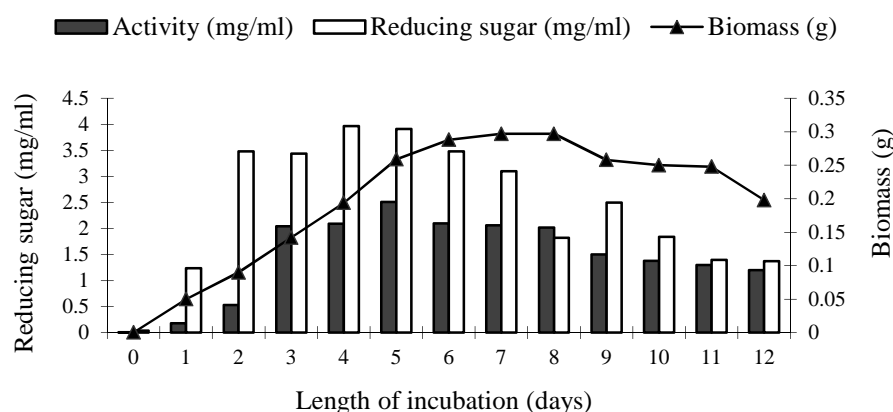


Figure 1. Profil of inulinase production by *A. clavatus* Gmn11.3. on medium containing inulin as the only carbon source

A. clavatus Gmn11.3 could potentially be used as a producer of inulinase, because the incubation time was relatively faster than the production of inulinase from *Fusarium oxysporum*, *Marinimicrobium sp.*, and *Aspergillus versicolor* [18,19,20]. Nakamura *et al.*, [21], produced inulinase from *A. niger* mutant 817 with a seven days incubation time. While Chainulfiffah *et al.*, [22], reported that optimum incubation time for the production of inulinase from local strain of *A. niger* Gmn11.1 was five days.

The production of inulinase is growth associated and reached the optimum near the stationary phase. The decrease in enzyme activity after 5 days fermentation could be either due to decrease in nutrient availability in the medium or catabolic repression of enzyme as reported by Kumar *et al.*, [23]. One of the most important factors that determined the cost of production in the fermentation process was the time of enzyme production. Therefore, many efforts needed to transform and accelerate the production time because microorganisms naturally produced the enzyme at slow speed.

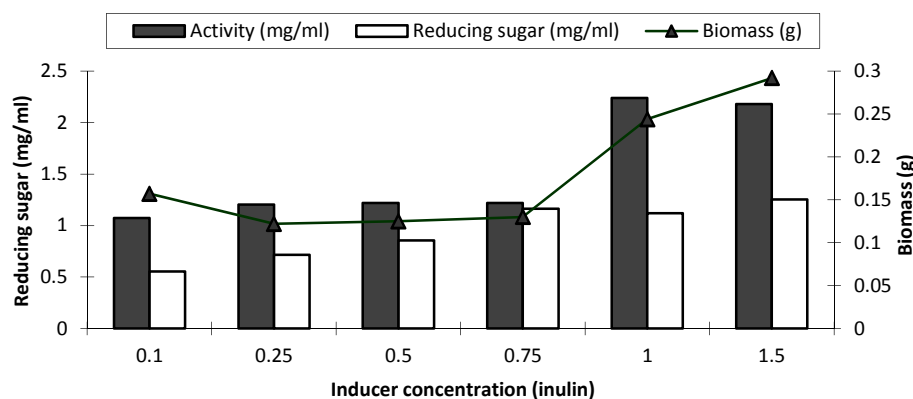


Figure 2. Inulinase activity, reducing sugar content, and biomass produced at various concentrations of inducers on inulinase production by *A. clavatus* Gmn11.3

Determination of inducer concentration

Determination of the appropriate inducer concentration for inulinase production by *A. clavatus* performed by varying the series of concentration of inulin 0.1%; 0.25%; 0.50%; 0.75%; 1.0%; and 1.5%. Inductive enzyme such as inulinase would be synthesized by a microorganism if there were inducers or inducer compounds. In Fig.2, the highest inulinase enzyme production was obtained at inducer concentration of 1.0% with activity of 2.24 mg/ml. The highest biomass was 0.044 g, also produced at the inulin concentration of 1.0%. While the highest reducing sugar level was obtained at the concentration of inducer 1.5%.

Inducer is a substrate or a similar compound used by enzymes to synthesize. Inulin was used as inducer, because inulin is a substrate for the inulinase enzyme. Kauret *al.* [24] also produced inulinase from *Fusarium oxysporum* using inulin from Chikory as inducer. According to Vandamme and Dirk [13], the use of inulin as a carbon source was a prerequisite in inulinase-producing microbial screening. *Kluyveromyces fragilis* used 3% inulin as inducer and carbon source to produce inulinase. The use of inulin was better than the other inducers such as raffinose, fructose, and sorbitol.

To determine whether inulinase produced was constitutive or inductive, replacement with a carbon source glycerol and lactose was performed. The highest inulinase activity was 2.37 mg/ml, produced using inulin as a carbon source. Biomass concentration was expressed as dry cell weight. As the carbon concentration increased, biomass concentration increased correspondingly. These results were in agreement with those obtained in previous study [25]. The highest biomass produced by fungi grown in medium containing 0.2963 g of lactose, while the highest reducing sugar lactose presented in the medium at 6.05 mg/ml as shown in Fig.3.

Inulinase generated by *A. clavatus* Gmn11.3 was inductive which inulin was the best inducer, although this enzyme could also be induced by other carbon sources such as sucrose, starch, and cellulose. Whilst, glycerol and lactose could not induce inulinase at all. Cruzguerrero *et al.*, [26], found that while inulin was a best source of carbon or inducers for inulinase production by *Kluyveromyces marxianus* CDBB-L-278, this fungus produced the same enzyme using glycerol as carbon source as well. On the other hand, a different result reported by Mahmoud *et al.* [27] that sucrose slightly favored inulinase production compared with pure inulin which suppressed enzyme.

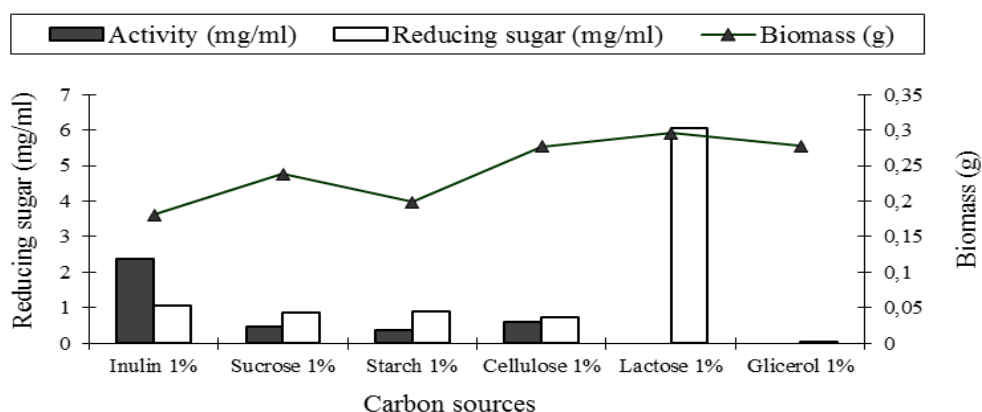


Figure3. Inulinase activity, reducing sugar, and biomass in inulinase produced by *A. clavatus* Gmn11.3 in various carbon sources

Based on the result of this study, inulin was a potential inducer of inulinase and it was in a good agreement with Gao *et al.* [25] which yielded inulinase 66% higher than fructose. In the previous study, fructose was believed to be the primary inducer of inulinase [28]. Fig.3 shows that *A. clavatus* Gmn11.3 produced high reducing sugar using lactose as a carbon source or inducer. This symptom indicated that *A. clavatus* Gmn11.3 was not only able to produce inulinase but also produced lactase or β -galactosidase when using lactose as inducer. Inulinase production by microbes was often followed by the activity of other enzymes such as invertase. Kaur *et al.*, [24] had identified four types of inulinase and four types of invertase with different properties on *Fusarium oxysporum* with inulin as carbon source. *Clostridium thermosuccinogenes* grown on inulin medium could produce six enzymes out of inulinase, namely lactate dehydrogenase, phosphoenol pyruvate carboxylase, malate dehydrogenase, fumarase, fumarate reductase, phosphor-transacetylase, acetate kinase, pyruvate kinase, and alcohol dehydrogenase [29].

Selection of growth medium composition for production inulinase

The highest activity and the highest reducing sugar content was obtained from medium no. 8 which was consisting of 1% inulin; 0.05% 0.05% $MgSO_4 \cdot 7H_2O$; 0.015% $FeSO_4$; 0.2% NaCl, 0.05% KCl, and 1% yeast extract. This medium was then used in the production of inulinase. However, the highest biomass was found in the medium No.

10 (Fig.4). Optimization of the enzyme production by a microbial was influenced by the formulation of the medium, pH, temperature, and the availability of specific nutrients properly during fermentation[30,31].

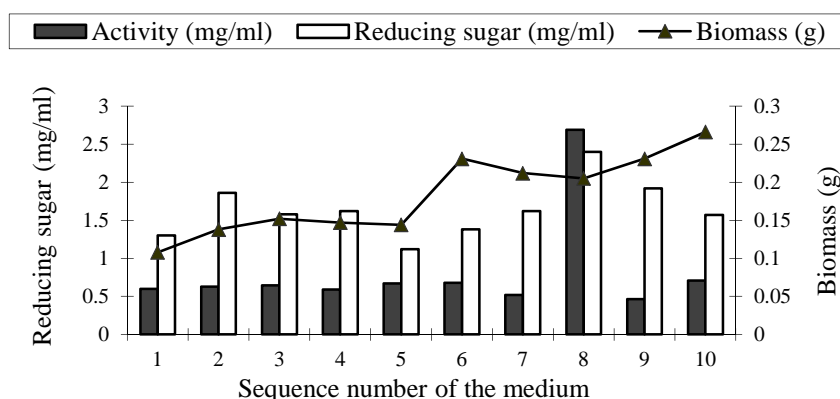


Figure4. Effect of medium composition on inulinase activity, reducing sugar content, and biomass produced by *A. clavatus* Gmn11.3

The composition of microbial growth media was different from each other depending on the type of microbes. Enzyme production by microbes also depended on the type of microbes and nutrients needs appropriately. *A. niger* Gmn11.1 isolated from Malang, East Java, showed high inulinase activity in the medium with inorganic nitrogen source, which consisted of 1% inulin; 0.5% $(\text{NH}_4)_2\text{HPO}_4$; 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ [22]). Another study [21] reported that inulinase produced from *A. niger* mutant 817 with medium composition 1% inulin; 0.5% peptone; 0.1% $(\text{NH}_4)_2\text{HPO}_4$; 0.05% KCl; 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; and 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$.

Determination of incubation temperature on enzyme production

The optimum fermentation temperature on inulinase production by *A. clavatus* Gmn11.3 was obtained by varying the temperature into 30°C, 35°C, 37°C, 40°C, 45°C and 50°C. Fig 5 showed the optimum inulinase and the optimum biomass activity were reached at temperature of 37°C with reducing sugar content of 1,97 mg/ml. It had a little different from Meenakshi *et al.*[32], who reported the optimum temperature for inulinase production by *Bacillus cereus* was at 30°C. Generally, inulinase formation would be reduced if the temperature kept increasing because temperature influenced the metabolic activity of cells. The optimum activity of inulinase production by the soil bacteria, *Enterococcus faecalis*, was achieved at a higher temperature of 55°C after incubation [33].

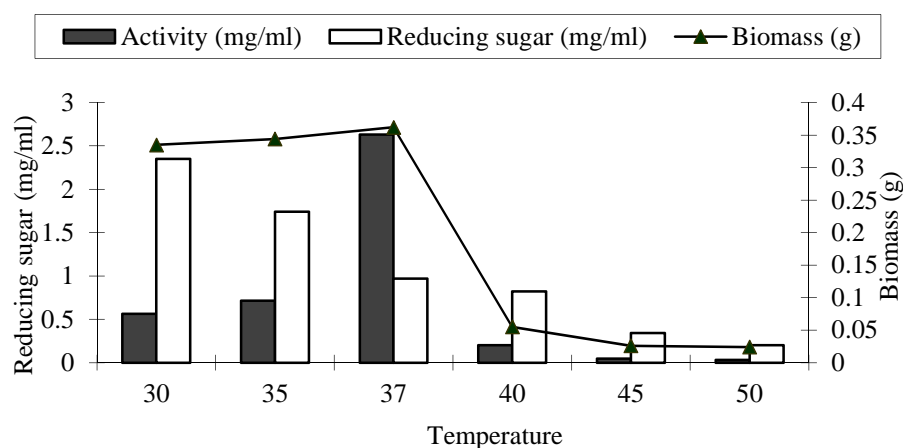


Figure5.Effect of incubation temperature on inulinase activity, reducing sugar content, and biomass produced by *A. clavatus* Gmn11.3

Determination of pH on Enzyme Production

The secretion of extracellular enzymes by a microbial growth medium is significantly affected by pH. Determination of the optimum pH for the inulinase production by *A. clavatus* Gmn11.3 conducted using the optimum temperature conditions which had been obtained. The optimum inulinase activity (2.55 mg/ml) was produced at pH 4.5. The highest reduction sugar (3.8mg/ml) was produced at pH 4 and the highest biomass (0.26 g) was obtained at pH 5, as shown in Fig.6. Production of inulinase by *Thielavia terrestris* NRRL 8126 and mesophile *Aspergillus foetidus* NRRL 337 had optimum pH at 4.5 to 5.5 [34]. On contrary to this, maximum production of inulinase from

inulin by *Xanthomonas campestris* was obtained at pH 7, which was also shown to be the optimal pH for biomass growth[35]. However, pH of extracellular enzyme production by a microbe is not always the same as pH of optimum cell growth, because pH of the enzyme production is closely related to the secretion of enzymes produced through the cell membrane [13].

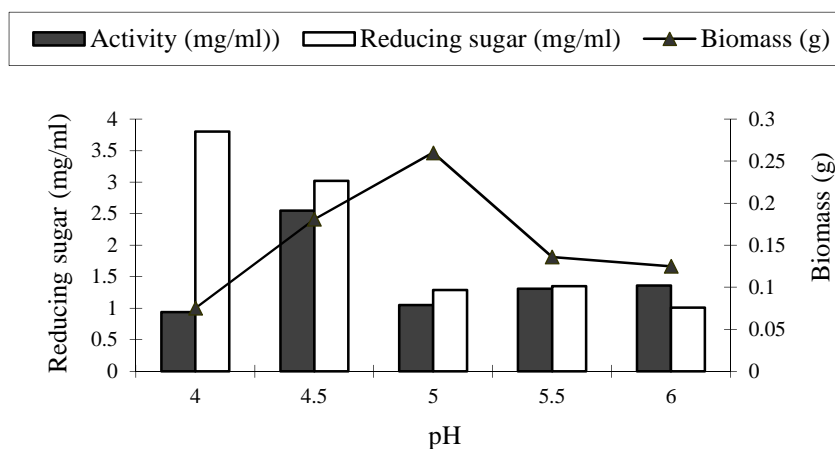


Figure 6. Effect of pH on inulinase activity, reducing sugar content, and biomass produced by *A. clavatus* Gmn11.3

Inulinase Fractionation

Based on the best condition which was obtained previously, inulinase production was carried out in a 300 ml medium at 37°C, pH 4.5, and agitation of 100 rpm for 5 days incubation. After separated using 5000 rpm centrifugation, the crude enzyme extract was yielded as much as 270 ml with activity of 0.144 U and 6 mg/ml protein content. Fractionation of crude enzyme extracts was performed using ammonium sulphate and Sephadex G75 gel filtration. Purification was followed by Sephadex G50 chromatography columns, the active fraction was purified further by DEAE-Cellulose anion exchange column chromatography.

Activity of 60-100% saturated $(\text{NH}_4)_2\text{SO}_4$ purified fraction was 117.3 mg/ml or equivalent to 0.651 units/ml and 4.56 mg/ml protein content. The purity level increased 5.91 times and recovery reached 33.5% as shown in Table 2. Inulinase produced by *A. clavatus* Gmn11.3 was further purified by gel filtration column chromatography using Sephadex G75 as a matrix. Sephadex G75 is able to filter out molecules with molecular weight of 30-70 kDa while inulinase was about 60 kDa [36].

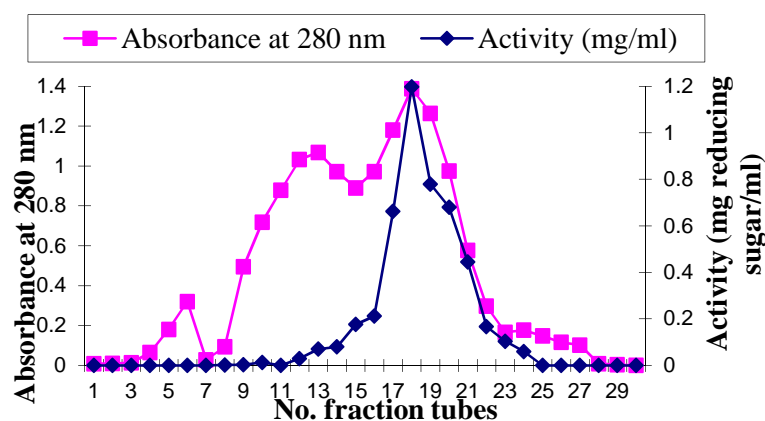


Figure 7. Purification using Sephadex G75 column chromatography (1.5 x 60 cm) of crude enzyme extract fraction at 60-100% saturation of $(\text{NH}_4)_2\text{SO}_4$, 0.01 M acetic acid as eluent at flow rate of 1 ml/hour

Separation using Sephadex G75 gel filtration chromatography showed three peaks of protein (Fig. 7). Inulinase was contained in the third peak which its activity was 354 mg reducing sugar/ml or equivalent to 0.651 units of activity, and the protein content of 3.83mg/ml. The enzyme moved out of the column at the end or third peak fraction could be caused by the enzyme molecules which passed through the pores of the gel in advance so that the protein having a molecular weight greater than 70 kDa came out first. Protein at third peak from fractions 15, 16, 17, 18, 19, 20, 21, and 22 which have inulinase activity were collected for subsequent purification stages. Electrophoresis results of the active fraction from Sephadex G75 gel filtration results still showed three protein stains. Two stains adjacent to each

other which were about 60-70 kDa while another stain was located quite a distance which was about 25 kDa. To separate the protein of *A. clavatus* Gmn11.3 located far apart on the electrophoresis gel, those were performed with gel filtration chromatography Sephadex G50, which can filter out molecules with a molecular weight of about 1.5 to 30 kDa.

Separation of proteins by Sephadex G50 column chromatography resulted in two major peaks. Inulinase activity presented in the first peak at fractions 5, 6, 7, 8, and 9 was obtained as much as 413.4 mg/ml, equivalent to 2.29 units (Fig 8). Results of chromatographic separation using Sephadex G50 obtained the inulinase activity in the first peak and moved out first because its molecular size was larger than the pores of the gel. The second peak has no inulinase activity and protein allegedly positioned at 25 kDa.

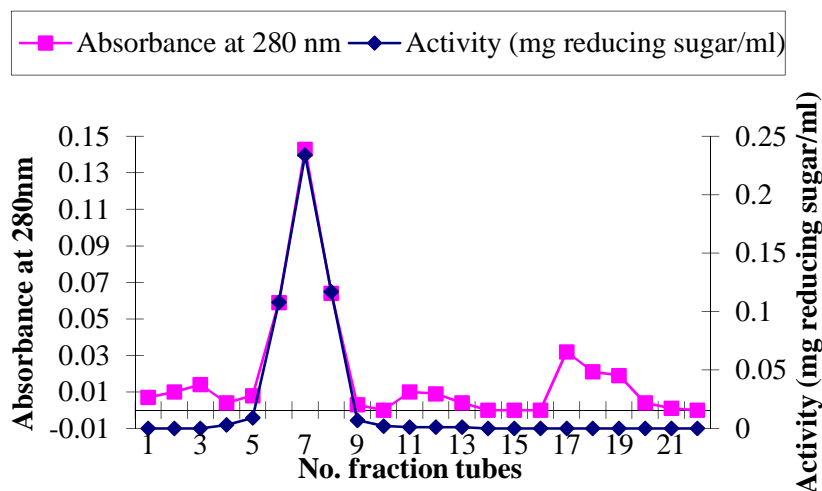


Figure 8. Patterns of protein and activity of fractions after chromatographic separation using Sephadex G50 column

SDS-polyacrylamide gel electrophoresis result of the protein from the active fractions of Sephadex G50 gel chromatography column showed the persistence of the two adjacent stains. Separation of both types of proteins was accomplished by ion exchange chromatography column with a matrix of DEAE cellulose which positively charged at pH below 9. This positive charge bound negatively charged proteins in the inulinase molecules. Inulinase having pH of about 5 negatively charged at pH below 9.

Protein content measurement of the fraction results of ion exchange chromatography showed two major peaks. The data were consistent with results of previous electrophoresis (Fig. 8), where the G50 gel filtration fraction results left two stains. Inulinase activity was shown at the second peak (Fig. 9), which was 435mg/ml or equivalent to 2.42 units with a protein content of 1.41mg/ml. This fraction electrophoresis showed one major stains, but there was still a thin shadow stain nearby. Thus, inulinase from *A. clavatus* Gmn11.3 was estimated to have two sub-units.

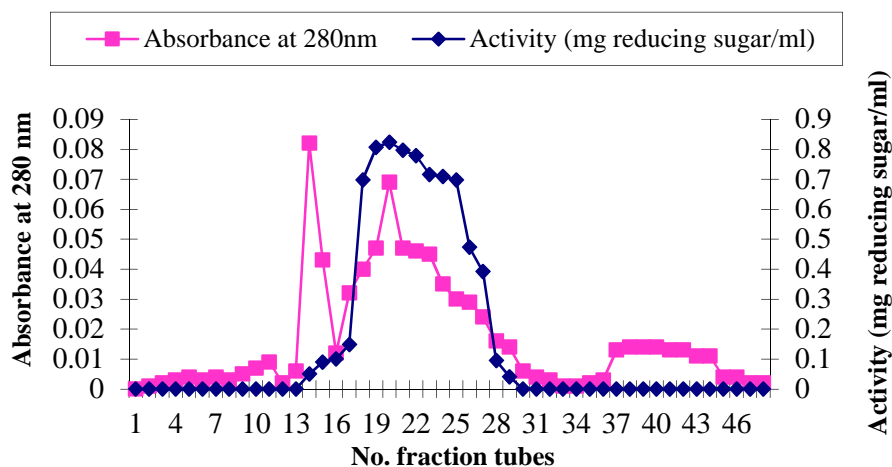


Figure 9. Patterns of protein and activity of fractions after ion exchange chromatographic separation using DEAE cellulose column

Separation of the two adjacent protein resulted from purification using Sephadex G50 was continued with DEAE-Toyopearl matrix chromatography. The chromatogram showed that there were four protein peaks (Fig. 10), where

inulinase activity was obtained at the third and fourth adjacent peak, so that this enzyme was thought to consist of two subunits. However, there was also a possibility that this fraction was composed of two types of inulinase. Nakamura *et al.*[21] Reported the existence of two types of inulinase of *Aspergillus niger* mutant 817 with a molecular weight of 70 kDa and 68 kDa. Whilst Gupta *et al.*[37] found that there were four types of inulinase produced by *Fusarium oxysporum* which each had different properties, such as pH optimum, Km values, and thermal stability.

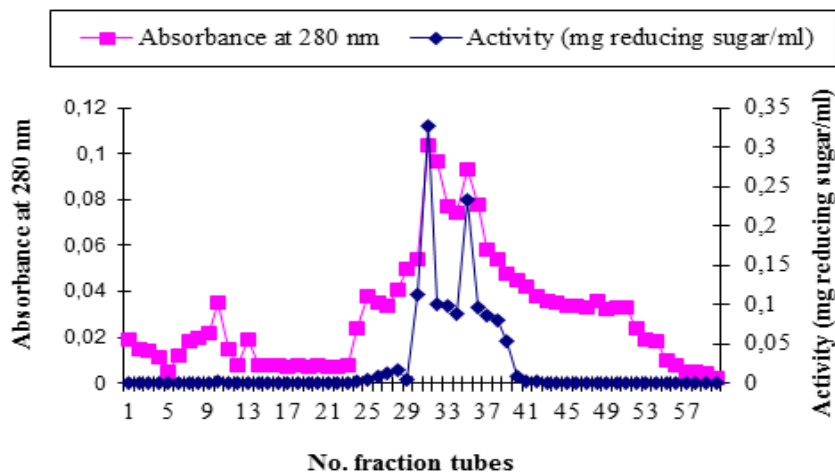


Figure 10. Patterns of protein and activity of fractions after ion exchange chromatographic separation using DEAE Toyopearl column

Based on all the purification steps that had been made, the protein content and enzyme activity were resumed in Table 2. The purity increased as the terrace purifications were stepped and it reached 71.66 fold after being purified with DEAE Cellulose matrix.

Table 2. Purification steps and its effects on protein content and enzyme activity

Purification steps	Total protein (mg)	Specific activity (U/mg)	Total unit	Yield (%)	Purity factor (fold)
Crude extract	1620	0.024	38.88	100	1.0
(NH ₄) ₂ SO ₄	91	0.142	13.02	33.5	5.91
Sephadex G75	22.98	0.510	11.76	30.25	21.25
Sephadex G50	7.6	1.21	9.18	23.55	50.42
DEAE Cellulose	4.23	1.72	7.26	18.19	71.66

Determination of inulinase molecular weight by SDS-PAGE electrophoresis

SDS-PAGE was performed on all stages of the purification of the enzyme. To determine the molecular weight of the purified enzyme, standard proteins was used and followed by the curve of % migration versus log molecular weight. Total length of gel used was 6 cm while samples inulinase moved on the gel as far as 1.6 cm and 1.7 cm, so that the migrations were 26.6% and 28.3% respectively (Fig. 11). If the percent migration of enzyme samples was plotted on a standard curve, the molecular weights were obtained of 65.2 kDa and 62.4 kDa. Inulinase molecular weight resulted from various microbes appeared to be dissimilar one to others. Inulinase produced by *Aspergillus* species generally showed the nearly molecular weight. Nakamura *et al.* [21] found endo- and exo-inulinase of *Aspergillus niger* mutant 817 having a molecular weight of 68 kDa and 70 kDa respectively. Fawzi[34], discovered an enzyme of *Aspergillus foetidus* NRRL 337 and *Thielavia terrestris* NRRL 8126 with molecular weight of 78 kDa and 70 kDa respectively.

To determine the value of Km and Vmax of inulinase from *A. clavatus* Gmn11.3, curve of 1/S vs 1/V was made. The transformation will produce Lineweaver-Burk curve to determine the price of Km and Vmax (Fig. 12 and Fig. 13). From Fig. 12, with pure inulin as substrate, yielding Km value of 7.1mg/ml or 0.039mM and Vmax values of 7.4 mg/ml/h or 0,041mM/h. While with dahlia uber extract as substrate (Fig. 13), generating Km value of 14.2 mg/ml or 0.078mM and Vmaks of 17.7 mg/m/h or 0,098mM/h.

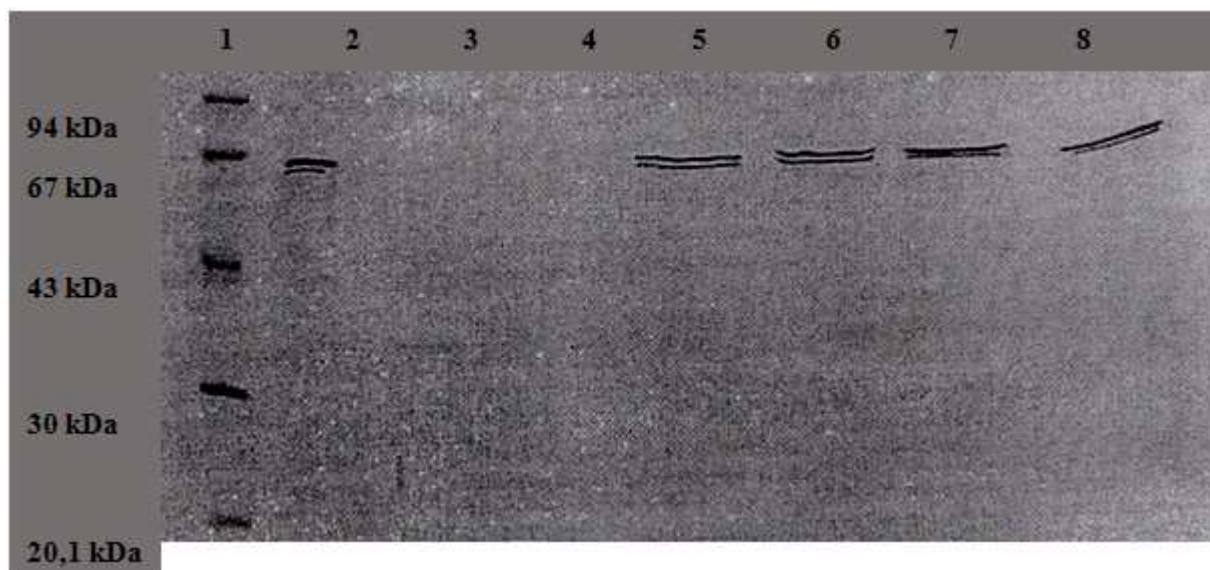


Figure 11. SDS-Page electrophoresis of inulinase from *A. clavatus* Gmn11.3, 1= standard 20 μ l, 2=DEAE-cellulose ion exchange chromatography, 3= 1st peak of DEAE-Toyopearl, 4= 2nd peak of DEAE-Toyopearl, 5 and 6= 3rd peak of DEAE-Toyopearl, 7 and 8= 4th peak of DEAE-Toyopearl.

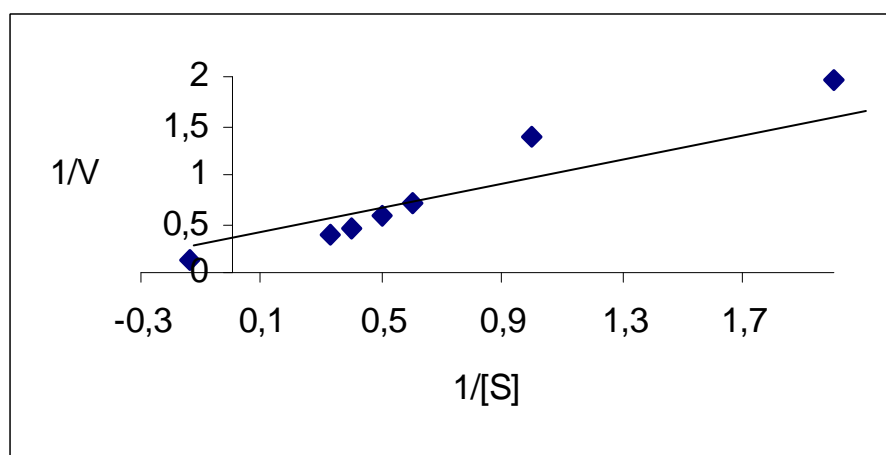


Figure 12. Lineweaver-Burk curve of inulinase from *A. clavatus* Gmn11.3 with pure inulin as substrate

Lower K_m and higher V_{max} indicated that this enzyme had a high reactivity. K_m value of *A. clavatus* Gmn11.3 inulinase was smaller than inulinase from *Bacillus subtilis* 430A that is equal to 8mM[38]. On the other hand, Nakamura *et al.* [21] reported K_m of inulinase from *A. niger* mutant 817 was 0.48mM. It showed that the lower value of K_m inulinase of *A. clavatus* Gmn11.3 meaning that this enzyme had a higher specificity towards their substrate compared with inulinase from *Bacillus subtilis* 430A and inulinase from *A. niger* Mutants 817.

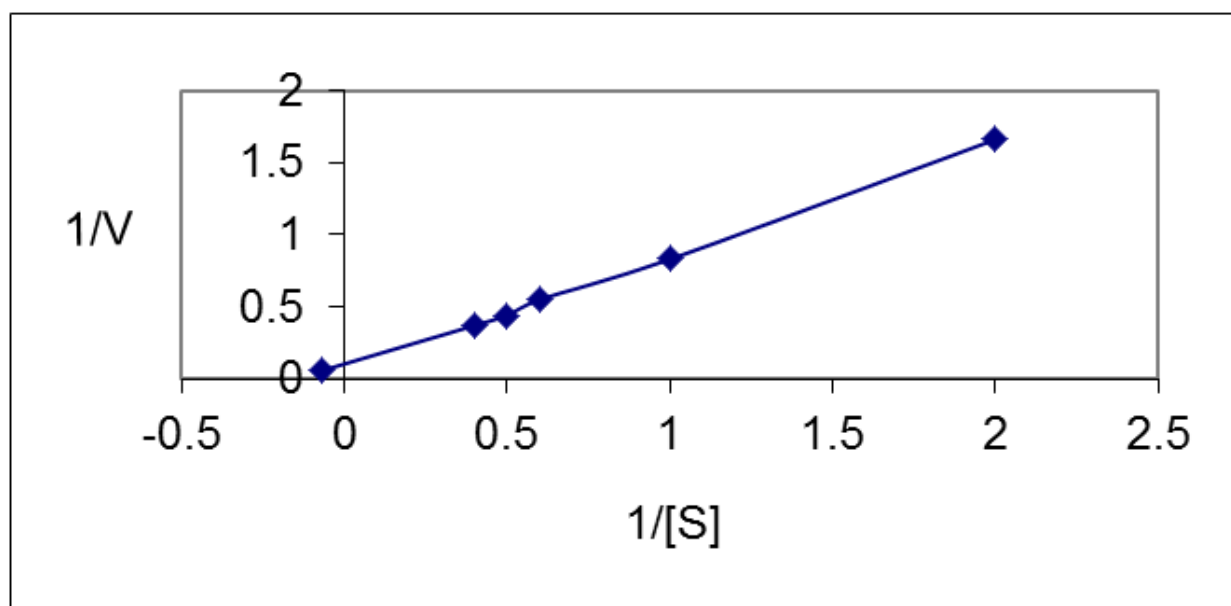


Figure 13. Lineweaver-Burk curve of inulinase from *A. clavatus* Gmn11.3 with dahlia tubers extract as substrate

CONCLUSION

Inulinase production was carried out using the best inducer; 1% inulin in a medium containing 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.015% FeSO_4 ; 0.2% NaCl, 0.05% KCl, and 1.0% yeast extract at 37°C , pH 4.5, and 5 days incubation (120 hours). The crude extract of extracellular enzyme was purified by ammonium sulphate precipitation, followed by Sephadex G75 and G50 gel filtration, DEAE cellulose and DEAE-Toyopearl anion exchanger-column chromatography. The purity of extracellular enzyme was monitored using SDS PAGE. Inulinase purity increased 5.91 fold after ammonium sulphate-fractionation, and 71.66 fold after purification using gel filtration-column chromatography and ion exchange-column chromatography. Inulinase from *A. clavatus* Gmn11.3 showed higher affinity than to its substrates with K_m 0.078 mM and 0.039 mM, and V_{\max} were 0.098 mM/h and 0.041 mM/h respectively at optimum pH 5.0; temperature 50°C at four hours-incubation time; and its molecular weight were estimated to be 65.2 KDa and 62.4KDa. Whilst inulinase from *A. niger* Gmn11.1, as a comparison, had a K_m and V_{\max} value 0.021 mM and 0.022 mM/h respectively at optimum pH 4.6; temperature 45°C with 15 hours incubation time; and its molecular weight was estimated to be 63 KDa.

REFERENCES

- [1] Pandey A; Soccol CR; Selvakumar P; Soccol VT; Krieger N; Fontana JD. *ApplBiochem Biotech*, **1999**, 81(1), 35-52.
- [2] Rocha JR; Catana R; Ferreira BS; Cabral JMS; Fernandes P. *Food Chem*, **2006**, 95,77–82.
- [3] Gill PK; Manhas RK; Singh P. *J Food Eng*, **2006**, 76, 369-375.
- [4] Nakamura T; Hoashi S. *Nippon NogeikagakuKaishi*, **1969**, 43, 599-605.
- [5] Kalil SJ; Maugeri-Filho F; Rodrigues M. *Process Biochem*, **2005**, 40, 581-586.
- [6] Haraguchi K; Kishimoto M; Seki K; Kobayashi S; Kainuma K. *AgricBiolChem*, **1988**, 52, 291-292.
- [7] Kawamura M; Takahashi S; Uchiyama T. *AgricBiolChem*, **1988**, 52, 3209-3210.
- [8] Yokota A; Enomoto K; Tomita F. *J Ferment Bioeng*, **1991**, 72, 262-265.
- [9] Haraguchi K; Yamanaka T; Ohtsubo K. *CarbohydrPolym*, **2002**, 52, 117-121.
- [10] Kang S; Kim W; Chang Y; Kim S. *Biosci Biotech Biochem*, **1998**, 62, 628-631.
- [11] Sévenier R; Hall RD; van der Meer I; Hakkert HJ; van Tunen AJ; Koops AJ. *Nature Biotechnol*, **1998**, 16, 843–846.
- [12] Saryono S; Reginawati H. UNRI Press, Pekanbaru, **2009**, 7-8.
- [13] Vandamme EJ; Derycke DG. *AdvApplMicrobiol*, **1983**, 29, 139-176.
- [14] Saryono; Chainulfiffah AM; Soekartadiredja EM; Supriatna; Hadiman. *Seri KajianIlmiahUnikaSoegijapranata*, **1999**, 9(2), 1-10.
- [15] Saryono; Atria M; Chainulfiffah AM. *Jurnal Natur Indonesia*, **2002**, 4(2), 171-177.
- [16] Byun SM., BH.Nahm. *J Food Sci*, **1978**, 43, 1871-1873.
- [17] Lawry O H; Rosenbrough NJ; Farr AL; Randall JR. *J Biol Chem*, 1951, 193, 265-275.
- [18] Byun SM., BH.Nahm. *J Food Sci*, **1978**, 43, 1871-1873.

- [19] Gupta AK; Rathore P; Kaur N; Singh R. *J Chem Tech Biotech*, **1990**, 45(1),245-251.
- [20] Ai-Xia Li; Li-ZhongGuo; Wei-Dong Lu. *World J MicrobBiot*, **2012**, 28(1): 81-89.
- [21] Nakamura T; Nagatomo Y; Hamada S; Nisino Y; Ohta K. *J Ferment Biotech*, **1994**, 78(2), 134-139.
- [22] Chainulfiffah AM; Jhon S; Saryono. LembagaPenelitianUniversitas Riau, Pekanbaru, **2000**, 3-4.
- [23] Kumar GP; Kunamneni A; Prabhakar T; Ellaiah P. *World J MicrobBiot*, **2005**, 21: 1359-1361.
- [24] Kaur N; ;Kaur M; Gupta AK; Singh R. *J Chem Tech Biotech*, **1992**, 53,279-284.
- [25] Gao J; Chen L; Yuan W. *J BioSci Biotech*, **2012**, 1(2), 155-161.
- [26] Cruzguerrero A; Garciapena I; Barzona E; Garciagaribay M; Gomezruiz L. *J Ferment Bioeng*, **1995**, 80(2), 159-163.
- [27] Mahmoud DAR; Mahdy EME; Shousha WG; Refaat W; Abdel-Fattah AF. *J Basic ApplSci*, **2011**, 5, 453-462.
- [28] Jing W; Zhengyu J; Bo J; Augustine A. *Process Biochem*, **2003**, 39, 5-11.
- [29] Sridhar J; Eiteman MA; Weigel JW. *Appl Environ Microb*, **2000**, 66(1), 246-251.
- [30] Smith JE. *Biotechnology, Studies in Biology*, 3rd edition, Cambridge University Press, Cambridge, **1997**, 60.
- [31] Sivakumar T; Shankar T; Vijayabaskar P; Ramasubramanian V. *Academic Journal of Plant Sciences*, **2012**, 5 (3), 102-109.
- [32] Meenakshi S; Umayaparvathi S; Manivasagan P; Arumugam M; Balasubramanian T. *Indian Journal of Geo-Marine Sciences*, **2013**. 42(4), 510-515.
- [33] Tinrat S; Wongsariya K; Chomnawang MT. Isolation of a Novel Inulinase Producing Bacteria from Soil, Proceeding on The 8th International Symposium on Biocontrol and Biotechnology, Bangkok, **2010**, 62-67.
- [34] Fawzi EM. *Braz J Microbiol*, **2011** , 42(2), 633-649.
- [35] Ayyachamy MK; Khelawan D; Pillay KP; Singh S. *Lett ApplMicrobiol*, **2007**, 45, 439-444.
- [36] Determann H. *Gel Chromatography, A Laboratory Handbook*, 2nded, Springer-Verlag, Berlin-Heidelberg-New York, **1969**.
- [37] Gupta AK; Kaur M; Kaur N; Singh R. *JChem Tech Biotech*, **1992**, 53, 293-296.
- [38] Vullo OL; Cellia EC; Faustino S. *Appl Environ Microb*, **1991**, 57(8), 2392-2394.