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OPTIMIZATION AND CHARACTERIZATION OF EXO-POLYGALACTURONASE BY *Aspergillus niger* CULTURED VIA SOLID STATE FERMENTATION

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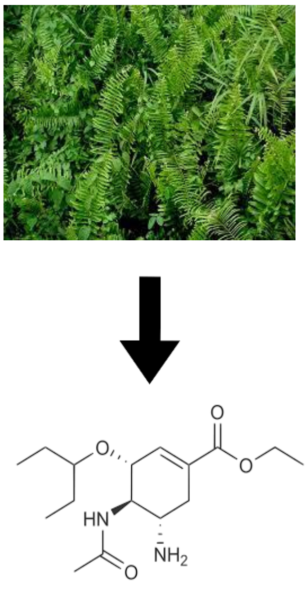
Graphical abstract



Exo-polygalacturonase

Abstract

Polygalacturonases represent an important member of pectinases group of enzymes with immense industrial applications. The activity of exo-polygalacturonase produced by *Aspergillus niger* was studied in solid state fermentation (SSF) using *Nephrolepis biserrata* leaves as substrate. Central composite design (CCD) was used to optimize four significant variables resulted from the screening process that has been initially analyzed for the production of exo-polygalacturonase which are incubation time, temperature, concentration of pectin and moisture content. The optimum exo-polygalacturonase production obtained was 54.64 U/g at 120 hours of incubation time, temperature at 34°C, 5.0 g/L of pectin concentration and 75.26% of moisture content. For partial characterization of exo-polygalacturonase, the optimum temperature and pH were obtained at 50°C and pH 4.0, respectively. SDS-PAGE analysis showed that molecular weight of exo-polygalacturonase were 35 and 71 kDa. This study has revealed a significant production of exo-polygalacturonase by *A. niger* under SSF using cheap and easily available substrate and thus could found immense potential application in industrial sectors and biotechnology.

Keywords: Nephrolepis biserrata, Exo-polygalacturonase, Solid-state fermentation, Response Surface Methodology and *Aspergillus niger*

Abstrak

Poligalakturonase merupakan ahli penting dalam kumpulan enzim pektinase dengan aplikasi besar dalam industri. Aktiviti ekso-poligalakturonase yang dihasilkan oleh *Aspergillus niger* telah dikaji dalam fermentasi keadaan pepejal (FKP) menggunakan daun *Nephrolepis biserrata* sebagai substrat. Reka bentuk komposit berpusat (RBKB) telah digunakan untuk mengoptimumkan empat pemboleh ubah yang didapati signifikan hasil daripada proses penyaringan awal yang telah dianalisa iaitu masa pengeraman, suhu, kepekatan pektin dan kelembapan medium. Penghasilan ekso-poligalakturonase optimum yang diperoleh adalah 54.64 U/g pada masa pengeraman 120 jam, suhu pada 34°C, 5.0 g/L kepekatan pektin dan 75.62% kandungan kelembapan medium. Bagi pencirian separa ekso-poligalakturonase, suhu dan pH optimum masing-masing diperolehi pada 50°C dan pH 4.0. Analisis SDS-PAGE menunjukkan bahawa jisim molekul ekso-poligalakturonase adalah 35 dan 71 kDa. Kajian ini telah membuktikan penghasilan signifikan ekso-poligalakturonase oleh *A. niger* daripada FKP menggunakan substrat murah dan mudah diperolehi sekali gus boleh menjadi potensi besar untuk aplikasi di sektor industri dan bioteknologi.

Kata kunci: Nephrolepis biserrata, ekso-poligalakturonase, fermentasi keadaan pepejal, Rekabentuk komposit berpusat, *Aspergillus niger*

1.0 INTRODUCTION

Pectinase (EC 3.2.1.15) are a group of enzymes that decompose the pectin from plant tissue into simple molecules such as galacturonic acids [1]. Pectinase, also known as pectic enzyme or pectinolytic enzyme is naturally produced by plants, filamentous fungi, bacteria and yeast [2]. The pectinases are one of the important upcoming enzymes of the commercial sector specially for fruit juice industry as a prerequisites for obtaining well clarified and stable juices with higher yields [3]. Based on pectinase mode of action, it can be divided into three types, namely 1) Protopectinase, 2) Esterases (pectin methylesterases and pectin acetylesterases), 3) Depolimerases, who broke the bands of α-(1-4) glycosidic bonds between galacturonic residues by hydrolysis (Polygalacturonase) and trans-elimination (Pectate lyase and pectin lyase) [4].

Fungi, yeast and bacterial are commonly express and secrete a variety of enzymes involved in the degradation and recycling of complex biopolymers using solid state fermentation process (SSF) [5] - [8]. *Aspergillus* sp. is one of the effective bioremediation agents [9]. *Aspergillus niger* was exploited primarily in the food industry and it is generally regarded as safe. In pectin and pectinase industry, application and preparation fungus has mostly obtained from *Aspergillus niger* [10].

Methods of fermentation such as solid-state fermentation (SSF) and submerged fermentation (SmF) are important for the production of microbial enzymes [11]. SSF is the most suitable fermentation technique involving fungi and microorganisms that require low moisture content [12]. Generally, pectinases from various microorganisms were characterized and the molecular masses is around 13 to 82 kDa [13].

Most of the previous studies uses pectin source from fruits and agricultural waste to produce pectinase. However, no study up to date has focus on using fern leaves as the substrate. Hence, in this study the production of pectinase from *Nephrolepis biserrata* leaves is being tested. *Nephrolepis biserrata* is a wild fern that easily grow under palm trees and along the hillsides in Malaysia and other tropical countries. A study by Essuman *et al*. (2014) shows that the *Nephrolepis biserrata* powder contains high carbohydrate content (43.01%, g/g) which indicate a good source for pectin [14].

The goal of this study is to optimize the production of exo-polygalacturonase by *Aspergillus niger* in solid state fermentation (SSF) using *Nephrolepis biserrata* leaves pretreated as the substrate and to partially characterize the enzymes. This study will reveal a significant production of exo-polygalacturonase by *A. niger* under SSF using cheap and easily available substrate and thus could found immense potential application in industrial sectors and biotechnology

2.0 METHODOLOGY

2.1 Sample

Fresh *Nephrolepis biserrata* leaves were collected within the vicinity of Universiti Teknologi Malaysia (UTM).

2.2 Inoculum Preparation

Fungal culture *Aspergillus niger* (ATCC 1010) was obtained from Bioprocess Engineering Laboratory, UTM. It was periodically sub-cultured on Potato-Dextrose Agar (PDA) medium and maintained at 4 °C. Fungus was grown on PDA plates for 5-7 days to obtain sufficient quantity of matured spores. These spores were scrapped out, suspended in 0.1% w/v Tween-80 and then transferred to sterile test tubes. These suspended spores were used as seed culture spores for inoculum preparation. All chemicals used were of analytical grades from Merck and Fisher Scientific unless otherwise stated.

2.3 Solid State Fermentation

Dried, grinded and sifted *Nephrolepis biserrata* leaves were used as the substrate. The leaves were weighed at 10 g/flask into a 500 ml Erlenmeyer flask. Nutrients, $MgSO_4.7H_2O$ at 0.5 g/L, KH_2PO_4 at 0.5 g/L, $FeSO_4.7H_2O$ at 0.005 g/L, pectin *Nephrolepis biserrata* leaves at 10 g/L, yeast at 1 g/L and glucose at 10 g/L were added to the flask. Next, 50 mM citrate buffer at pH 5.0 was added so that the moisture content reaches 70% moisture. The substrate mixture was autoclaved for 1 hour at 121°C and cooled to room temperature (27°C±1) before inoculated with the fungus. Solid medium and steriled substrate were inoculated with 10% (v/v) from 1 x 10^7 spores/mL. Spore suspension was transferred to the flask containing the solid substrate medium, mixed evenly, and incubated in an incubator at temperature and time intervals optimized by the design of experiments. The experiment was conducted in two conical flasks in which each flask was sampled three times. Sampling was performed every 24 hours on the first day to 192 hours of incubation. For each sampling, 1 g was removed and was collected for every 24 hours.

2.4 Exo-polygalacturonase Activity Assay

For each sample, 1 g samples were removed then mixed with 10 mL of citrate buffer (pH 5.0). The exo-polygalacturonase activity was assayed by measuring the release of reducing sugars by the DNS method [15]. Glucose was used as the standard. In a test tube, 0.5 ml of 1.0% (w/v) pectin (Sigma) in 0.5 M citrate buffer (pH 5.0) was added to 0.5 ml of diluted enzyme solution. After 15 min incubation at 50°C, the reaction was stopped by the addition of 1 ml of DNS and heated in boiling water for 5 min. Next, 5 ml of distilled water was added to each sample. Samples were read at OD 540 nm in a spectrophotometer. One unit of Exo- polygalacturonase enzymes is defined as the number of µM reducing sugars that are measured in terms of glucose, produced because of the action of the enzyme extract 1.0 ml in 1 min at 35° C ± 1°C [16]. The total soluble protein was determined by Lowry method [17] using BSA (*Bovine serum albumin*) as standard.

2.5 Experimental Design and Statistical Analysis

The experimental design and statistical analysis were made using Design Expert Version 6.0.4 (Stat-Ease, Inc., Minneapolis, MN, USA) software. The RSM used in the present study is a central composite experimental design (CCD) [18] involving four different factors. Experiments were conducted in a randomized fashion. The CCD contains a total of 30 experimental trials involving the replications of the central points. The dependent variables selected for this study were exo-polygalacturonase activity (U/g). The independent variables chosen were incubation time (h), X_1; temperature (°C), X_2; pectin (g/L), X_3 and moisture content (%), X_4. Once the experiments were performed, a second order polynomial equation (1) shown below was used to describe the effect of variables in terms of linear, quadratic and cross product terms. In CCD, the range and levels of the variables investigated in this study are as given in the Table 1.

Table 1 Code and actual values of the factors in central composite design

Var.	Parameter	Level				
		-a	-1	0	1	+a
X_1	Incubation time (h)	14.40	24	72	120	129.60
X_2	Temperature (°C)	25.20	26	30	34	34.80
X_3	Pectin conc. (g/L)	4	5	10	15	16
X_4	Moisture content (%)	74	75	80	85	86

Experimental data were analyzed to fit the following regression model with interaction terms are given below:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 + b_{22x}X_2^2 + b_{33}X_3^2 + b_{44x}X_4^2 + X_1X_2 + X_1X_3 + X_1X_4 + X_2X_3 + X_2X_4 + X_3X_4 \quad (1)$$

Where, Y represents exo-polygalacturonase activity (U/g), while the X_1, X_2, X_3 and X_4 represent temperature, time of incubation, concentration of pectin, and moisture content of the substrate, respectively. The regression model was generated by the Design Expert software after considering all the variables. The model has one designation offset, 4 linear terms, quadratic terms 4, and 6 interactions.

2.6 Characterization of Crude of Exo-polygalacturonase

2.6.1 *Effect of pH on Exo-polygalacturonase Activity and Stability*

The effect of pH on exo-polygalacturonase activity was determined by incubating the reaction mixture at pH values ranging from 4.0 to 6.0, under standard enzyme assay conditions. The pH stability of the enzyme was evaluated by measuring the residual activity, under standard enzyme assay conditions, after incubating the enzyme for 24 h at 4°C at various pH from 2.5 to 7.5. The buffers employed in these measurements were citrate/phosphate buffer (pH 3.0 and 6.0 - 9.0). All the experiments were conducted in triplicates and the results show the mean values of the activities.

2.6.2 Effect of Temperature on Exo-polygalacturonase Stability

The thermostability of the enzyme was determined by measuring the residual activity, under standard enzyme assay conditions, after incubating the enzyme solution for 60 min at various temperatures from 50°C to 70°C, at pH optimum. All the experiments were conducted in triplicates and the results show the mean values of the activities.

2.7 SDS-Page Analysis

Determination of molecular weight of exo-polygalacturonase was performed by using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) [19]. This method is an important way to carry out protein analysis and characterization. Each protein differs from one another by the amino acid sequence. The amino acid sequences give each protein a unique charge, size and shape. A total of 12% (v/v) gel separator and 5% (v/v) gel compiler is provided in the set of gel plates. Samples of 20 µL were mixed and 5 µL of sample buffer as ballast (20 µL+5 µL) in the Eppendorf tube. The sample was heated at 100°C for 5 minutes and placed on ice. The protein solution was thrown for 1 second in a sprayer at 4,000 rpm. The sample (20 µL) was loaded into the container. For reference, 10 µL molecular weight marker (Protein Marker) was loaded into separate containers. The electrophoresis was carried out at 90 V for 60 minutes. After the process completed, the gel is colored with Coomassie blue. The gel was then dried. The partial characterization of raw exo-polygalacturonase by *Aspergillus niger* is shown in Figure 7.

3.0 RESULTS AND DISCUSSION

3.1 Optimization of Exo-polygalacturonase

The probability value (P-value) for each parameter was shown in Table 2. The P-value of less than 0.05 defines the factors as significant. The model is significant with probability of <0.0001. The regression coefficient, R^2 value of 0.9748 indicates the model fit with the experimental data. The optimum level for each variable factor was determined by constructing three-dimensional surface plot based on the mathematical model equation that have been issued. The production of exo-polygalacturonase was achieved at 54.64 U/g. This plot also represents the interaction between two variables while maintaining the third variable (Figure 1 to 2).

Table 2 Regression analysis (ANOVA) for the Exo-polygalacturonase activity using 2 – level factorial design

Source	Sum of Squares	Degree of Freedom	Mean Square	F-value	p -value	*R*
Model	2821.69	14	201.55	41.46[a]	<0.0001[b]	0.9748
X_1	2069.02	1	2069.02	425.64	<0.0001	
X_2	0.87	1	0.87	0.18	0.6780	
X_3	56.27	1	56.27	11.58	0.0039	
X_4	160.88	1	160.88	33.10	<0.0001	
X_1^2	25.62	1	25.62	5.27	0.0065	
X_2^2	55.36	1	55.36	11.39	0.0042	
X_3^2	16.06	1	16.06	3.30	0.0891	
X_4^2	4.10	1	4.10	0.84	0.3729	
$X_1 X_2$	258.21	1	258.21	53.12	<0.0001	
X_1X_3	26.68	1	26.68	5.49	0.0333	
X_1X_4	130.66	1	130.66	26.88	0,0001	
X_2X_3	11.58	1	11.58	2.38	0.1435	
X2X4	11.04	1	11.04	2.27	0.1526	
X_3X_4	0.42	1	0.42	0.086	0.7737	
Residual	72.91	15	4.86	-	-	-
Lack of Fit	55.47	10	5.55	1.59	0.3175[c]	-
Pure error	17.44	5	3.49	-	-	-
Co Total	2894.60	29	-	-	-	-

[a] *F*-Value is significant.
[b]Model is significant, with p> F lower than 0.05.
[c] model is fit due to insignificant *F*-value.
Standard deviation is 20.2

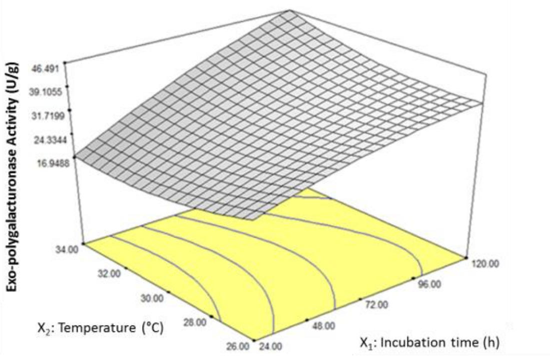


Figure 1 Response Surface plot of exo-polygalacturonase activity: a) effect of incubation time and temperature

Figure 1 shows the effect of incubation time and temperature with the moisture content being radiated at the midpoint value (50%). Exo-polygalacturonase activity increased in tandem with incubation time from 24 hours to 120 hours and temperatures ranging from 26-34°C. Exo-polygalacturonase production is plotted as a function of incubation time and temperature (Figure 1) and the variable shows a significant production of exo-polygalacturonase (ANOVA Table 2). In this study, the optimum incubation time obtained was 120 hours. The screening of incubation time is important for optimizing the enzyme production process. If less incubation time is used, it may cause low enzyme synthesis and subsequently low enzyme activity due to insufficient amount of enzyme produced. However, overtime incubation will increase the risk of contamination [20] - [22]. Pectinase production can be obtained starting from the incubation period of 2 days to 9 days [23]. Previously, the optimal pectinase production was obtained at different incubation time of 96 hours [24], 48 hours [25, 26] and 120 hours [27].

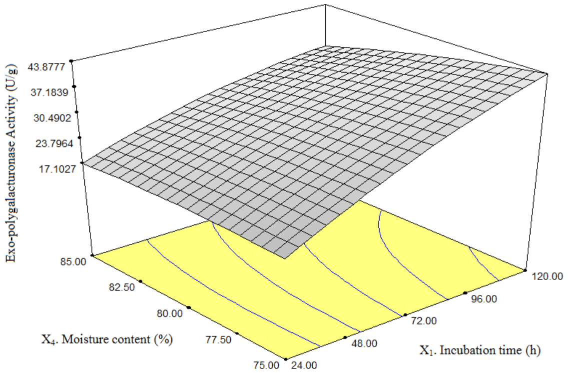


Figure 2 Response Surface plot of exo-polygalacturonase activity: (b) effect of incubation time and moisture content

Furthermore, the effect of incubation time and moisture content of the substrate with temperature which is radiated at midpoint value (50%) is shown in Figure 2. Exo-polygalacturonase production is plotted as a function of incubation time and moisture content to show the relationship between both variables. The effect of interaction between incubation time and moisture content was significant with temperature (30°C) and pectin concentration (10 g/L) was observed at the midpoint. In this study, the optimum moisture content was obtained at 75.26%.

Ahmed and Mostafa (2013) state that moisture content is one of the most important parameters in the SSF process [28]. This is because a very high or very low substrate humidity can reduce the production of exo-polygalacturonase due to the disruption of the growth of microorganisms used. The microorganisms require a certain amount of moisture in order to grow well. Therefore, the production of exo-polygalacturonase will be low when the moisture content decreases or exceeds the optimal optimum level [29]. El-Shishtawy *et al*. (2014) also reported that at high moisture content, the substrate prevents penetration of oxygen and facilitates pollution, while low levels of humidity can inhibit enzyme activity as well as access to nutrients [30]. Generally, the 70% moisture content of the substrate responded well to all substrates that uses *Aspergillus niger* and subsequently produce optimum enzyme activity.

3.2 Optimum Temperature and Temperature Stability for Exo-polygalacturonase Activity

Temperature is identified as a critical factor that affects the enzyme and substrate responses. Each enzyme has its optimum temperature to be able to react effectively. The influence of temperature on the activity and stability of exo-polygalacturonase is shown in Figure 3 and Figure 4. In this study, the optimum temperature of exo-polygalacturonase was obtained at 50°C as shown in Figure 3. The increased plot in Figure 3 is known as the activation of temperature. At 60°C, the exo-polygalacturonase activity has begun to decline from 100% relative activity at 50°C to 97.9% due to enzyme inactivation. Each enzymes has its own optimum temperature range. Temperature exceeding this optimum range will cause the protein to denature and hence losses its activity and becomes inactive.

The optimum temperature of 50°C obtained for exo-polygalacturonase activity in this study is similar to the exo-polygalacturonase activity resulting from *Aspergillus niger* by Mrudula and Anithraraj (2011) and Aspergillus fumigatus by Phutela *et al*. (2005), [24, 31]. Other report also states that the optimum temperature on exo-polygalacturonase activity by *Aspergillus niger* is at temperature ranging from 40°C to 60°C [32]. The optimum temperature of polygalacturonase enzyme activity was achieved at 40°C by *Aspergillus sojae* [33]. In contrast, another study showed that the optimum temperature for polygalacturonase production was at 55°C by *Aspergillus sojae* fungi [34] and *Aspergillus niger* [35, 36]. In addition, Mrudula and Anithraraj (2011) report that the optimum temperature for exo-polygalacturonase activity of *Aspergillus niger* is at 60°C [24]. Therefore, from these discussions and comparisons it is clear that the optimum temperature for exo-polygalacturonase activity of the genus *Aspergillus* is within the range of 40°C to 50°C

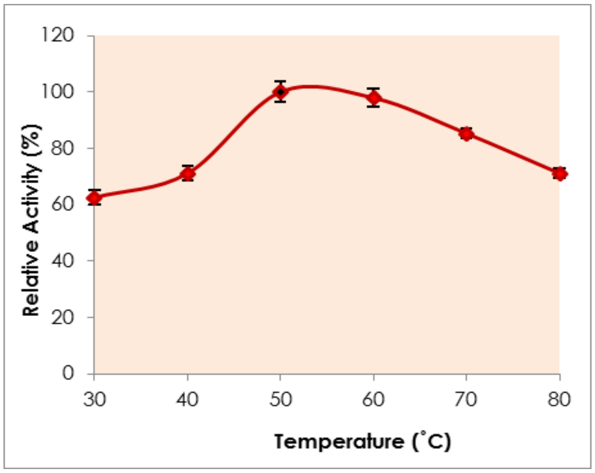


Figure 3 Relative activity of exo-polygalacturonase by *Aspergillus niger* at different temperatures

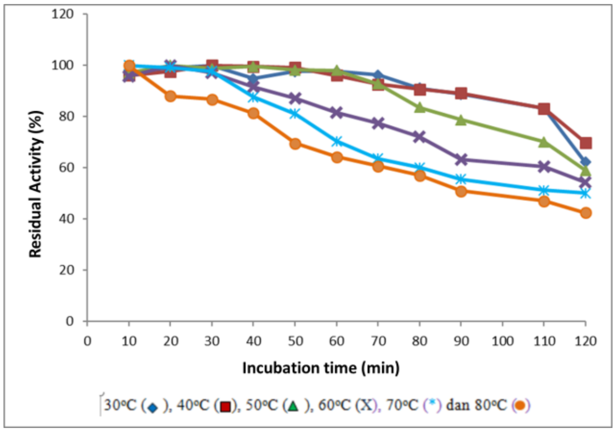


Figure 4 Temperature stability studies on raw exo-polygalacturonase by *Aspergillus niger* at different temperature

Temperature stability studies on raw exo-polygalacturonase by *Aspergillus niger* are shown in Figure 4. From the diagram, exo-polygalacturonase activity was stable at incubation time up to 60 min at temperatures of 40°C and 50°C before the relative activity decline below 95%. For temperatures of 30°C, 60°C and 70°C, exo-polygalacturonase activity was stable until the incubation time reached 30 min before subsequently reduced drastically. Whereas for temperature 80°C, a decrease of 13% residual activity occurred during the first incubation of 10 to 20 min. After 120 minutes, exo-polygalacturonase activity was reduced from 100% to 62.21% (30°C), 69.79% (40°C), 59.04% (50°C), 54.34% (60°C), 50.08% 70°C) and 42.45% (80°C). This result shows that the increase in temperature from 30 to 80°C in the range of 10 to 120 min has reduced the stability of enzymes, in which the hydrogen bond interactions do not work properly and caused protein refraction [37]. Exposure of enzymes to high temperatures has led to the abatement of the proteins function. This also strongly depends on the duration of enzymes exposed to the high temperatures. However, in this study, the studied exo-polygalacturonase has proved to be stable at long incubation time (120 min) where it still exhibiting exo-polygalacturonase activity at relative activity of 50% or higher. The enzymes require longer reaction times and high temperatures to bond more strongly with the substrate on the active site [38]. Thermostable temperatures can be divided into 3 groups, i.e. medium thermostable (45-65°C), thermostable (65-85°C) and highly thermostable (> 85°C) [38]. This exo-polygalacturonase by *Aspergillus niger* has managed to maintain its lowest residual activity of 59% at temperature of 50°C and thus making this enzyme in the thermostable group of enzymes.

3.3 pH Optimum and pH Stability of Exo-polygalacturonase Activity

The effect of pH on exo-polygalacturonase crude activity by *Aspergillus niger* was studied at pH 3.0 to pH 10.0 as shown in Figure 5. From this study, the optimum pH for exo-polygalacturonase is obtained at pH 4.0. The pH value obtained in this study is equivalent to the results obtained by Rashmi *et al*., (2008) who also reported the exo-polygalacturonase by *Aspergillus niger* showed optimum pH at 4.0 [39]. In this study, the exo-polygalacturonase activity obtained 100% relative activity at pH 4.0 and started to decrease at pH 5.0 up to pH 10.0. Based on Figure 5, the relative activity of exo-polygalacturonase investigated was 76.34% at pH 3 and increased 100% of its relative activity at pH 4.0. Relatively exo-polygalacturonase activity decreased at pH 5.0 (90%), pH 6 (73.9%), pH 7 (69.2%), pH 8 (56.9%), pH 9 (52.4%) and pH 10.0 (46.8%) after 120 minutes incubation at 50 ° C. This is because pH changes can affect ionization of active sites of amino acids and caused them to be affected and the occurrence of enzyme disassembly [40].

The study was consistent with other reports showing that the optimum pH of exo-polygalacturonase activity by *Fusarium oxysporum*, *Aspergillus niger* and *Mucor circinelloides* was in the range of 4.0 to 5.0 [41, 42].

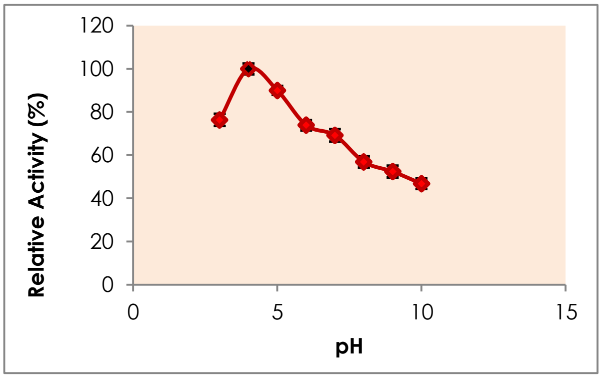


Figure 5 Relative activity of exo-polygalacturonase by *Aspergillus niger* at different pH with optimum temperature of 50 ° C

Other studies reported that the optimum pH of exo-polygalacturonase activity from *Kluyveromyces wickerhamii* [43] and by *Trichoderma reesei* [44] was at pH 5.0. The exo-polygalacturonase enzyme produced in the present study with the optimum pH range is believed to have potential applications in the fruit juices and wine manufacturing industries [36].

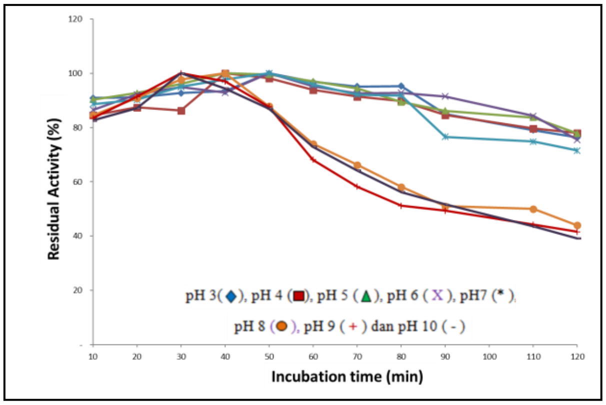


Figure 6 pH stability of exo-polygalacturonase from *Aspergillus niger* at different pH (3-10)

The pH stability of exo-polygalacturonase activity by *Aspergillus niger* at an optimum temperature of 50°C at different pH within the range of pH 3.0 to pH 10.0 during 120 minutes of incubation is shown in Figure 6. At pH range of 3.0 to 7.0 indicates that the activity of exo-polygalacturonase maintained stable above 70% until after 120 minutes of incubation time. At incubation time of 70 minutes, the stability of exo-polygalacturonase activity at pH range 8.0 to 10.0 decreased by less than 70%, respectively. From this observation, exo-polygalacturonase by *Aspergillus niger* produced in the present study was stable within the range of pH 3 to pH 7. Whereas for alkaline pH (8.0 to 10.0), the exo-polygalacturonase activity decreased after incubation time of 40 min until it reached 40% of residual activity after 120 min incubation time.

3. 4 Characterization of Exo-polygalacturonase

3.4.1 Molecular Weight Determination of Exo-polygalacturonase

In this study, two protein bands with molecular weight 35 and 71 kDa is observed from the SDS-PAGE analysis (Figure 7). These proteins are labeled as exo-polygalacturonase from *Aspergillus niger*. Buga *et al*., (2010) reported that the polygalacturonase enzyme from *Aspergillus niger* isolated in the SDS-PAGE gel showed the existence of endo-exo-polygalacturonase at 35 kDa and exo-polygalacturonase at 40 kDa [45]. It was also found that polygalacturonase enzyme molecules from *Penicillium sp*. is at 35 kDa [46], 31 kDa by *Rhyzopus oryzae* [47] and 31 kDa by *Penicillium chrysogenum* [48]. Arijit *et al*., (2013) found that the pectinase from *Aspergilus giganteus* was 71 kDa [49, 50]. Other study has also reported that the molecular weight of the exo-polygalacturonase enzyme by *Klebsiella sp*. is at 72 kDa [51].

Meanwhile, different molecular weights of the exo-polygalacturonase enzyme (two strips) were observed from *Bacillus sp* which was 36 and 72 kDa [52], 36 and 38 kDa by *Aspgillus niger* [53], 38 and 61 kDa by *Aspergillus niger* [54], and 38 and 65 kDa by *Aspergillus japonicus* [55], respectively. Gummadi *et al*., (2007) have reported that the molecular weight of exo-polygalacturonase enzymes from various sources such as citrus fruits, apples and plums differ from 25 to 350 kDa [56].

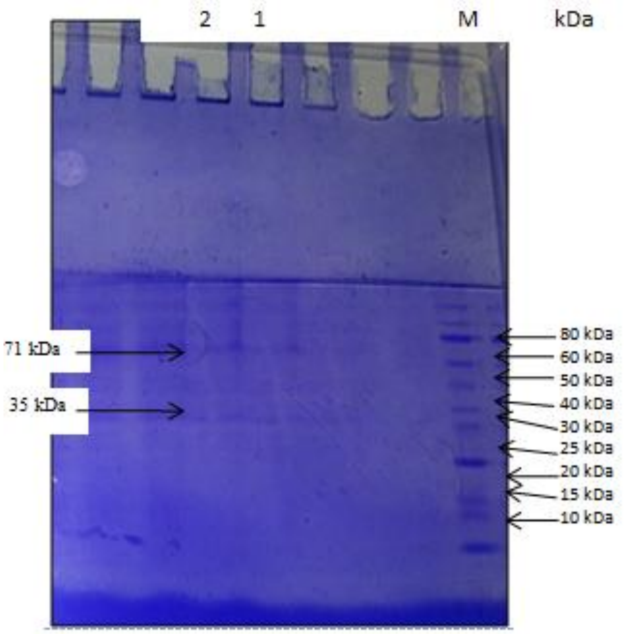


Figure 7 SDS-PAGE analysis of crude exo-polygalacturonase by *Aspergillus niger.* (lane 1, 2: crude of exo-polygalacturonase, M: marker)

4.0 CONCLUSION

Based on the screening process that has been analyzed for the production of exo-polygalacturonase, the optimal conditions was achieved at incubation time 120 h, temperature 34°C, pectin concentration 5.01 g/L and moisture content 75.62%. From the optimization process by the CCD, the production of exo-polygalacturonase activity by *Aspergillus niger* was achieved at 54.64 U/g using *Nephrolepis biserrata* leaves. The crude exo-polygalacturonase by *Aspergillus niger* was partially characterized. The optimum temperature and pH were 50°C and 4.0, respectivley. Based on protein visualization by the SDS-PAGE, the molecular weights of the crude exo-polygalacturonase were identified at 35 kDa and 71 kDa.

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