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Pectinase Production from Banana Peel Biomass via the Optimization of the Solid-state Fermentation Conditions of *Aspergillus niger* Strain

Nazaitulshila Rasit[1,2]*, Yong Sin Sze[2], Mohd Ali Hassan[1], Ooi Chee Kuan[2], Sofiah Hamzah[2], Wan Rafizah Wan Abdullah@Wan Abd. Rahman[2] and Md. Nurul Islam Siddique[2]

[1]*Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*
[2]*Faculty of Ocean Engineering Technology and Informatics, Universiti Malaysia Terengganu, 21030 UMT, Kuala Nerus, Terengganu, Malaysia*

ABSTRACT

In this study, the biomass of banana peel was used to produce pectinase via optimization of solid-state fermentation conditions of the filamentous fungi *Aspergillus niger (A. niger)*. The operating conditions of solid-state fermentation were optimized using the method of full factorial design with incubation temperature ranging between 25 °C and 35 °C, moisture content between 40% and 60%, and inoculum size between 1.6 x 10^6 spores/mL and 1.4 x 10^7 spores/mL. Optimizing the solid-state fermentation conditions appeared crucial to minimize the sample used in this experimental design and determine the significant correlation between the operating conditions. A relatively high maximal pectinase production of 27 UmL-1 was attained at 35° C of incubation, 60% of moisture content, and 1.6 x 10^6 spores/mL of inoculum size with a relatively low amount of substrate (5 g). Given that the production of pectinase with other substrates (e.g., pineapple waste, lemon peel, cassava waste, and wheat bran) generally ranges between 3 U/mL and 16 U/mL (Abdullah et al., 2018; Handa et al., 2016; Melnichuk et al., 2020;

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E-mail addresses:
nazaitulshila@umt.edu.my (Nazaitulshila Rasit)
p3312@pps.umt.edu.my (Yong Sin Sze)
alihas@upm.edu.my (Mohd Ali Hassan)
9w2ckq@gmail.com (Ooi Chee Kuan)
sofiah@umt.edu.my (Sofiah Hamzah)
wanrafizah@umt.edu.my (Wan Rafizah Wan Abdullah@Wan Abd. Rahman)
m.nurul@umt.edu.my (Md. Nurul Islam Siddique)
*Corresponding author

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Thangaratham and Manimegalai, 2014; Salim et al., 2017), thus the yield of pectinase derived from the banana peel in this study (27 U/mL) was considered moderately high. The findings of this study indicated that the biomass of banana peel would be a potential substrate for pectinase production via the solid-state fermentation of *A. niger*.

Keywords: Banana peel, biomass, full factorial design, optimization, pectinase, solid-state fermentation

INTRODUCTION

The pectinolytic enzymes, or more commonly known as pectinases, are normally found in plant cell walls. They break down the polysaccharide pectin through the reactions known as hydrolysis and de-esterification (Sudeep et al., 2020). Based on the mechanism of actions of their pectin molecules, they are classified into three groups, namely pectolyase, pectozyme, and polygalacturonase (Jayani et al., 2005). Given its capability to degrade plant materials, pectinase has a wide range of industrial uses, such as in the food processing industry (Ribeiro et al., 2010), textile manufacturing (Aggarwal et al., 2020), agricultural application (Al-Rousan et al., 2019) and environmental cares (Kamaruddin et al., 2019). However, the key role for the application is the capability of the enzyme to act as a catalyzing reaction in degrading a long and complex molecule presented in the cell wall of a plant cell, thus improving further related processes (Jayani et al., 2005; Ribeiro et al., 2010).

In the food processing industry, pectinase is combined with other enzymes, such as xylanases and cellulases, to increase juice extraction and clarity (Ribeiro et al., 2010, Dal Magro et al., 2018; Cerreti et al., 2016). Meanwhile, in the textile industry, pectinase is used to pre-treat the cotton fibers by bioscouring to increase their absorbency, making them suitable for subsequent staining (Aggarwal et al., 2020; Erdem & Bahtiyari, 2018). Furthermore, in agricultural application, pectinase is widely used in the extraction of olive oil to prevent emulsification by dispersing the oils droplets from peel extracts, thus facilitating its extraction later in the aqueous process (Al-Rousan et al., 2019; Caporaso, 2016). On the other hand, in the environmental application, pectinase is commonly used in wastewater treatment, whereby pretreatment of wastewater with pectinase could remove oil and grease from kitchen greywater, thereby reducing its biological oxygen demand (Kamaruddin et al., 2019).

Although pectinase occurs naturally in many terrestrial plants, in industrial applications, the microbial systems are used for pectinase production because they provide a higher yield at less cost (Ribeiro et al., 2010; Sudeep et al., 2020). There are various pectinase-producing microorganisms, such as bacteria, fungi, nematodes, and protozoans (Sudeep et al., 2020). The most widely used microorganism in pectinase production is the filamentous fungi, particularly the *Aspergillus niger* that the United States Food and Drug Administration (FDA) has classified under the Generally Recognized as Safe (GRAS) category (Jayani

et al., 2005). Also, the development of the fermentation technique, known as solid-state fermentation (SSF), has helped *A. niger* gain wide acceptance in industrial applications (Kapilan, 2015).

The SSF is an economical fermentation technique because the amount of water used is low, thereby reducing the chance of contamination. Also, the yield of SSF is higher than that of the conventional methods, such as liquid and submerged fermentation, because a high biomass ratio is used in the SSF (Doriya et al., 2007; Webb & Manan, 2017). In SSF fermentation, the substrate provides the nutrients that act as carbon and nitrogen sources for the microorganisms to grow on the culture medium (Kapilan, 2015). Thus, in the production of industrial pectinase, the selection of substrate and its availability has become a crucial factor in determining the production cost and efficiency (Abdullah et al., 2018; El Enshasy et al., 2018; Handa et al., 2016; Thangaratham & Manimegalai, 2014). In this respect, cheap carbon sources are commonly acquired from agro-industrial waste because they are highly abundant (Melnichuk et al., 2020; Salim et al., 2017). In this study, the biomass of banana peel, an underutilized agro-waste, was used as a substrate for pectinase production. The biological and physico-chemical factors are important in affecting the performance of SSF. Among the factors are temperature, moisture content, pH, aeration rate, and particle size of the substrate. The development of the SSF method varies according to the factors used, and each factor selected has its advantages and disadvantages (Webb & Manan, 2017).

In Malaysia, banana is the second-largest cultivated fruit, with a total production of about 530,000 metric tons in 2007, together with a substantial amount of banana peel (Mekhilef et al., 2011). The banana peel, comprising approximately 40% of the banana's weight, contains a relatively high amount of pectin (10-21% of its total biochemical content) (Mohapatra et al., 2010), making it a suitable substrate for the production of pectinase. In this study, the fungi *A. niger* was used to hydrolyze pectin in the banana peel for the production of pectinase using the SSF method. The objective of this study was to determine the optimal operating conditions for the SSF of *A. niger* in producing the maximum yield of pectinase at a specific amount of substrate. Thus, the use of banana peels would contribute toward more effective sustainable management of agro-waste in the country.

MATERIALS AND METHODS

The Experimental Design and Optimization of Solid-state Fermentation

In this study, the SSF with *A.niger* was used to produce pectinase, and the operating conditions of SSF were optimized for the maximum production of the enzyme. Three major factors influence the performance of SSF: biological factors, physico-chemical factors, and mechanical factors. However, only the biological and physico-chemical factors are frequently manipulated to enhance the productivity of SSF. Meanwhile, the latter can be further designed during the commercial stage (Webb & Manan, 2017).

Thus, this study selected three fermentation parameters for optimization, namely moisture content, temperature, and inoculum size. They are among the most commonly reported contributory biological and physico-chemical factors that control the dynamics of enzyme production. For example, the moisture content is known as the limiting factor for which *A. niger* grows optimally in the moisture content of 40% - 60% (Jayani et al., 2005; Manpreet et al., 2005) and in the inoculum size of 1.59 x 10^6 spores/ml to 1.43 x 10^7 spores/ml (Sandoval-Contreras et al., 2017). Meanwhile, the enzymatic reactions are dependent on incubating temperatures with high reactivity between 25 °C and 35 °C (Kapilan, 2015; Pandey et al., 2001).

The full factorial design incorporated in the Minitab Statistical Software *(Minitab LLC, Sydney, Australia)* was used to list all the possible interactive combinations of SSF parameters while determining the number of samples to be run. In addition, this study employed the 2k-level design, where k denoted the number of parameters examined in the experiment (Awad et al., 2011; Bouchekara et al., 2011). Altogether, this study experimented with 24 samples run in triplicates on three parameters.

The Substrate, Microorganisms, and Culture Conditions

In this study, the biomass of banana peel served as the substrate for pectinase production. The banana peel collected from the local market was washed with tap water, sliced, and dried in an oven at 45 °C for 24 hours to remove the water content. The dried peel was grounded into fine masses, sieved through a standard mesh sieve of 75 µm, and stored at room temperature before using it as the solid substrate. Meanwhile, the CFR335 strain of *A. niger* used in this study to hydrolyze pectin in the substrate was obtained from the microbiological laboratory of Universiti Malaysia Terengganu. The fungi strain was inoculated on the potato-dextrose agar medium and incubated at 30 °C for six days to produce enough mature spores. Spores were then collected by flooding the surface of PDA with distilled water. Then, they were removed by scratching away from the PDA gently with a spatula and diluting with 500 mL of distilled water. The spores in the suspension were calculated as an inoculum size using a Neubauer Chamber (Sandoval-Contreras et al., 2017). These spores were then stored at 4 °C before they were used for pectinase production.

The Preparation of *A. niger* for Pectinase Production via Solid-State Fermentation

The SSF technique used in this study was similar to the method described in Mansor et al. (2019). A series of 24 aqueous fermentation media with varying moisture contents were prepared. In the preparation of each fermentation medium, 5 g solid substrates were placed in each 100-mL Erlenmeyer flask and mixed with the corresponding amount of distilled water to produce the projected moisture content. Specifically, 5.5 mL and 7.5 mL of distilled water were added to generate a moisture content of 40% and 60%, respectively. These fermentation media were then sterilized at 121 °C for 20 minutes. Each fermentation

medium was inoculated with 2 mL of diluted *A. niger* at varying inoculum sizes upon cooling. Specifically, the fermentation medium of 40% and 60% were inoculated with *A. niger* at an inoculum size of 1.59 x 10^6 spores/ml to 1.43 x 10^7 spores/ml, respectively. The inoculum size of the mature spores *A. niger* was prepared by diluting 1 petri dish in 500 mL of distilled water and was calculated as 1.59 x 10^6 spores/mL. Meanwhile, spores were collected from the PDA of 9 petri dishes, diluted with 500 mL of distilled water, and calculated as 1.43 x 10^7 spores/ml. These inoculated fermentation media were then incubated at different temperatures for 120 hours. Specifically, 40% and 60% fermented medium were incubated at 25 °C and 35 °C, respectively. Table 1 shows the design matrix of the factors in the binary systems, and the coded values for the selected parameters are randomized. For each selected parameter, the low level and high level were coded -1 and +1, respectively.

Table 1

The design matrix of aquoes fermentation for the optimization of pectinase

Run Number	Coded Values of Variables			Pectinase Activity (U/ml)
	Temperature	Moisture Content	Inoculum Size	
1	-1	-1	-1	4.264
2	+1	-1	-1	16.116
3	-1	+1	-1	6.251
4	+1	+1	-1	27.678
5	-1	-1	+1	5.348
6	+1	-1	+1	20.813
7	-1	+1	+1	2.909
8	+1	+1	+1	16.477
9	-1	-1	-1	5.348
10	+1	-1	-1	15.935
11	-1	+1	-1	9.413
12	+1	+1	-1	23.704
13	-1	-1	+1	4.354
14	+1	-1	+1	17.019
15	-1	+1	+1	3.631
16	+1	+1	+1	19.368
17	-1	-1	-1	5.980
18	+1	-1	-1	17.019
19	-1	+1	-1	9.142
20	+1	+1	-1	25.510
21	-1	-1	+1	3.722
22	+1	-1	+1	19.368
23	-1	+1	+1	4.444
24	+1	+1	+1	18.284

Pectinase Extraction and Activity

Upon the completion of fermentation, each of the inoculated fermentation mediums was added with 20 mL of 0.01 M sodium acetate buffer at pH 5.8. The Erlenmeyer flasks containing fermentation media were shaken at 120 rpm for 30 minutes. The suspension of each flask was filtered, and each filtrate was transferred into a separate Falcon tube. The cultured filtrate was cooled to 4 °C and centrifuged at 4000 rpm for 15 minutes. The supernatants were collected as crude pectinase. The extracted pectinase was analyzed for enzymatic activity using the method of dinitrosalicylic acid (DNS). The enzymatic concentration was defined as the amount of enzyme that catalyzes the μmol of substrate per minute under the assay condition (U/mL). Pectinase produced catalyzes the degradation of pectic substances in two reactions, which are depolymerization and desertification (Miller, 1959). Pectinase produced catalyzes the degradation of pectic substances in two reactions, which are depolymerization and desertification. The biological factors used in this study are related to the reproduction and metabolic process of the microorganism used in the SSF. On the other hand, the physico-chemical factors are related to the conditions inside the bioreactors during the SSF process.

Statistical Analyses, the Relative Importance, and Standardized Effects of Individual Parameter and their Interactions with other Parameters

The underlying assumptions of ANOVA and outliers were examined with the residual plot, evaluating the difference between the predicted response value and observed response value (El-Zaher & Mahrouse, 2013). Also, the normal probability plot of residual analysis was used to check the normality of data (Hasan et al., 2009).

Meanwhile, the relative importance of each parameter was assessed with a Pareto chart, in which a statistical-based acceptance limit equivalent to 95% confidence level was used for ranking the effect of each parameter and their interactions (Van Hecke, 2016). Also, the normal probability plot was used to examine if the effect of a parameter by itself or in interaction with others was significant. The effect of a parameter or an interaction is significant if the plot is close to the normal line, but if the plot is centered on the zero value, the effect is deemed negligible (Kukreja et al., 2011; Regti et al., 2017). Besides, the main effect plot was used to determine the magnitude of each parameter for the optimized production of pectinase (Kukreja et al., 2011). On the other hand, the response surface and contour plots were used to determine the relationship and interaction between all the parameters on their responses to pectinase activity while establishing the desirable response values and operating conditions (Hank et al., 2014). Finally, the experimental results were validated with the optimization plots from Minitab Statistical Software.

RESULTS AND DISCUSSION

The Individual Effect and Interaction of Parameters

The main effect plot in Figure 1 shows that the plots for all the three parameters, namely incubation temperature, moisture content, and inoculum size, were not parallel with the reference line, suggesting that these parameters would affect the pectinase production. For the temperature plot, the highest pectinase activity was attained at 35 °C, and raising the incubation temperature from 25 °C to 35 °C increased the pectinase activity by 14 U/mL. Meanwhile, the moisture content of 60% yielded a pectinase activity of about 4 U/mL higher than the moisture content of 40%. Besides, an increase of inoculum size from 1.59 x 10^6 spores/ml to 1.43 x 10^7 spores/ml resulted in a decrease of 3 U/mL of pectinase activity. Together, the incubation temperature appeared to have the greatest effect on pectinase activity with the steepest plot slope than the moisture content and inoculum size.

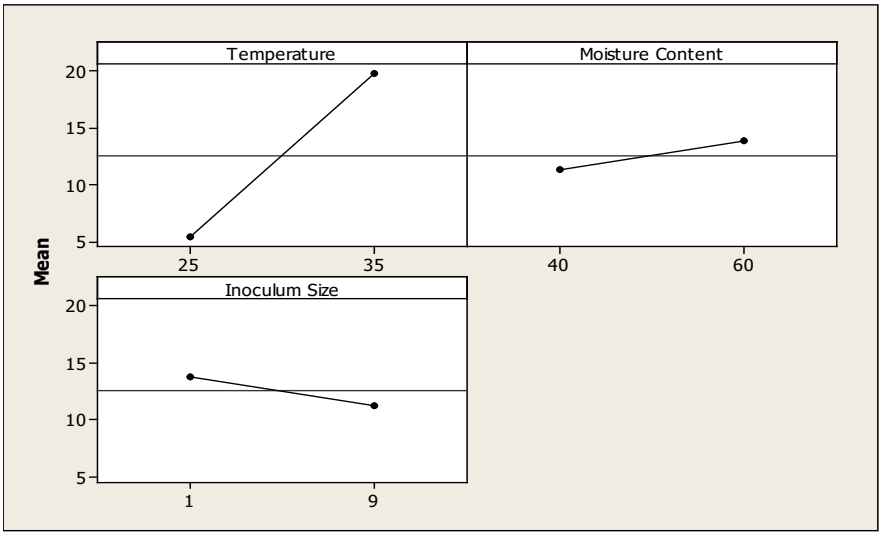


Figure 1. The main effect plots for pectinase activity

Meanwhile, the interaction between moisture content and inoculum size, with plots deviating more from parallel (Figure 2, bottom right), was greater than the interaction between incubation temperature and moisture content (Regti et al., 2017). Besides, the interaction plot of temperature and moisture content (Figure 2, top left) shows that the maximum pectinase activity was attained at an incubation temperature of 35 °C and a moisture content of 60%. For the interaction plot of moisture content and inoculum size, the highest pectinase activity was reached at a moisture content of 60% and an inoculum size of 1.59 x 10^6 spores/ml. On the other hand, the interaction between incubation temperature and inoculum size was insignificant, with two nearly parallel lines (Figure 2, top right) (Hank et al., 2014).

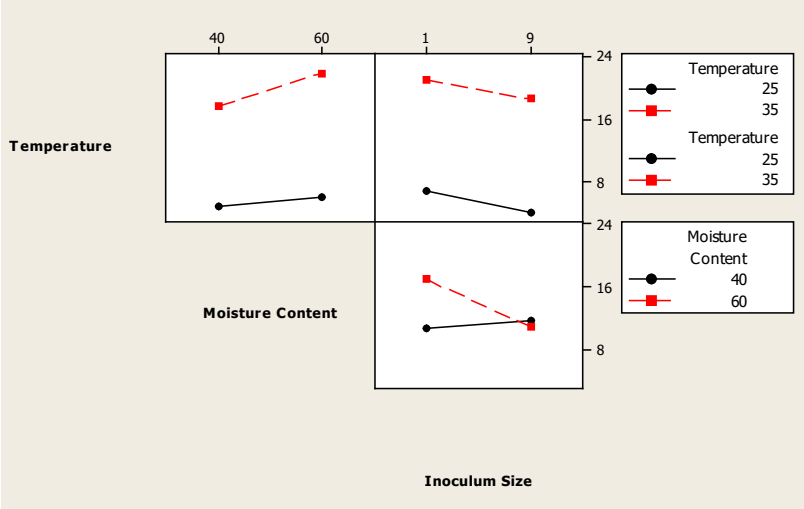


Figure 2. The interaction plots for pectinase activity

Statistical Analysis, Residual Analysis, and the Relative Importance of Various Parameters

Pectinase produced from *A.niger* by using 5g banana peel as solid substrate in 24 experiment runs were conducted as suggested by the design matrix of Minitab for the selected parameter. The selected parameters with their corresponding low and high level were moisture content (40%, 60%), inoculum size (1.59 x 10^6 spores/ml, 1.43 x 10^7 spores/ml) and incubation temperature (25 °C, 35 °C). Table 2 shows the effects of the individual parameter (X_1, X2, and X_3) and their interactions (X_1X_2, X_1X_3, X_2X_3, and $X_1X_2X_3$), the regression coefficients (RCs), standard error of RCs, and *P* value, i.e., the probability of statistical tests. Except for the interaction between temperature and inoculum size, the effects of each parameter and the remainders of their interactions were significant with *P* values <0.05. Equation 1 gives the regression of the interacting parameters with a coefficient of determination (R^2) of 97.9% and a predicted R^2 of 95.3%.

$$Y=12.587+7.187X_1+1.314X_2-1.276X_3+0.749X_1X_2+0.057X_1X_3-1.773X_2X_3-0.802X_1X_2X_3 \quad (1)$$

The residuals of ANOVA show a normal distribution with a straight line with no outliers, skewness, or unknown variables detected (Figure 3). Furthermore, the histogram of residuals shows a similar pattern with a bell-shaped curve, suggesting that the errors were normally distributed with a mean value of zero. On the other hand, the plot of residuals versus fitted values shows that the residuals were scattered randomly around zero, indicating a constant variance for the errors. Finally, the plot of residuals in the

order of corresponding observation shows that the residuals ranged between 2 and -2 and were randomly scattered around zero. Thus, the residual analysis suggested that the model adequately explained the data.

Table 2

The estimated effects and regression coefficients for the optimization of pectinase

Term	Effect	Regression Coefficient (RC)	SE of RC*	T**	*P* value
Constant (average value of the enzyme yield from the total run)		12.587	0.2812	44.76	0
Temperature (X_1)	14.374	7.187	0.2812	25.56	0
Moisture Content (X_2)	2.627	1.314	0.2812	4.67	0
Inoculum Size (X_3)	-2.552	-1.276	0.2812	-4.54	0
X_1X_2	1.498	0.749	0.2812	2.66	0.017
X_1X_3	0.113	0.057	0.2812	0.2	0.843
X_2X_3	-3.546	-1.773	0.2812	-6.3	0
$X_1X_2X_3$	-1.603	-0.802	0.2812	-2.85	0.012
S=1.37761***			PRESS=68.3210§		

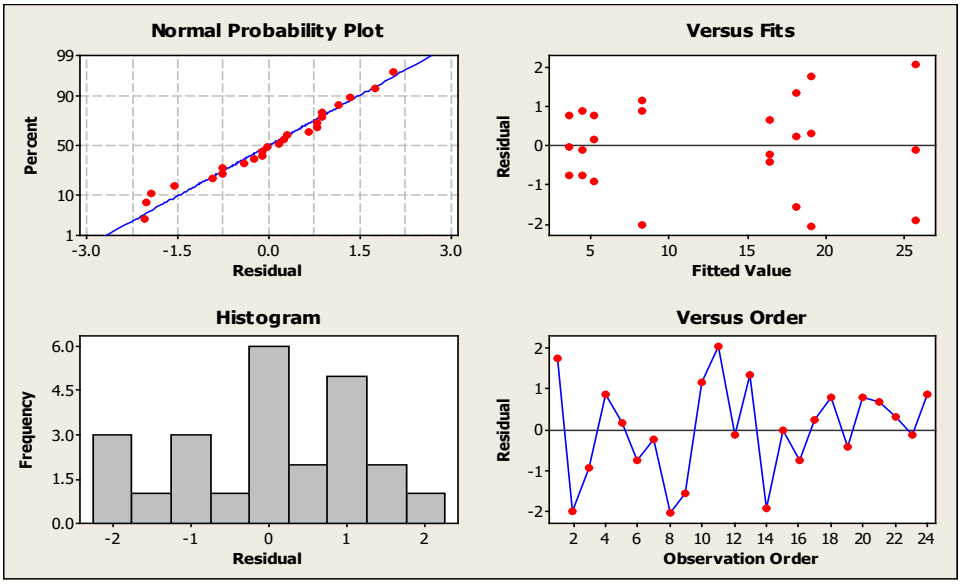
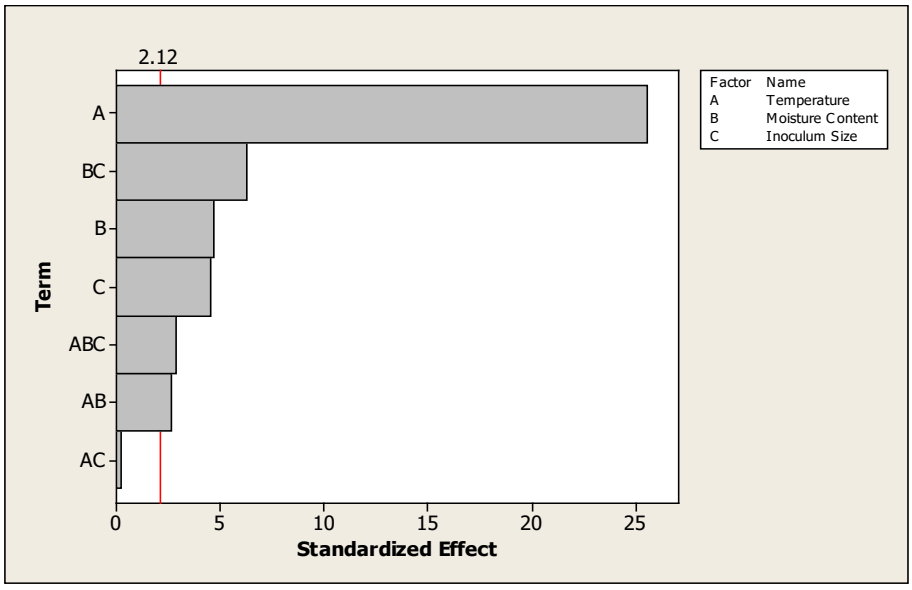


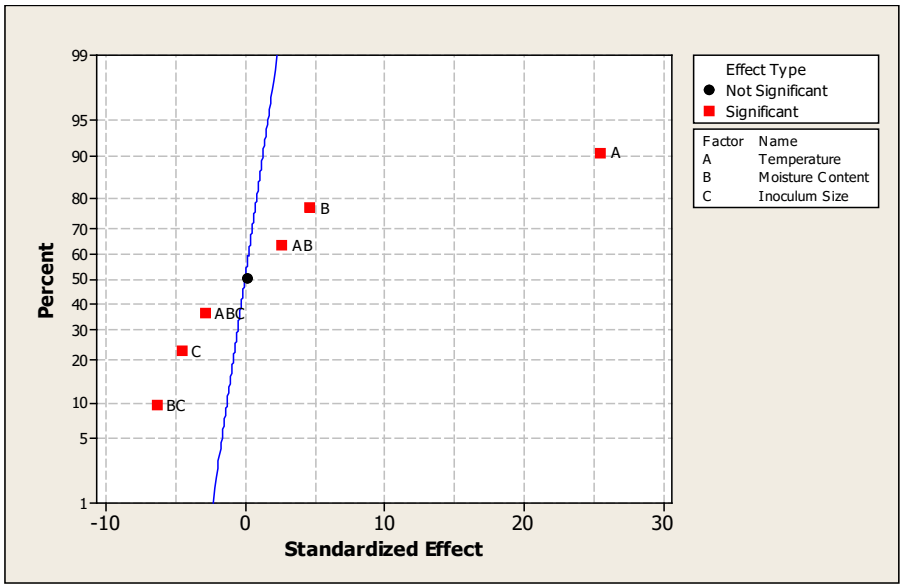
Figure 3. The residual plots for pectinase activity

Meanwhile, the Pareto chart (Figure 4a) shows a similar result to the Full Factorial Analysis (Table 2), i.e., except for the interaction between temperature and inoculum size (AC), all other standardized effects, inclusive each parameter by itself and its interaction with other parameters, were significant with values exceeding the threshold of 2.1 at 95% confidence level. Also, the Pareto chart indicates that the incubation temperature (A) was the most influencing parameter on the pectinase activity, followed by the interaction of moisture content and inoculum size (BC), moisture content by itself (B), inoculum size by itself (C), the interaction of temperature, moisture content, and inoculum (ABC), and interaction between temperature and moisture content (AB).

Also, the results of the normal probability plot (Figure 4b) were in congruence with that of the statistical tests and the Pareto chart, i.e., except for the interaction between temperature and inoculum size (AC), all other standardized effects, inclusive a parameter by itself and its interaction with other parameters, were significant with plots centered around the zero value. Besides, the incubation temperature (A), with the largest plot value and locating the farthest from the normal line, appeared to have the greatest effect on the SSF. In addition, the standardized effects of incubation temperature (A), moisture content (B), and interaction between incubation temperature and moisture content (AB) occurred on the right of the normal line, indicating a positive effect of these factors on the SSF. In contrast, the standardized effects of inoculum size (C), interactions of incubation temperature, moisture content, and inoculum size (ABC), and interaction between moisture content and inoculum size (BC) occurred on the left of the normal line, showing a negative effect of these factors on the SSF.



(a)



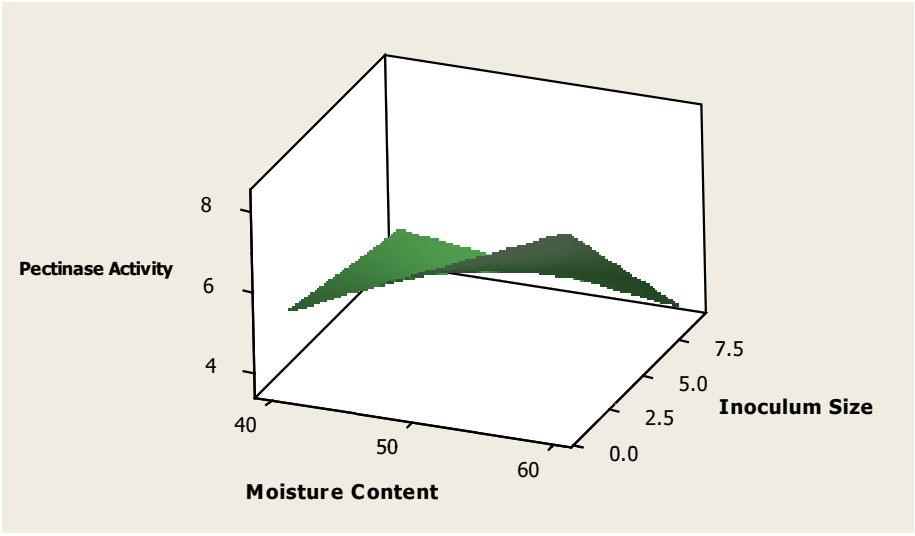
(b)

Figure 4. The (a) Pareto chart; and (b) normal probability plot of standardized effects

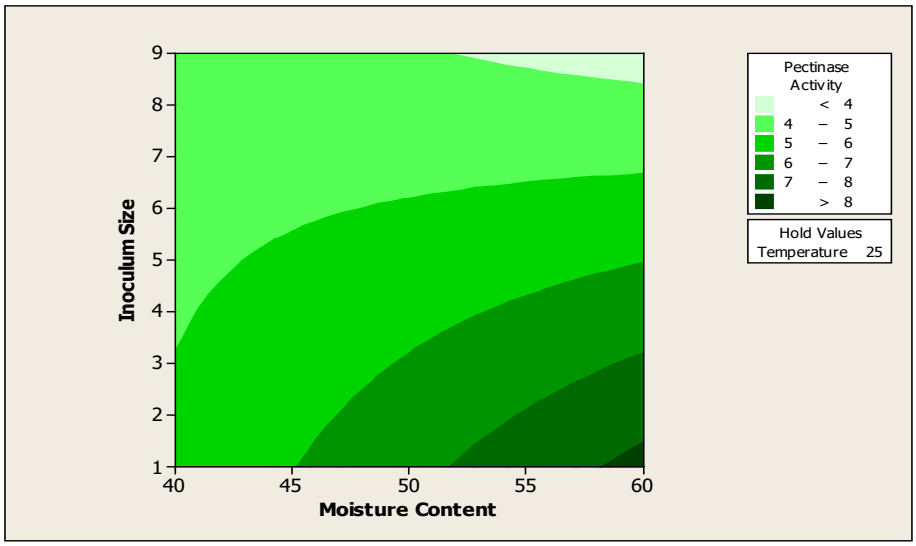
The Response Surface, Contour Plots, and Optimization

As a starting point, the hold value of response surface for each parameter was set to the lowest level, i.e., 25 °C for incubation temperature, 40% for moisture content, and 1.59 x 10^6 spores/ml for inoculum size. For the interaction between moisture content and inoculum size, a relatively high pectinase activity was attained at a combination of high moisture content of 60% and low inoculum size of 1.59 x 10^6 spores/ml (Figure 5a). A similar trend is shown in the contour plot of this interaction (Figure 5b). The maximum pectinase activity was attained at increasing moisture content in combination with decreasing inoculum size. Thus, the responses of moisture content appeared to be inhibited by high inoculum size, i.e., however high the moisture content increased, the maximal pectinase activity would remain unachievable if the inoculum size were not reduced.

Meanwhile, the surface plot for the interaction (Figure 6a) between temperature and moisture content shows that the maximum pectinase activity was achieved when the moisture content and incubation temperature were at their respective highest magnitude, namely 60% and 35 °C. Also, the contour plot for this interaction (Figure 6b) shows a directly proportional relationship, and the pectinase activity was the highest at 60% moisture content and 35 °C incubation temperature. Thus, the temperature plays an important role in the SSF process because it will determine the occurrence of protein denaturation, enzymatic inhibition, and cell death. Furthermore, the temperature will affect the germination of fungi, production of primary and secondary metabolites, and sporulation (Melnichuk et al., 2020; Thangaratham & Manimegalai, 2014).

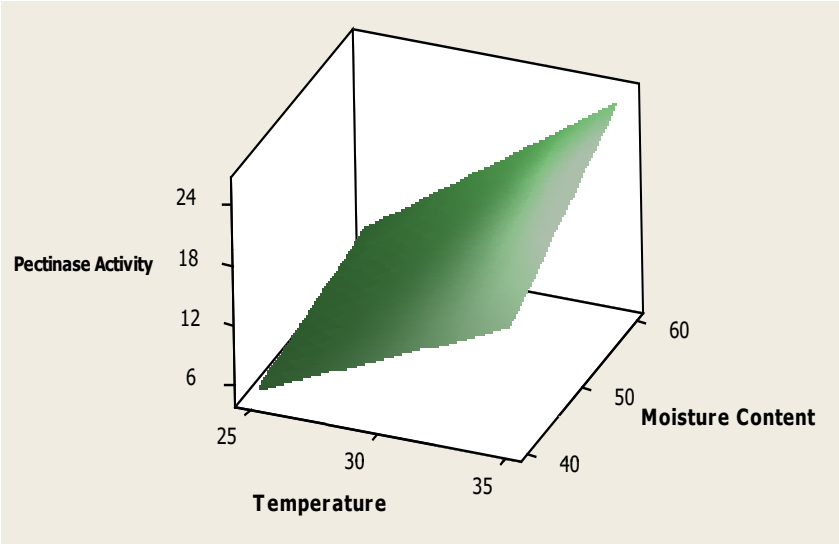


(a)

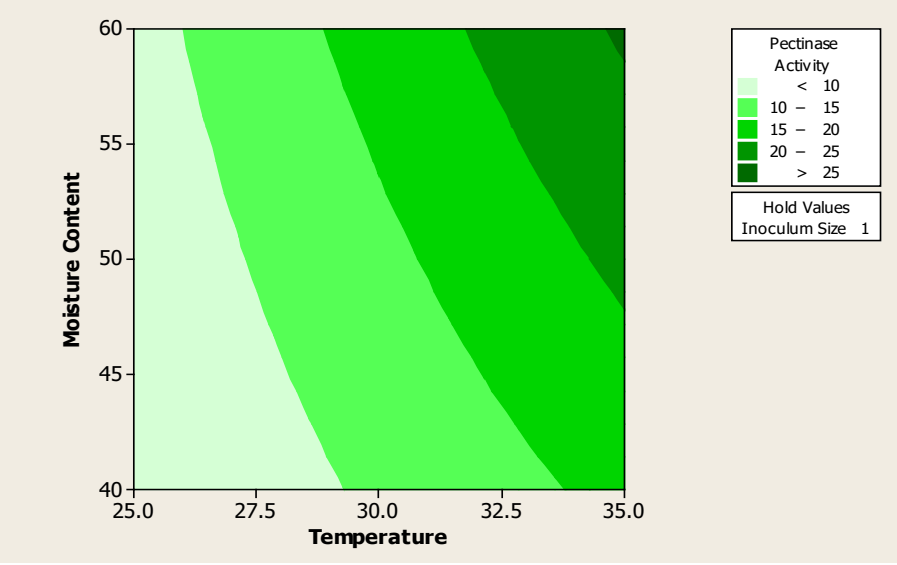


(b)

Figure 5. The surface plot (a) and contour plot (b) for the interaction between moisture content and inoculum size on pectinase activity



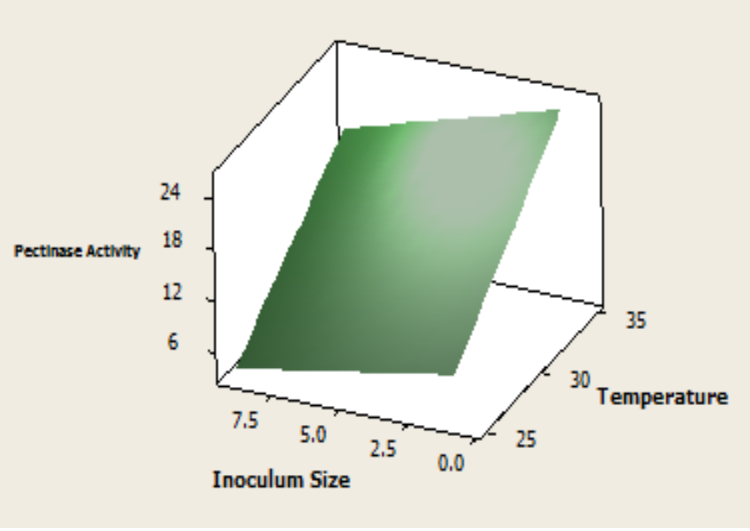
(a)



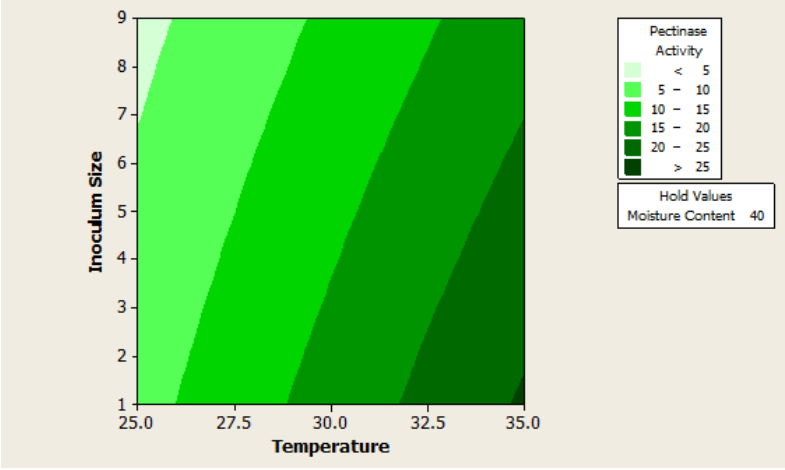
(b)

Figure 6. The surface plot (a) and contour plot (b) for the interaction between moisture content and temperature on pectinase activity

On the other hand, the surface plot for the interaction between inoculum size and temperature (Figure 7a) shows that a combination of low inoculum size (1.59 x 10^6 spores/ml) and high temperature (35 °C) yielded high pectinase activity. The contour plot for this interaction (Figure 7b) shows that the highest pectinase activity was achieved at 35 °C incubation temperature and 1.59 x 10^6 spores/ml inoculum size. However, the interaction between incubation temperature and inoculum size was relatively mild since the curvature of its plot (Figure 7b) was not as pronounced as those of the other two interactions (Figures 5b and 6b). Together, the optimum conditions for the maximum pectinase activity were 35 °C incubation temperature, 60% moisture content, and 1.59 x 10^6 spores/ml of inoculum size.



(a)



(b)

Figure 7. The surface plot (a) and contour plot (b) for the interaction between temperature and inoculum size on pectinase activity.

Finally, in the validation of experimental results, the minimum and maximum pectinase activity were set to 20 U/ml and 30 U/ml, respectively, yielding an average of 25 U/ml as the target value (Ayed et al., 2012). The optimization plot (Figure 8) shows that the target pectinase activity was predicted to achieve at 35 °C incubation temperature, 58.6% moisture content, and 1.59 x 10^6 spores/ml of inoculum size, and the desirability to achieve the target was 1. Besides, the plot's desirability shape shows that a higher temperature and moisture content with a lower inoculum size was optimal for a maximum yield of pectinase production. On the other hand, other than banana peel biomass, agro-industrial residues used in the SSF are sugar cane bagasse, wheat bran, rice husk, coconut coir pith, banana waste, tea waste, apple pomace, and many more (Kapilan, 2015). Therefore, other than the parameters used in the study, a few factors affect the selection of substrate for producing an enzyme: the cost and availability of substrate (Pandey et al., 2001).

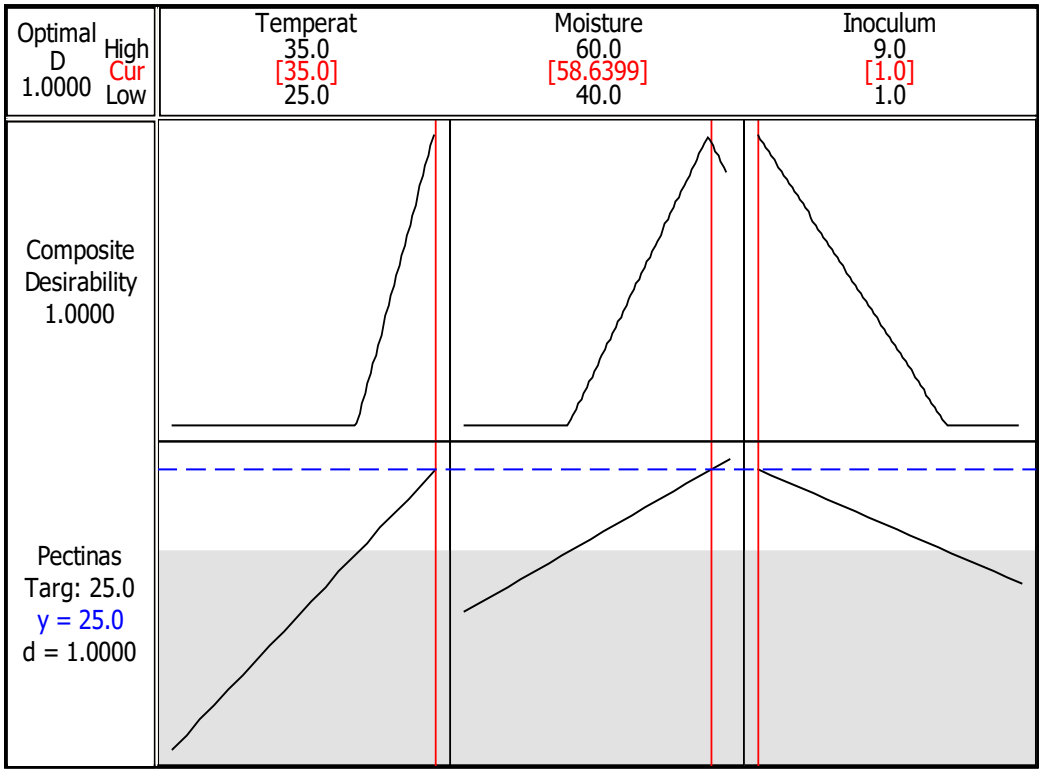


Figure 8. The process optimization curve

CONCLUSION

In this study, the incubation temperature was the most crucial parameter in driving for the maximum production of pectinase. However, the interaction between incubation temperature and inoculum size did not affect the pectinase activity. A weak interaction was found between the incubation temperature and inoculum size, whereas a strong interaction occurred between the moisture content and inoculum size. Therefore, the optimum conditions for the pectinase production in SSF were 35 °C incubation temperature, 60% moisture content, and 1.59 x 10^6 spores/ml of inoculum size, with the highest pectinase activity at 27 U/mL. Given that the production of pectinase with other substrates (e.g., pineapple waste, lemon peel, cassava waste, and wheat bran) generally ranges between 3 U/mL and 16 U/mL (Abdullah et al., 2018; Handa et al., 2016; Melnichuk et al., 2020; Thangaratham & Manimegalai, 2014; Salim et al., 2017), thus the yield of pectinase derived from the banana peel in this study (27 U/mL) was considered moderately high. Therefore, the biomass of banana peel has the potential to be used as a substrate in SSF for pectinase production. If this design of optimum operating conditions could be commercialized, it would enhance the efficiency of managing agro-industrial waste for sustainable development.

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