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PRODUCTION OF THERMOSTABLE PULLULANASE FROM *Bacillus Flavothermus* KWF-1: MEDIUM DEVELOPMENT USING EXPERIMENTAL DESIGN WITH DATA TRANSFORMATION

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Abstract. The extracellular production of pullulanase from *Bacillus flavothermus* KWF-1 using experimental design was studied. Two-level full factorial design was applied to enhance these nutrient constituents with respect to pullulanase production. Statistical analysis (ANOVA) have identified that tapioca starch was the most significant factor affecting the enzyme production. Diagnostic plots obtained from ANOVA revealed abnormalities with respect to the response data. Therefore, data transformation was advantageous in order to make the distribution of the response variable closer to the normal distribution and to stabilize the variance of the response, as well as to improve the fit of the model to the data. An improved model was attained with higher coefficient of determination ($R^2 = 0.9523$) compared to original R^2 of 0.9280. The enhanced medium consisted of (% w/v): 3.0 tapioca starch, 0.11 peptone from casein, 0.08 KH_2PO_4, 0.02 $MnSO_4$ and an initial pH of 9.0, produced 3.498 U/ml pullulanase, which was approximately 4.5 fold increment than that obtained from initial medium (0.791 U/ml).

Keywords: Medium development; full factorial design; data transformation; pullulanase; *bacillus flavothermus*

Abstrak. Kajian ini dilaksanakan untuk meningkatkan penghasilan pullulanase melalui kaedah reka bentuk eksperimen. Dengan bantuan perisian Design-Expert 6.0.4, peningkatan penghasilan enzim dilakukan menerusi reka bentuk penuh faktorial dua peringkat. Analisis statistik (ANOVA) mengenal pasti bahawa kanji ubi kayu adalah faktor yang memberikan kesan yang paling besar di dalam penghasilan enzim ini. Menerusi plot-plot diagnostik, didapati bahawa terdapat masalah terhadap kenormalan dan kesamaan variasi data. Penjelmaan data secara log membolehkan model yang lebih tepat diperolehi ($R^2 = 0.9523$). Medium yang terdiri daripada 3.0% (b/i) kanji ubi kayu, 0.11% (b/i) pepton dari kasein, 0.08% (b/i) KH_2PO_4, 0.02% (b/i) $MnSO_4$ and pH awalan 9.0 berjaya meningkatkan penghasilan enzim pullulanase sebanyak kira-kira 4.5 kali kali ganda berbanding penggunaan medium asal.

Kata kunci: Pullulanase; medium pengkulturan; *bacillus flavothermus* KWF-1

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1.0 INTRODUCTION

Pullulanase (EC 3.2.1.41, pullulan-6-glucanohydrolase) is an amylolytic enzyme that capable of cleaving α-1,6-glycosidic bonds in polysaccharides such as starch, pullulan, amylopectin and related oligosachharides. This enzyme became of interest due to its debranching capability. However, for industrial purposes, the more stable enzymes are in high demand, leading to various researches done on producing thermostable pullulanase from thermophilic and hyperthermophilic organisms [1]. This enzyme is widely used in starch-processing industry mainly for producing smaller products such as high fructose syrup and maltose [2]. Thermostable pullulanase is necessary in the reaction of converting starch to different sugars used in combination with other amylolytic enzymes. It enhances the reaction rate thus speeds up the production of sugars resulting in cost reduction of producing sugars from starch [3].

Pullulanase is produced by a variety of microorganisms, such as mesophiles - *Bacillus macerans* [4] and *Bacillus circulans* F-2 [5]; thermophiles - *Thermoactinomyces thalpophilus* [6] and *Thermus* sp. AMD33 [7]; and hyperthermophiles - *Pyrococcus woesei* [8], *Thermotoga maritima* [9] and *Rhodothermus marinus* [10]. *Bacillus sp.* US149 [11] and *Thermoanaerobacter* strain B6A [12] produced acidic pullulanase while *Bacillus* sp. S-1 [13] and *Bacillus* sp. KSM-1378 [14] secreted alkaline pullulanase.

Medium development plays an important role in order to enhance the production of pullulanase. It is a process that can be used to identify the ideal growth and production environment of a cell [15]. Therefore, the appropriate fermentation medium should be developed because medium composition can significantly affect product concentration and yield. The conventional method of medium development (one-factor-at-a-time or OFAT strategy) is time consuming and inaccurate, especially when interactions between medium components exist. Statistical experimental designs allow simultaneous, systematic and efficient variation of all components [16].

Previously, we conducted a preliminary characterization on crude pullulanase produced from *Bacillus flavothermus* KWF-1 and found out that this enzyme was a thermostable enzyme [17]. In this study, we have carried out experiments to enhance the production medium using experimental design approach. Data transformation was also carried out in order to improve the fit of the model to the data.

2.0 MATERIALS AND METHODS

2.1 Preparation of Bacterial Inoculum

Bacillus flavothermus KWF-1 was maintained on solid medium consisted of (g/l): 5.0 pullulan (Fluka), 3.0 yeast extract (Merck), 10.0 peptone (Amresco), 1.0 NH_4Cl, 0.3 KH_2PO_4, 2.67 $Na_2HPO_4.12H_2O$, 0.2 $MgCl_2.6H_2O$ and 20.0 agar, for 24 h at 50 °C. Subsequently, a colony of the culture was transferred aseptically into a flask containing 50 ml of medium composed of (g/l): 20.0 soluble starch, 17.5 peptone, 0.5 yeast

extract, 2.0 KH_2PO_4 and 0.4 $MgSO_4$. The culture was incubated for 18 h at 50 °C with shaking at 200 rpm. The cells were then centrifuged at 5000 rpm for 5 minutes, washed with normal saline solution, 0.85% (w/v) NaCl, to give an optical density (OD) reading of 0.5 at 550 nm. The media were autoclaved at 121 °C for 15 minutes prior to inoculation [17]. All the chemicals used in this study were of analytical grade.

2.2 Preparation of Crude Enzyme

10% (v/v) of the bacteria inoculum was inoculated into the same medium in a conical flask. The culture was incubated at 50 °C for 24 hours in an orbital shaker at 200 rpm. The incubation period was chosen based on previous works (data not shown). After the incubation, the culture was centrifuged at 10,000 rpm for 10 minutes [17]. The clear supernatant was used for enzyme assay and protein content determination.

2.3 Pullulanase Activity Assay

Pullulanase activity was assayed by measuring the amount of reducing sugar released from pullulan. A reaction mixture containing 0.1 ml supernatant (crude enzyme) and 0.5 ml 1% (w/v) pullulan in 100 mM glycine-NaOH buffer pH 10.0 was incubated at 80 °C for 30 min [22]. The reaction was quenched by cooling on ice and the amount of reducing sugar was measured by the dinitrosalicylic (DNS) acid procedure with slight modification [18]. DNS reagent (0.6ml) was added into the mixture followed by 60 ml 0.1N NaOH. The mixture was then incubated at 100 °C for 5 minutes. Subsequently, the reaction was stopped by cooling on ice. Distilled water (5 ml) was added and the color intensity was measured at 540 nm. The non-enzymatic release of sugars was corrected by setting up a separate blank for each sample. One unit (U) of pullulanase activity was defined as the amount of enzyme that produced one mmol of reducing sugar as glucose per minute under the assay conditions.

2.4 Fermentation Medium Development

Medium development was carried out using experimental design approach. The matrix design of the experiment was developed based on the design in the Design Expert Software Version 6.0.4. A 2^5 full factorial design was used to show the statistical significance of the medium composition (Table 1). The factors involved in this design were selected from the screening process. The selections of minimum and maximum values were based on the investigation of single factors and literature (data not shown). A total of 32 experiments were employed to determine the significant factors affecting the production of pullulanase. The statistical significance of the model equation was determined by F-value and the proportion variance explained by the model obtained was given by the multiple coefficient of determination, R^2.

Table 1 Factors (variables) studied and their concentration levels[a]

Variables	Unit	Actual Value		Coded Value	
		Low	High	Low	High
A: Tapioca starch	%w/v	0.50	3.00	–1	1
B: Peptone (casein)	%w/v	0.10	2.50	–1	1
C: Initial pH	-	7.0	9.0	–1	1
D: $MnSO_4$	%w/v	0.002	0.03	–1	1
E: KH_2PO_4	%w/v	0.02	0.20	–1	1

[a]The selections of minimum and maximum values were based on the investigation of single factors and literature (data not shown)

3.0 RESULTS AND DISCUSSION

3.1 Experimental design

The effects of tapioca starch, peptone from casein, $MnSO_4$, KH_2PO_4 and medium initial pH were studied using two-level full factorial design. Table 2 represents the results obtained from the experimental works. Analysis of variance (ANOVA) was employed for the determination of significant variables. The F value was the ratio of mean square due to regression to the mean square due to residual and it indicated the influence of each factor on tested model. Generally, the calculated F value should be several times the tabulated value if the model is a good predictor of the experimental results. Additionally, the P value corresponding to the F value signified the probability that differences between calculated and tabulated statistics was due only to random experimental error.

From Table 3, the confidence level was greater than 95% ($P < 0.05$) in pullulanase production with the F and P value of the model of 66.9794 and 0.0001, respectively. The P value of less than 0.0001 implied that the effect of tapioca starch concentration was highly significant to the production of pullulanase and there were two other terms that exhibited P value of less than 0.05. Manganese sulfate (D) and interaction between pH and peptone from casein (BC) were also significant based on their P values. The equation of the model generated based on the regression analysis is as follows:

$$Y = -2.1466 + 0.9473A + 1.0158B + 0.2250C + 11.2522D - 0.1242BC \quad (1)$$

where Y was pullulanase activity (U/ml) and A, B, C, D and BC were tapioca starch, peptone from casein, medium initial pH, $MnSO_4$, and peptone (casein)-pH interaction. In order for the model to be hierarchically correct, Factor B and C were also included in the model in spite of their insignificance [19].

The determination coefficient (R^2) value provides a measure of how much of the variability in the observed response values can be explained by the experimental

Table 2 Experimental design of 2^5 full factorial design (actual and predicted values)

Run	Coded level					Pullulanase activity (actual)[a]	Pullulanase activity (predicted)	Pullulanase activity (actual, log–transformed)	Pullulanase activity (predicted, log–transformed)
	A	B	C	D	E				
1	–1	–1	–1	–1	–1	0.216	–0.061	–0.666	–0.634
2	1	–1	–1	–1	–1	2.012	2.308	0.304	0.286
3	–1	1	–1	–1	–1	0.182	0.291	–0.740	–0.521
4	1	1	–1	–1	–1	2.611	2.659	0.417	0.399
5	–1	–1	1	–1	–1	0.350	0.365	–0.456	–0.451
6	1	–1	1	–1	–1	2.827	2.733	0.451	0.469
7	–1	1	1	–1	–1	0.177	0.120	–0.752	–0.577
8	1	1	1	–1	–1	1.270	2.488	0.104	0.343
9	–1	–1	–1	1	–1	0.201	0.255	–0.697	–0.542
10	1	–1	–1	1	–1	2.036	2.623	0.309	0.378
11	–1	1	–1	1	–1	0.494	0.606	–0.306	–0.429
12	1	1	–1	1	–1	3.056	2.974	0.485	0.491
13	–1	–1	1	1	–1	0.647	0.680	–0.189	–0.359
14	1	–1	1	1	–1	2.995	3.048	0.476	0.561
15	–1	1	1	1	–1	0.316	0.435	–0.500	–0.485
16	1	1	1	1	–1	3.138	2.803	0.497	0.435
17	–1	–1	–1	–1	1	0.326	–0.061	–0.487	–0.634
18	1	–1	–1	–1	1	1.941	2.308	0.288	0.286
19	–1	1	–1	–1	1	0.350	0.291	–0.456	–0.521
20	1	1	–1	–1	1	2.899	2.659	0.462	0.399
21	–1	–1	1	–1	1	0.388	0.365	–0.411	–0.451
22	1	–1	1	–1	1	2.779	2.733	0.444	0.469
23	–1	1	1	–1	1	0.436	0.120	–0.361	–0.577
24	1	1	1	–1	1	3.043	2.488	0.483	0.343
25	–1	–1	–1	1	1	0.259	0.255	–0.587	–0.542
26	1	–1	–1	1	1	3.258	2.623	0.513	0.378
27	–1	1	–1	1	1	0.331	0.606	–0.480	–0.429
28	1	1	–1	1	1	3.138	2.974	0.497	0.491
29	–1	–1	1	1	1	0.407	0.680	–0.390	–0.359
30	1	–1	1	1	1	3.258	3.048	0.513	0.561
31	–1	1	1	1	1	0.302	0.435	–0.520	–0.485
32	1	1	1	1	1	3.012	2.803	0.479	0.435

[a]The pullulanase activity (U/ml) of each design was measured after 24 hours of incubation at 200 rpm, 50°C

Table 3 Regression analysis of the 2^5 full factorial design (before and after log transformation)

Factor	Before transformation		After transformation	
	F Value	Probability > F (*P* value)	F Value	Probability > F (*P* value)
Model	66.9794	< 0.0001[a]	103.8770	< 0.0001[a]
A: Tapioca starch	322.9683	< 0.0001[a]	503.4643	< 0.0001[a]
B: Peptone (casein)	0.1644	0.6884[b]	0.0267	0.8715[b]
C: Initial pH	0.9316	0.3433[b]	2.3782	0.1351[b]
D: $MnSO_4$	5.7164	0.0243 [a]	5.0451	0.0334[a]
BC (Interaction)	5.1161	0.0323 [a]	8.4706	0.0073[a]
Adeq. Precision			23.79	
R^2	0.9280		0.9523	
R	0.9633		0.9759	

[a]Significant factor ($P < 0.05$)

[b]Factor B and C were included in order to correct the hierarchy of the model despite their insignificance

factors and their interactions [37]. A value of $R^2 > 0.75$ indicates the aptness of the model. The R^2 value of 0.9280 was obtained from the model. This value indicated that the model could explain 92.80% of the variability in the production of pullulanase.

3.2 Model Validation via Diagnostic Plots

In order to further validate the model, several additional diagnostic plots have been examined. The plots were normal probabilty plot of the studentized residuals (to check for normality of residuals), studentized residuals versus predicted values (to check for constant error), outlier T versus run order plot (to look for outliers).

The outlier T plot was used to observe if any point stands out, that was, fall outside the limit lines. Only the extreme values (T > 3.5 or T < -3.5) should be considered outliers. Figure 1 exhibits the outlier T plot. The plot revealed one potential outlier in the experimental data. The easiest way to deal with this data is to exclude it from the analysis but there is a chance that the problem maybe lies in how the data is modeled, not in the data itself [20]. Therefore, further inspections on other diagnostic plots would be advantageous.

In order for the model to be valid, the residuals should be approximately normally distributed. Normal plot of residuals evaluates the behavior of the residuals in comparison to a normal distribution or non-normality in the error term [19]. Figure 2 shows an abnormality in the plot. There was one point that fell far from the line. Moreover, there was fairly an "S" shape curve formed by the other points.

Figure 3 depicts residuals versus predicted values plot which also revealed a non-normality. A megaphone ("<") shape in the plot clearly showed a problem with non-constant variances of the residuals, that is, the variability of the residuals increased

Table 4 Summary of medium development using experimental design approach for pullulanase production from *Bacillus flavothermus* KWF-1

Fermentation medium	Nutrients (% w/v)	Pullulanase activity[a] (U/ml)
Initial medium	2.0 soluble starch 1.75 peptone 0.5 yeast extract 0.1 KH_2PO_4 0.02 $MgSO_4.7H_2O$ pH 7.02	0.791
Improved medium	3.0 tapioca starch 0.11 peptone from casein 0.08 KH_2PO_4 0.02 $MnSO_4$ pH 9.0	3.498

[a]Pullulanase activity was measured after 24 hours of incubation at 200 rpm, 50°C

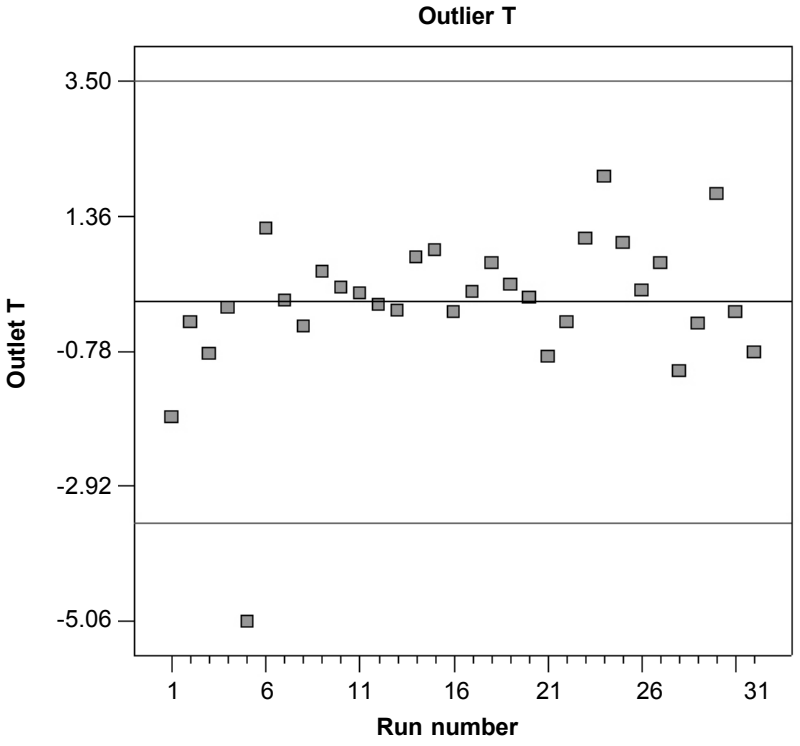

Figure 1 Outlier T plot. (One potential outlier revealed by the plot, which fell at –5.06 standard deviation from its expected value)

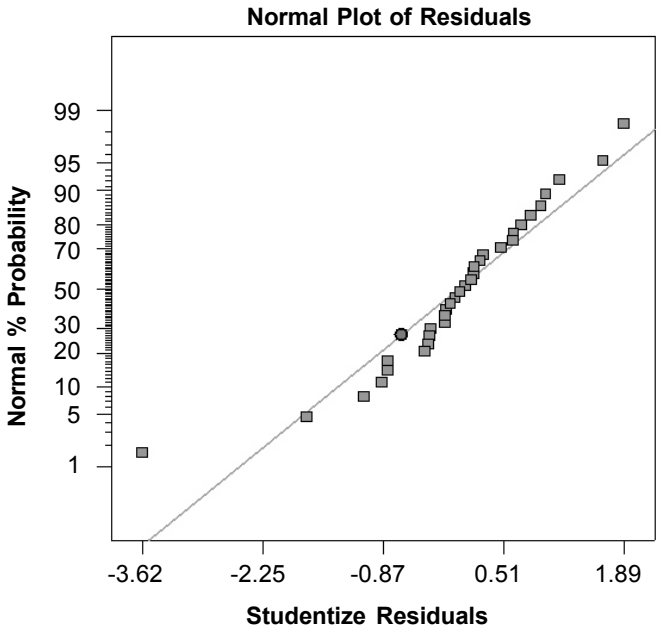

Figure 2 Normal plot of residuals. (There was one point that fell far from the line at the left side of the plot. An "S" shape formed by the other points exhibited problems with normality)

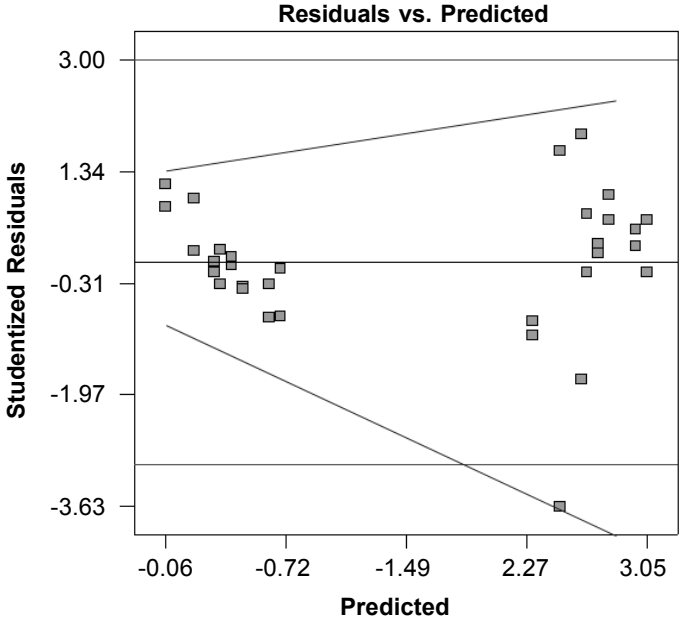

Figure 3 Plot of residuals versus predicted values. (A megaphone ("<") shape exhibited in the plot. This pattern often occurs with data that varies in a broad range and it needs to be transformed to improve the fit of the model)

as the response increased. These two patterns are very typical of data for which a transformation of the response will improve the fit of the model [19]. It often occurs with data that vary over such a broad range [20].

3.3 Data Transformation

Anderson and Whitcomb [20] discussed a report on improving the life of a deep-groove rolling bearing that exhibited the "S" and megaphone shape on the normal plot of residuals and residuals versus predicted values plot, respectively. Based on preliminary results, points that were detected as outliers represented a breakthrough in improvement in bearing life. Therefore, they applied a log transformation on their data and found out that the points were no longer outliers following the transformation. Daniel [21] describes a 2^4 factorial design that observed the same situation in which log transformation of the response data was applied and thus obtained a much simpler model. Generally, transformations are used for three purposes; stabilizing response variance, making the distribution of the response variable closer to the normal distribution and improving the fit of the model to the data [22].

Table 3 also summarizes ANOVA following the log transformation. The F and P values obtained from the improved model were 103.8770 and 0.0001, respectively. The final equation of the improved model is as follows:

$$\mathrm{Log}_{10}(Y) = -1.5028 + 0.3680A + 0.3950B + 0.0962C + 3.2889D - 0.0497BC \quad (2)$$

where Y was pullulanase activity (U/ml) and A, B, C, D and BC were tapioca starch, peptone from casein, medium initial pH, MnSO4, and peptone (casein)-pH interaction.

The R^2 value of 0.9523 was obtained from the improved model, which was higher than the previous model (0.9280). This proved that a better-fitted model was obtained following the transformation of data. The improved model now could explain 95.23% of the variability in the production of pullulanase from *Bacillus flavothermus* KWF-1. At the same time, the signal to noise ratio was 23.79 (Adeq. Precision). This is desirable because a ratio of greater than 4 indicates a strong signal which means that the readings are not influenced by noise (errors and disturbances). This further implies that the model obtained from the software could be accepted with high confidence [23].

Figure 4, 5 and 6 present, respectively, a normal probability plot of the residuals, plot of residuals versus predicted values and outlier T plot following the log transformation. These plots were now satisfactory. It was noticeable that a normal pattern exhibited in the normal probability plot (Figure 4). The points were normally distributed along the straight line. There were points of residuals that seemed detached from the "body" of the residuals. However, the departure of the residuals from normality was not serious hence the plot was acceptable [19].

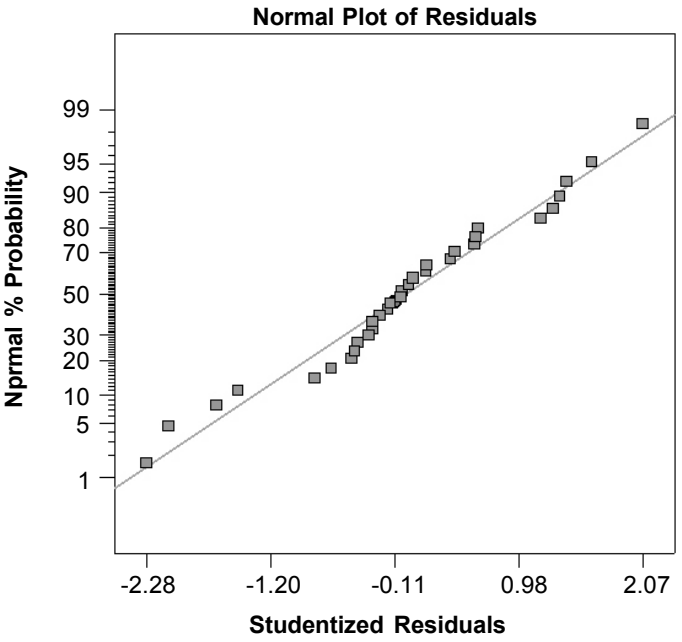

Figure 4 Normal plot of residuals after log transformation. (The points were normally distributed along the straight line, exhibiting a normal pattern)

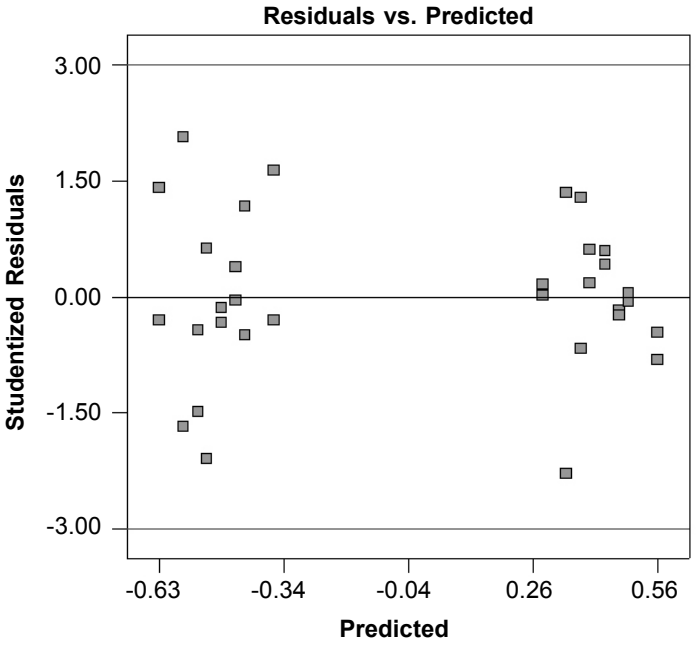

Figure 5 Plot of residuals versus predicted values after log transformation. (No more pattern formed indicating non-constant variance for the residuals)

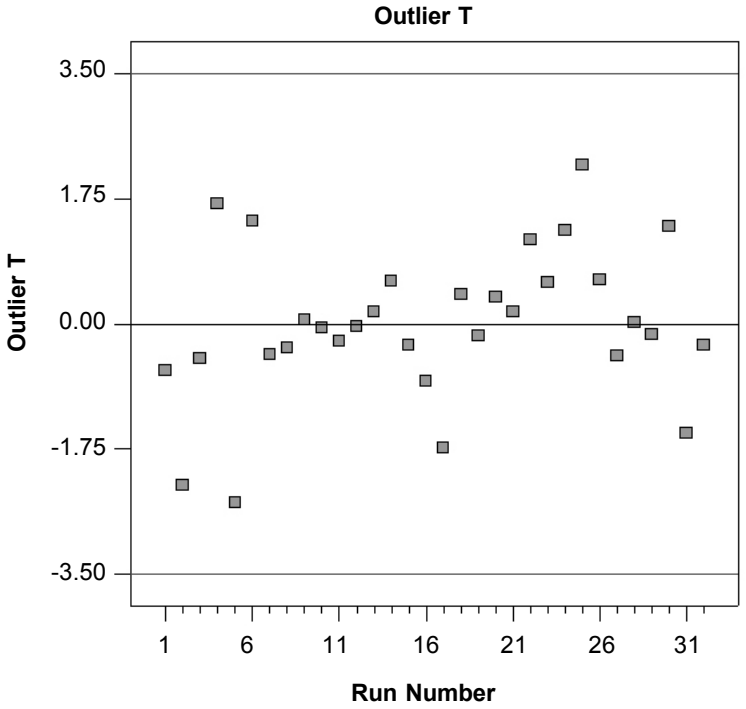

Figure 6 Outlier T plot after log transformation. (The suspected outlier now fell into line with the other points)

In the residuals versus predicted values plot (Figure 5), there was no longer any pattern indicating non-constant variance for the residuals. With respect to Figure 4 and 5, Daniel [21] also obtained the same and more satisfactory diagnostic plots. Figure 6 presents the outlier T plot. Following log transformation, outlier point revealed previously was transposed into standard deviation limits. Clearly, log transformation of the data produced a good model by improving the behavior of the residual values (minimizing the residuals) as the residuals show how well the model represents the data [19]. Meanwhile, Figure 7 exhibits the plot of predicted values versus actual values. This plot complemented the R value of 0.9759 (Table 6). High R value implied a good agreement between experimental and predicted values of response. In other words, it also signified that residuals between actual and predicted values were small.

Following obtaining the improved model, suggested medium composition for pullulanase production from *Bacillus flavothermus* KWF-1 was 3.0% (w/v) tapioca starch, 0.11% (w/v) peptone from casein, 0.08% (w/v) KH_2PO_4, 0.02% (w/v) $MnSO_4$ and an initial pH of 9.0. The predicted amount of pullulanase obtained from the equation was 3.365 U/ml. Experimental rechecking was performed using medium composition suggested and actual amount of pullulanase observed from the

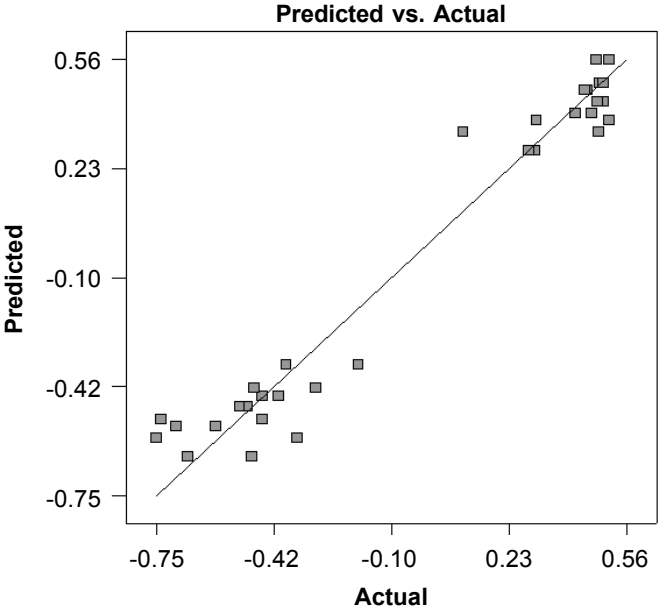

Figure 7 Plot of actual values versus predicted values. (The points scattered along the straight line indicating a good agreement between the actual and predicted values)

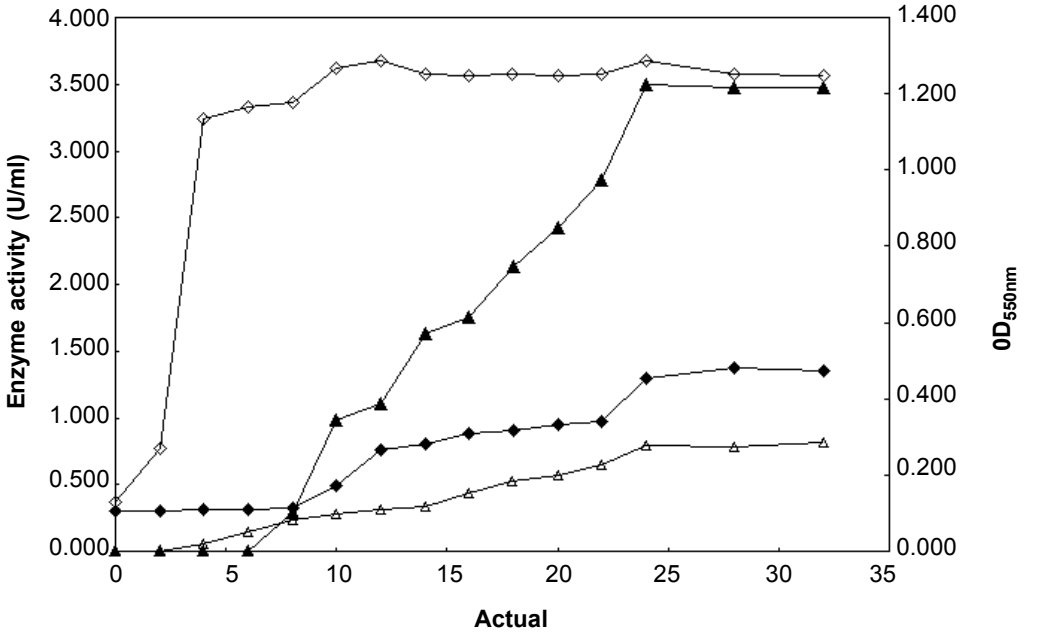

Figure 8 Growth profile and pullulanase production from *Bacillus flavothermus* KWF-1 using initial medium (growth, ◇ ; pullulanase, △) and enhanced medium (growth, ◆; pullulanase, ▲)

experiment was 3.498 U/ml. Table 7 summarizes the medium development using experimental design approach for pullulanase production from *Bacillus flavothermus* KWF-1. Improved medium yielded approximately 4.5 fold amount of pullulanase compared to initial medium. Concisely, experimental design has been a useful tool in providing a more systematic and thorough experiments with as few measurements as possible.

3.4 Pullulanase Production Profile

The time course of pullulanase production from *B. flavothermus* KWF-1 was studied with the initial and enhanced medium for 32 hours (Figure 10). Using the initial medium, the strain started to secrete pullulanase after two hours of incubation. Meanwhile, pullulanase was secreted after six hours of incubation in the enhanced medium due to its relatively high viscosity. However, there was approximately 4.5 fold increment in pullulanase production from *B. flavothermus* KWF-1 at 24 h of incubation in the enhanced medium. *Rhodothermus marinus* ITI 990 produced 2.1 fold higher pullulanase amounts using optimized medium [10] and *Clostridium thermosulforogenes* SV2 produced 10% more pullulanase in the nutritionally optimized solid-state fermentation medium [24].

4.0 CONCLUSION

Experimental design, particularly two-level full factorial design for five variables, has been successfully employed in the development of production medium for the production of pullulanase from *B. flavothermus* KWF-1. Based on statistical analysis, abnormalities were revealed in several diagnostic plots. Normal plot of residuals exhibited a fairly "S" shape along the straight line while a megaphone ("<") shape was depicted in residuals versus predicted values plot. Moreover, outlier T plot revealed one potential outlier in the experimental data. Abnormalities shown by the plots suggested that transformation of response data would be advantageous to improve the fit of the model. Log transformation was done to the response data and an improved model was obtained with higher R^2 (0.9523). The enhanced medium resulted in 4.5 fold higher amount of pullulanase (3.498 U/ml) compared to initial medium (0.791 U/ml).

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REFERENCES

[1] Saha, B. C. and J. G. Zeikus. 1989. Novel Highly Thermostable Pullulanase from Thermophiles. *TIBTECH*. 7: 234-239.

[2] Norman, B.E. 1982. A Novel Debranching-enzyme for Application in the Glucose Syrups Industry. *Starch*. 34**:** 340-346.

[3] Crabb, W. D. and C. Mitchinson. 1997. Enzymes Involved in the Processing of Starch to Sugars. *TIBTECH*. 15: 349-352.

[4] Adams, K. R. and F.G. Priest. 1977. Extracellular Pullulanase Synthesis in *Bacillus macerans. FEMS Microbiol. Lett*. 1: 269-273.

[5] Sata, H., M. Umeda, C. H. Kim, K. Taniguchi and Y. Maruyama. 1989. Amylase-Pullulanase Enzyme Produced by *B. circulans* F-2. *Biochim. Biophys. Acta*. 991: 388-394.

[6] Odibo, F. J. C. and S. K. C. Obi. 1990. Optimum Culture Conditions for the Production of the Extracellular Pullulanase of *Thermoactinomyces thalpophilus. Biological Wastes*. 32: 9-15.

[7] Nakamura, N., N. Sashihara, H. Nagayama and K. Horikoshi. 1989. Characterization of Pullulanase and a-Amylase of a *Thermus* sp. AMD33. *Starch*. 3: 112-117.

[8] Rüdiger, A., P. L. Jorgensen and G. Antranikian. 1995. Isolation and Characterization of a Heat-Stable Pullulanase from the Hyperthermophilic Archaeon *Pyrococcus woesei* after Cloning and Expression of Its Gene in *Escherichia coli. Appl. Environ. Microbiol*. 612: 567-575.

[9] Kriegshäuser, G. and W. Liebl. 2000. Pullulanase from the Hyperthermophilic Bacterium *Thermotoga maritima:* Purification by b-cyclodextrin Affinity Chromatography. *J. Chromatogr. B.* 737: 245-251.

[10] Gomes, I., J. Gomes and W. Steiner. 2003. Highly Thermostable Amylase and Pullulanase of the Extreme Thermophilic Eubacterium *Rhodothermus marinus:* Production and Partial Characterization. *Bioresource Technol*. 90: 207-214.

[11] Roy, A., E. B. Messaoud and S. Bejar. 2003. Isolation and Purification of an Acidic Pullulanase Type II from Newly Isolated *Bacillus* sp. US149. *Enzyme Microb. Technol*. 33: 720-724.

[12] Saha, B. C., R. Lamed, C. Y. Lee, S. P. Mathupala and J. G. Zeikus. 1990. Characterization of an *Endo*-Acting Amylopullulanase from *Thermoanaerobacter* Strain B6A. *Appl. Environ. Microbiol*. 564: 881-886.

[13] Lee, M. J., Y. C. Lee. and C. H. Kim. 1997. Intracellular and Extracellular Forms of Alkaline Pullulanase from an Alkaliphilic *Bacillus* sp. S-1. *Archives of Biochem. Biophys*. 3372: 308-316.

[14] Ara, K., K. Saeki, K. Igarashi, M. Takaiwa, T. Uemura, H. Hagihara, S. Kawai and S. Ito. 1995. Purification and Characterization of an Alkaline Amylopullulanase with both a-1,4 and a-1,6 Hydrolytic Activity from Alkalophilic *Bacillus* sp. KSM-1378. *Biochim. Biophys. Acta*. 1243: 315-324.

[15] Hunt, C. 2004. Production Enhancement Through Media Optimization. *BD Newsletter*. 12: 1-2.

[16] Box, G. P., W. G. Hunter and J. S. Hunter.1978. *Statistics for Experiments: An Introduction to Design, Data Analysis and Model Building*. New York: John Wiley and Sons.

[17] Rozaimi A. S., M. I. Rosli, H. Osman, M. S. Madihah, A. A. Suraini, A. R. Roshanida and K. Kamarulzaman. 2004. Thermostable Pullulanase from *Bacillus flavothermus* KWF-1. *Proc. Symposium of the Malaysian Society for Microbiology*. 84.

[18] Miller, G. L. 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem*. 313: 426-428.

[19] Haaland, P. D. 1989. *Experimental Design in Biotechnology*. New York: Marcell Dekker.

[20] Anderson, M. J. and P. J. Whitcomb. 2003. *How to Use Graphs to Diagnose and Deal with Bad Experimental Data*. Minneapolis: Stat-Ease, Inc.

[21] Daniel, C. 1959. *Applications of Statistics to Industrial Experimentation*. New York: John Wiley and Sons.

[22] Montgomery, D. C. 2001. *Design and Analysis of Experiments*. 5th ed.New York: John Wiley and Sons.

[23] Anderson, M. J. dan P. J. Whitcomb. 1997. *Breakthrough Improvements with Experiment Design*. Minneapolis: Stat-Ease, Inc.

[24] Reddy, P. R. M., G. Reddy and G. Seenayya. 1999. Production of Thermostable b-Amylase and Pullulanase by *Clostridium thermosulfurogenes* SV2 in Solid-State Fermentation: Screening of Nutrients Using Plackett-Burman Design. *Bioprocess Eng*. 21: 175-179.