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Auto-activated Thrombin Expression from *Pichia pastoris*SMD1168 Optimized by Plackett-Burman Design and Response Surface Methodology

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Abstract: *Recombinant thrombin (rThrombin) has been used topically as one component of fibrin sealant as the tissue adhesive. Recombinant prethrombin-2 is used as the precursor to generate rThrombin which is then activated by ecarin. Autoactivated thrombin (autoTrm) can be produced by altering four amino acids (EDGE) in prethrombin-2 thus obviate the use of ecarin and simplify the purification process. In this study, Pichia pastorisSMD1168 was used to producing autoTrm in flask culture. Influencing factors on P. pastorisSMD1168 culture in producing autoTRM were screened by Plackett-Burman (PB) design and the optimization of was carried out by Response Surface Methodology. The medium used for cultivation was BMGH for growth phase and Pichia Trace Minerals (PTM_1) supplemented Basal Salt Medium (BSM) for induction phase. The influencing factors (PTM_1 concentration, methanol concentration, induction temperature,induction time, and pH) on autoTrmexpression werescreenedby PB design then were optimized by Response Surface Methodology (RSM) with Central Composite Design.It was revealed that PTM_1 concentration (%) and pH had significant influence ($P < 0.05$) on autoTrm expression level based on PB design. The optimum%PTM_1 and pH revealed by (RSM) was 2.05% and 4.2, respectively.*

Keywords*: Autoactivated thrombin, P. pastoris SMD1168, PTM_1 supplemented medium, Placket-Burman design, Response SurfaceMethodology*

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I. INTRODUCTION

Thrombin is a protease that converts fibrinogen into fibrin to support blood clotting. It can be used topically as one component of fibrin sealant as tissue adhesive to replace suture in ophthalmologic surgery(Enus *et al.*, 2011). Recombinant thrombin has been used to replace the use of thrombin from other sources i.e. bovine and plasma. Yonemura *et al*. (2009) have produced recombinant thrombin from recombinant prethrombin-2 which is activated by ecarin, a snake venom-derived protease. Later study, performed by Pozzi *et al.* (2011, 2013), showed auto-activated thrombin can be produced by altering four amino acids (EDGE) in prethrombin-2, hence eliminates the zymogen activation since it is auto-activated upon refolding. Nevertheless, the auto-activated thrombin (autoTrm) had functional properties toward substrates similar to the wild-type enzyme.

Pichia pastoris has been used as a common and an effective host to produce secreted recombinant protein. It uses methanol as the sole carbon source and inducer for recombinant proteins production. As a host for heterologous protein production, *P. pastoris* can perform post-translational modifications and can be grown in the inexpensive and defined medium such as pure carbon sources (glycerol and methanol), salts, trace minerals, biotin, and water. Contamination by other micro organism has less likely happened since its culture is in relatively low pH and methanol (Creg*et al*., 2000). *P. pastoris* SMD1168 is a protease-deficient strain that has been used to reduce proteolysis of heterologous protein production during cultivation. This strain successfully produced biologically-active recombinant human fibrinogen (Tojo*et al*., 2008). Yu *et al.* (2014) has used this strain for the expression of human lysozyme and has been succeeded to optimize the production by employing Plackett-Burman (PB) design and Response Surface Methodology (RSM).

In our previous study, a synthetic autoTrm gene was inserted into pD912 and was expressed from *P. pastoris* SMD1168 yet satisfied result had not been met. In this study, the autoTrm gene was transferred from pD912 to the pPICZαB plasmid. Two cultivation mediums in flask culture were used i.e. BMGH-BMMH medium and PTM_1-supplemented medium before expression optimization. This study also showed the optimization evaluation result of environmental parameters influence on autoTrm production employing PB design followed by RSM.

II. EXPERIMENTAL

Strain construction

P. pastoris SMD1168 dan pPICZαB was purchased from Invitrogen/Life Technologies (Singapore). The synthetic autoTrm gene for expression in *P. pastoris* was designed based on the prethrombin-2 amino acid and nucleotide sequences of *Homo sapiens'* coagulation factor II (thrombin) (F2), which is available in GenBank (Accession number NM_000506.3). It was constructed as prethrombin-2 with EDGE mutation based on the study of Pozzi et al. (2013) in pD912 plasmid originated from DNA2.0 (California) when purchased (Subroto *et al*., 2016). The autoTrm gene was isolated from the pD912 plasmid by PCR with additional introduction of EcoRI and SacII restriction site to the gene in 5'- and 3'-end, respectively. The isolated autoTrm with additional EcoRI and SacII restriction site was inserted to expression plasmid pPICZαB. The recombinant plasmid was transformed into *P. pastoris* SMD1168 by electroporation method with Eppendorf Multiporator (catalogue number 4309000019). All media used for cultivation are listed in Table 1.

Table 1 Media for cultivation.

Media	Compositions
YPD	10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} dextrose
BMGH (+ sorbitol)	100 mM phosphate buffer pH 6.0; 20 g L^{-1} peptone; 3.4 g L^{-1} YNB with ammonium sulphate and without amino acids; 0.4mg L^{-1} biotin; 40mg L^{-1} histidine; 10 g L^{-1} glycerol; 20 g L^{-1} sorbitol
BMMH	Identical to BMGH except for 5.0 mL L−1 methanol instead of 10 g L^{-1} glycerol
BSM	20 g L^{-1} ammonium sulphate; 12 g L^{-1} potassium dihydrogen phosphate; 4.7 g L^{-1} magnesium sulphate.7H2O; 0.36 g L^{-1} calcium chloride.2H_2O, 20 g L^{-1} glycerol
PTM_1	6.0 g L^{-1} cupric (II) sulphate.5H2O; 0.08 g L^{-1} sodium iodide; 3.0 g L^{-1}manganese sulphate.H2O; 0.2 g L^{-1} sodium molybdate.2H2O; 0.02 g L^{-1} boric acid; 0.5 g L^{-1} cobalt chloride, 20 g L^{-1} zinc chloride, 65 g L^{-1} ferrous (II) sulphate.7H2O; 5 ml sulphuric acid; 0.2 g L^{-1} biotin

Expression test

Expression test in BMGH-BMMH medium

A single colony of the recombinant *P. pastoris* was inoculated into 2,5 mL YPD medium and was incubated overnight at 30°C, pH 6 with 250 rpm shaking. The culture was transferred into247,5 mL BMGH medium and was cultivated until reached OD_{600}11. The cell pellet was harvested from BMGH medium by centrifugation at 6,000×g for 5 minutes at room temperature. The pellet was then resuspended for induction in BMMH mediumusing the one-tenth volume of the original BMGH culture. The final methanol concentration was adjusted to 1.5% every 24 hours during 96 hours. One mL of culture sample was collected and characterized by SDS-PAGE.

Expression test in PTM_1suplemented medium.

This expression strategy was adapted from fermentor-scaled production published by Wan Seman*et al*., (2014). Pre-culture was prepared by transferring a single fresh colony into 1 mL YPD medium and shaken at 250 rpm overnight at 30°C, pH 6. The overnight culture inoculated 9 mL BMGH at 30°C with shaking at 250 rpm for 48 h. The culture was then added into 90 mL Basal Salts Medium (BSM) containing 8.7 mL L^{-1} of PTM_1 per liter BSM for 24 h. Glycerol feed [50% (v/v) glycerol + 7.2 mL PTM_1 per litre glycerol) was transferred into BSM culture. The cultivation was continued for another 12 h. Induction was performed by adding 100% methanol (containing 7.2 mL PTM_1 per L methanol) to a final concentration 1,5% every 24 hours during 96 hours. One mL of culture sample was collected and characterized by SDS-PAGE.

Experimental design and statistical analysis

Plackett–Burman (PB) design

The experimental design was generated by Minitab 15.0 software. The effects of five factors (trace minerals concentration, induction temperature, induction time, pH and methanol concentration) on autoTrm production were investigated using PB design consist of two levels (high and low levels) investigated variables (Yu *et al*. 2014; Plackett& Burman, 1946). The generated variables and the experimental design are given in Table 2 and Table 3, respectively.

Table 2 Factors and levels of PB design

Parameter	Abbreviation	Code	Levels	
			-	+
Trace mineral concentration (% v/v)	% PTM_1	X1	0.73	1.46
Methanol concentration (% v/v)	% MeOH	X2	1	2
Temperature (°C)	T	X3	23	30
Induction time (hour)	T	X4	96	120
pH	pH	X5	4.5	6.0

Table 3 Experimental design of PB

Run Number	Block	X1	X2	X3	X4	X5
1	2	+	-	+	+	-
2	2	+	+	-	+	+
3	2	+	-	+	-	-
4	2	-	+	+	-	+
5	2	+	-	-	-	+
6	2	+	+	-	+	-
7	2	-	-	+	+	+
8	2	-	+	+	+	-
9	2	-	-	-	+	+
10	2	+	+	+	+	+
11	2	-	-	-	-	-
12	2	-	+	-	-	-
13	1	+	+	+	-	+
14	1	-	+	-	-	-
15	1	-	-	-	-	-
16	1	-	-	-	+	+
17	1	+	+	-	+	-
18	1	+	+	-	+	+
19	1	-	-	+	+	+
20	1	+	-	+	+	-
21	1	-	+	+	+	-
22	1	-	+	+	-	+
23	1	+	-	+	-	-
24	1	+	-	-	-	+

Response Surface Methodology (RSM)

The value of significant factors based on the results of PB design was optimized further by RSM design through Central Composite Design (CCD). RSM was used as mathematical and statistical methods combination to investigate the response values through modeling and analysis of multiple independent variables affecting the response (Yu *et al*., 2014; Bas *et al*., 2007).

Activity measurement of autoTrm

The activity of autoTrm was determined by measuring the hydrolysis of a chromogenic substrate S-2238 (Aglyco, Beijing) at OD_{405} as described in Yonemura*et al*., (2009). Lyophilized thrombin (Sigma-Aldrich, Singapore) was used as standard with dilution to 0.1, 0.2, 0.4, 0.5, 0.8, 1.0 and 2.0 U/mL.

III. RESULTS AND DISCUSSION

Expression test

P. pastoris is considered as one of the most important bio factories for the production of different types of recombinant proteins (Sarmidi& El Enshasy, 2012). It is a well-known host used to produce the heterologus protein with the ability of post-translational modification, e.g glycosylation and disulphide bond (Cereghino*et al*., 2002). It is needed in the production of autoTrm since it has one glycosylation site and three disulfide bond. The smd1168 strain is not as vigorous as wild-type strains and slower growth rate (Creg*et al*., 2000) yet it is included in deficient-protease strain showed to potentially reduce the proteolytic degradation during production as in Fibrinogen production compare to GTS115 strain (Tojo*et al*., 2008).

The autoTrm gene, consist of 933 bp, was transferred from the pD912 plasmid into pPIZαB since the expression result observed was more consistent in the latter expression plasmid (data not were shown). pPICZαB was known contains the inducible promoter AOX1, α-factor secretion signal, and a transcription termination signal. The selection of the recombinant *P. pastoris* SMD1168 transformed with pPICZαB-autoTrm gave 50 transformants. *P. pastoris* SMD1168 transformant containing pPICZαB-autoTrm was grown in two different medium, (1) BMGH-BMMH medium and (2) PTM_1-supplemented medium. The result of secreted autoTrm in both media was shown in Figure 1. Secreted autoTrm was seen to be secreted better when the transformant was grown in the PTM_1-supplemented medium.

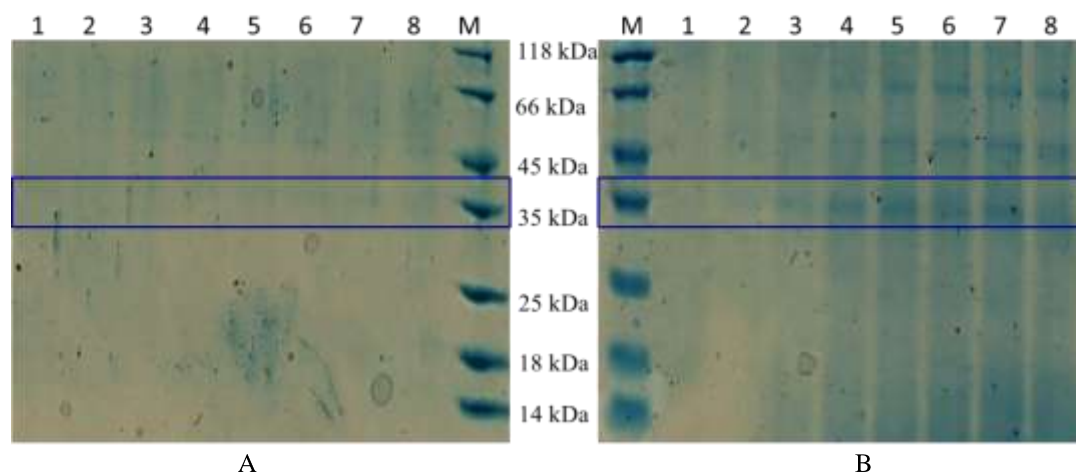


Figure 1 SDS-PAGE profile of secreted autoTrm. *P. pastoris*transformant was cultivated in BMGH-BMMH medium (A) and PTM_1-supplemented medium (B). Lane M: marker, lane 1-8: induction time at 0, 24, 48, 72, 96, 120, 144, 168 hours, respectively.

The using of pPICZαB containing α-factor signal peptide helped the expression of autoTrm secreted into culture medium. This study showed the secreted production of autoTrm in two mediums differentiated in the composition of trace minerals and the growth phase strategy of the host. In bioprocess, the cultivation medium and condition should be studied to optimize different process variables, include physical process parameters (temperature, pH, mixing, etc) and nutritional requirements (C-source, N-source, trace elements, vitamins, etc.), (Sarmidi& El Enshasy, 2012). Our previous study showed autoTrm could be expressed from *P. pastoris* in BMGH-BMMH medium (Subroto *et al.*, 2016) yet the result still needs to be improved. This study sought an alternative medium and growth strategy to use for *P. pastoris* cultivation in secreting autoTrm. Nutritional contents in the medium are the most important factor for healthy cell growth. accommodate elements, such as C, H and O, and macro- and microelements are needed by the cell (Sarmidi& El Enshasy, 2012). Cultivation medium supplemented with trace mineral has been generally used for cultivating *P. pastoris* in fermenter scale. The medium and strategy of *P. pastoris* cultivation using *P. pastoris* trace mineral (PTM_1) supplementation in fermenter scale was adapted in shaken flask cultivation. SDS-PAGE of autoTrm expressed in PTM_1-supplemented medium showed thicker band than autoTrm expressed in the BMGH-BMMH medium. The supplementation of PTM_1 into mediumhad been applied in fed-batch fermentation and shaken flask culture to produce recombinant frutalin from *P. transfor*mant. It became an important and significant strategy to increase the production of supplemented (Wanderley*et al.*, 2013).

Determining the significant factors on autoTrm expression using PB design

In order to gain better production result, optimization of autoTrm production was carried out by statistical design. PB design was used to screen the factors predicted affecting the autoTrm production significantly while RSM using CCD was used to determine the optimum value of the significant factors to generate optimum activity as the response. A 2-level method was employed in PB, applicable to determine significant factors affecting the experiment for a quick and efficient screening using the least experiments (Yu et al., 2014).

Five factors were investigated to study the influencing parameter that affects the expression of autoTrm in *P. pastoris* SMD1168. Minitab 15.0 was used as the software to generate the experimental design. All experiments were carried out in 100 mL shake flask containing 15 mL culture. The experimental design was carried out duplicate separated into 2 blocks based on the design generated by the software. The result of the experimental design was shown by Table 4, hence the significant factors were determined and the analysis of variance was performed (Table 5). By screening the influencing factors in autoTrm production with PB, it was

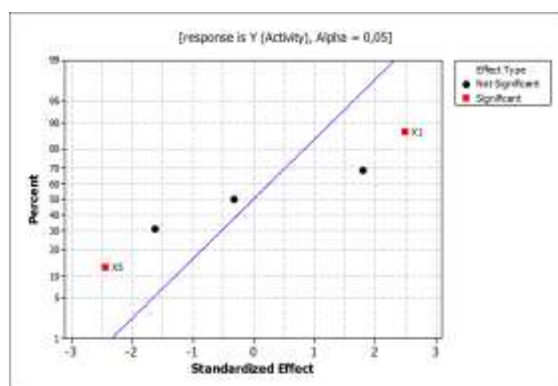
found that %PTM and pH were the significant factors ($P < 0.05$). The values of P-value indicated the significant levels of the model and the factors. The rank of influential factors was X1 > X5 > X3 > X4 > X2. Figure 2 shows that X1 and X5 (%PTM_1 and pH) had significant effects ($P < 0.05$) on autoTrm production in tested range and were chosen for further optimization.

Table 4 Experimental Result of PB

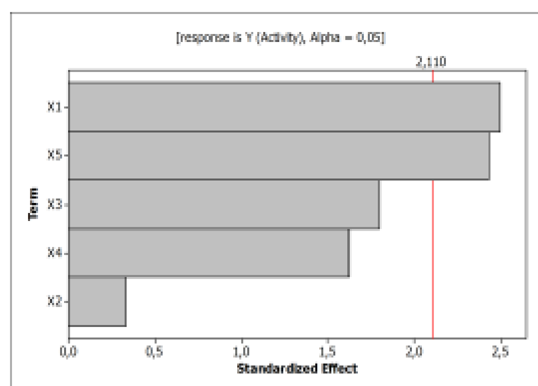
RunOrder	Blocks	X1	X2	X3	X4	X5	Y
		% PTM_1	% MeOH	T	t	pH	Activity (U/mL)
1	2	14.6	1	30	120	4.5	0.165
2	2	14.6	2	23	120	6.0	0.119
3	2	14.6	1	30	96	4.5	0.169
4	2	7.3	2	30	96	6.0	0.115
5	2	14.6	1	23	96	6.0	0.117
6	2	14.6	2	23	120	4.5	0.154
7	2	7.3	1	30	120	6.0	0.132
8	2	7.3	2	30	120	4.5	0.152
9	2	7.3	1	23	120	6.0	0.111
10	2	14.6	2	30	96	6.0	0.150
11	2	7.3	1	23	96	4.5	0.140
12	2	7.3	2	23	96	4.5	0.149
13	1	14.6	2	30	96	6.0	0.162
14	1	7.3	2	23	96	4.5	0.121
15	1	7.3	1	23	96	4.5	0.150
16	1	7.3	1	23	120	6.0	0.104
17	1	14.6	2	23	120	4.5	0.108
18	1	14.6	2	23	120	6.0	0.140
19	1	7.3	1	30	120	6.0	0.115
20	1	14.6	1	30	120	4.5	0.148
21	1	7.3	2	30	120	4.5	0.119
22	1	7.3	2	30	96	6.0	0.125
23	1	14.6	1	30	96	4.5	0.144
24	1	14.6	1	23	96	6.0	0.145

Table 5 Analysis of the results of PB design

Factor	P value	Significant
X1	0.023	1
X2	0.744	5
X3	0.090	3
X4	0.122	4
X5	0.027	2



A



B

Figure 2 Graphics of standardized effects. (A) Normal plot. (B) Pareto chart.

Optimization of the significant factors by RSM

Yu *et al.*, (2014) had used PB design and RSM using Box-Behnken design to optimize the production of human lysozyme from the same host and strain as used in this study. The study screened seven factors, i.e. inoculation volume, culture volume, growth time, induction temperature, induction time, initial pH and methanol concentration. Induction temperature, induction time and culture volume were the significant factors. Box-Behnken design (BBD) was employed to determine the optimum value of each significant factors and showed induction temperature, induction time and culture volume to be 23.5°C, 90 h, and 48 mL respectively. In this study, CCD was employed instead of BBD since only 2 significant factors generated by PB whilst BBD accommodate minimum 3 significant factors. These results showed parameters need to be considered for further bioprocess optimization in fermenter scale of autoTrm production.

As shown by the result of PB design in this study, %PTM_1 and pH (encoded as A and B, respectively) had the significant influence on the response values. CCD was then used to investigate the interactions and the optimal levels of both factors. Each factor had five coded levels (-1.4; -1; 0; 1; 1.4) and 12 runs were performed with 4 center points based on the experimental design generated by the software (Table 6). Low and high %PTM_1 value set into the design was 0.6 and 1.8, respectively, whilst low and high pH value set was 4.50 and 6.00, respectively. Experiment in 100 mL shaken flask containing 15 mL culture was carried out based on the generated design. Subsequently, the actual activity of autoTrm from the experiment was analyzed and gave mathematical model as follows

$Y = 0{,}6635 – 0{,}0130\ A – 0{,}1525\ B – 0{,}0026\ AB + 0{,}0132\ A^2 + 0{,}0105\ B^2$

Y was the response as predicted autoTrm activity, A and B were coded for %PTM_1 and pH, respectively.

Table 6 RS design through CCD generating model predicted values of autoTrm activity.

Run Order	Factors		Response (Y): autoTrm activity (U/mL)	
	A: %PTM_1	B: pH	Actual value	Predicted value
1	1.20 (0)	4.19 (-1.4)	0.219	0.199
2	1.80 (1)	4.50 (-1)	0.181	0.188
3	0.35 (-1.4)	5.25 (0)	0.148	0.145
4	0.60 (-1)	6.00 (1)	0.133	0.114
5	1.20 (0)	5.25 (0)	0.124	0.139
6	1.20 (0)	5.25 (0)	0.138	0.139
7	1.80 (1)	6.00 (1)	0.143	0.118
8	0.60 (-1)	4.50 (-1)	0.167	0.180
9	1.20 (0)	5.25 (0)	0.143	0.139
10	2.05 (+1.4)	5.25 (0)	0.148	0.153
11	1.20 (0)	6.31 (+1.4)	0.081	0.103
12	1.20 (0)	5.25 (0)	0.162	0.139

Predicted activity value was then generated by setting the value of each factor into the equation of mathematical model. The effect of the variables on autoTrm activity was observed by contour plot and surface plot. The highest predicted value is placed at the darkest area in the contour diagram and at the highest point in the 3D surface plot (Figure 3). The optimum condition predicted by optimization plot (Figure 4) to generate maximum autoTrm activity was found to be %PTM_1 2.0 and pH 4.2.

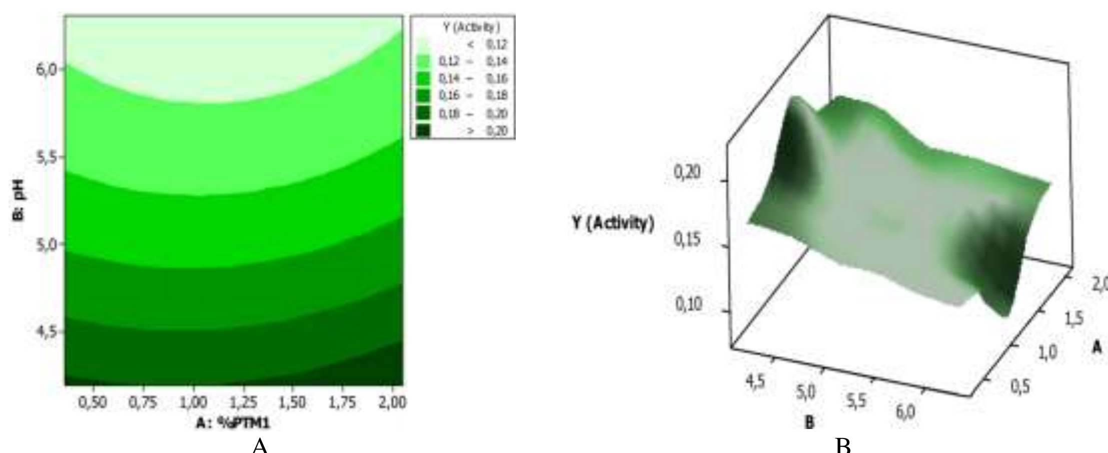


Figure 3 Contour plot (A) and 3D surface plot (B) of activity vs %PTM_1; pH.

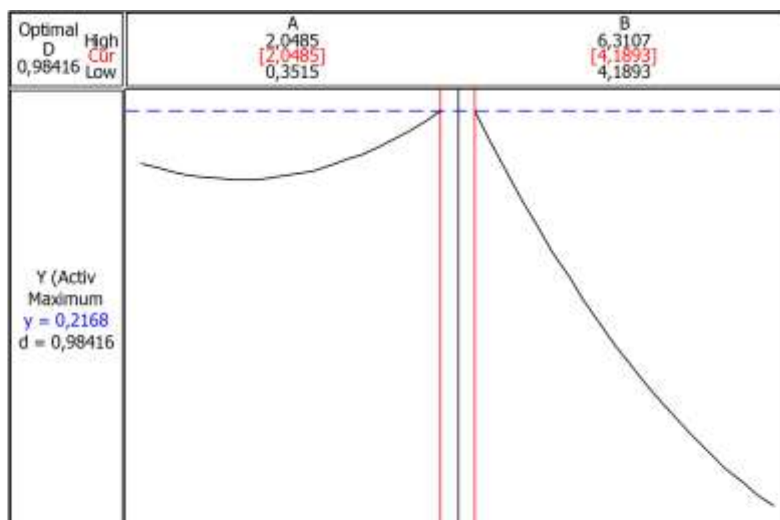


Figure 4 Optimization plot of %PTM$_1$ (A) and pH (B) to produce maximum autoTrm activity.

Predicted optimum activity generated from the equation by inserting the optimum %PTM$_1$ and pH was 0.2168 U/mL. This result was verified by conducting five experiments simultaneously with optimum %PTM$_1$ and pH. The average activity resulted from five experiments was 0.2110 ± 0.0132 (Table 7). Results comparison between predicted and experimental optimum activity showed similar value so that the equation can be used to predict the generated activity. Furthermore, SDS-PAGE (Fig. 5) showed thicker band (~36 kDa) resulted from optimized condition compare to the band from non-optimized one (autoTrm activity 0.1289 U/mL).

Table 7 Experimental autoTrm activity compare to predicted activity based on equation with optimum PTM and pH.

No.	autoTrmactivity (U/mL)	Average of experimental activity (U/mL)	Predicted activity (U/mL)
1.	0,2104		
2.	0,2256		
3.	0,2226	0,2110 ± 0,0132	0,2168
4.	0,2012		
5.	0,1951		

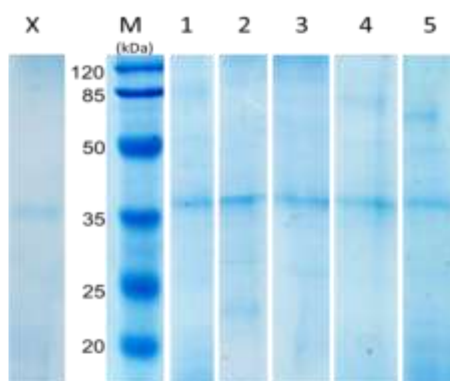


Figure 5 SDS-PAGE profile of expressed autoTrm with optimum %PTM and pH. Lane 1-5: autoTrm expression in optimized condition for 5 replications. Lane X: autoTrm expression in non-optimized condition.

IV. CONCLUSIONS

Expression of autoTrm gave better result by using PTM$_1$supplemented medium and could be optimized by employing PB design and RSM. The optimized condition based on mathematical equation generated had been proved by 5 replicates experiment. This strategy could be used to produce predicted autoTrm activity rely on the mathematical modeling.

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