Range Operation Studies of Microbial Fermentation for Biopharmaceutical Applications

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Abstract: The production of biopharmaceutical in lab scale differs from in pilot plant-scale particularly in process control or conditions control aspects. For larger volume of production, there is a chance of having deviation on controlling the operating conditions that is likely to have effects on specifications of biopharmaceuticals. Thus, this work focused on the optimization and robustness study of growth conditions on microbial fermentation using design of experiment methodology (DoE) and statistical analysis approach. Three factors for designing experiment were temperature, pH and % dissolved oxygen (DO). A high-throughput MRT-24 microbioreactor and *Pichia pastoris* KM71H which expresses Japanese Encephalitis virus envelope protein were used as a model study and the measured output was OD_{600}. After 17 hours of fermentation, the data was analyzed and the conditions where temperature was 28.0 °C, pH was 6.37 and DO was 30% were calculated to be the optimized value. Then the robustness study was carried out in which 5 % loss of the highest yield was set as a criterion. The results predicted that the robust range was temperature of 28^0Cto 29.5°C and pH of 6.0 to 6.5.

Key words: Design of experiment, microbioreactor, *Pichiapastoris*, robustness, ranges of operations.

1. Introduction

Biopharmaceuticals production has become significantly of interest among global healthcare-product market in the world where the sale market reaches 116 million US dollars in 2009 and it is expected to have 11.2% increase by 2016 [1], [2]. In Thailand, biopharmaceuticals production is still in the early beginning phase where researchers are developing the best candidates for cure which is in a lab-scale study [3]. However, the lab-scale production of biopharmaceutical differs from that of in pilot-scale particularly in process control or conditions control aspects. With the larger volume of production, there is a chance of having deviation on controlling the operating conditions that is likely to have effects to the specifications of biopharmaceuticals and consequently the qualities of the product maybe questioned. For example, the temperature of pilot-scale bioreactor is hard to control because the distribution of heat in larger volume is lower than the distribution of heat in lab-scale [4], [5]. Thus, the temperature of pilot-scale bioreactor is not stable at all-time [5]. Moreover, there is a few literatures reviewing on range of operating conditions and lab-scale research mainly focuses on optimal conditions while biopharmaceutical industry requires the information to support the operating conditions to assure the products remain good qualities. Therefore, the industry has to focus on their range of operating conditions and find significant parameters to control the process.

Herein we demonstrated the ranges of operations of growing *Pichia pastoris* using a strainKM71H which contains plasmids encoding Japanese Encephalitis Virus envelope protein as a case study. *Pichia pastoris* is one of the most common strains used in biopharmaceutical productions due to a number of advantages including its high growth rate coupled with ease of high cell-density fermentation, a simple and cheap growth medium to use and high levels of protein productivities and post translational modification applications [6]-[12].

The study was performed by using MRT-24 microbioreactors where parallel growth conditions can be high-throughput analyzed. The ranges were studied for factors including temperature, pH and %Dissolved Oxygen (%DO).

2. Materials and Methods

2.1. Cell Stocks Preparation

Pichia pastoris KM71H containing Japanese Encephalitis Virus (JEV) envelope protein gene was obtained as a gift from Assist. Prof. Anan Tongta and Dr. Jorgen Borg at KMUTT. The glycerol stocks were prepared in YPD and 50% glycerol then stored at -80°C for further use.

2.2. Media, Buffer, and Chemicals Preparation

YPD and BMGY were prepared as described in [4]. 10% Pluronic-68 used as antifoam reagent was prepared by diluting with deionized water then sterilized by an autoclave at 121°C for 20 minutes. 15% NH_4OH was also prepared by dilution manner then was poured into a pressurized vessel for ammonia vapor production used in pH adjustment during fermentation in MRT-24 microbioreactor.

2.3. Design of Experiment (DoE) and Statistical Analysis

Minitab program was used to conduct a Box-Behnken Design (BBD) model for three factors which are temperature (T, °C), pH and %Dissolved Oxygen (%DO). Levels of BBD design was chosen based on the growth characteristics of *Pichia pastoris* and JEV envelope protein properties. Table 1 displays high, medium and low levels for each factor that were used to create the 15 BBD experimental runs shown in Table 2.

Table 1. Factors and Their Levels

Factors	Level		
	Low	Medium	High
T (°C)	28	30	32
pH	5.5	6.0	6.5
%DO	10	20	30

The predictive model from the results of experiments was achieved by using Minitab program and shown in equation 1 where some terms were removed regarding the p-values. The summary of fit, ANOVA and response surface analysis were also demonstrated in table below.

2.4. Yeast Culturing Conditions and Microbioreactor Set up

Overnight cultivation of *Pichia pastoris* KM71H was conducted in a shake flask manner at 30°C and 300 rpm. Cells were harvested when the OD_{600} reaches 8 to 10 by centrifugation at 5000 rpm. The pellets were then resuspended in BMGY with its corresponding pH conditions to the OD_{600} of 1. The resuspension was transferred to MRT-24 plates to their corresponding assigned positions where a mixture of anti-foam and BMGY were pre-loaded. pH and DO optical sensors, in the bottom of the plate, were calibrated before the cell resuspension was transferred to the plate. PALL MRT-24 microbioreactor was employed for culturing MRT-24 plates overnight at designed experimental runs listed in Table 2. The growth of *Pichia pastoris* KM71Hexpressing JEV envelope protein was then checked by an off-line OD_{600} measurement. The Mrt-24 microbioreactor was set to agitate at 500 rpm and gases flows were 10 sccm for CO_2 and NH_4OH and 20 sccm for O_2.

3. Results and Discussion

BBD was chosen for this study as comparing to other models as BBD creates a reduced experimental run numbers that are sufficient to cover factors to be investigated [13]-[16]. 15 experimental runs had been created and the responses were OD_{600} measurements shown in Table 2 below. The responses were varied from 13.4 to 35.6 among which there were three runs marked with double asterisks where the DO sensor did not show any responses while the oxygen had been feeding in. The center point experiments were also indicated with an asterisk in Table 2.

Table 2. Experimental Runs and Their Responses

Randomized run no.	T (°C)	pH	%DO	**OD_{600}**		
4	28	6	30	32.4	31.88	32.7
6	28	6	10	31.55	29.2	31.45
9	28	6.5	20	19.1**	24.45	21.8
15	28	5.5	20	19.5	23.2	20.55
3*	28	6	20	29.55	27.19	22.75
5	30	5.5	10	24.05	23.1	29.25
7*	30	6	20	29.55	27.19	22.75
8	30	5.5	30	23.55	23.55	17.95
10	30	6.5	10	19.9	20.85	19.7
12	30	6.5	30	22.2	35.6	34.25
14*	30	6	20	29.55	27.19	22.75
1	32	6.5	20	20.75	21.2	20.65
2	32	6	30	22.9**	20.35	20.2
11	32	6	10	17.5	20.75	21
13	32	5.5	20	20.1**	19.9	13.4

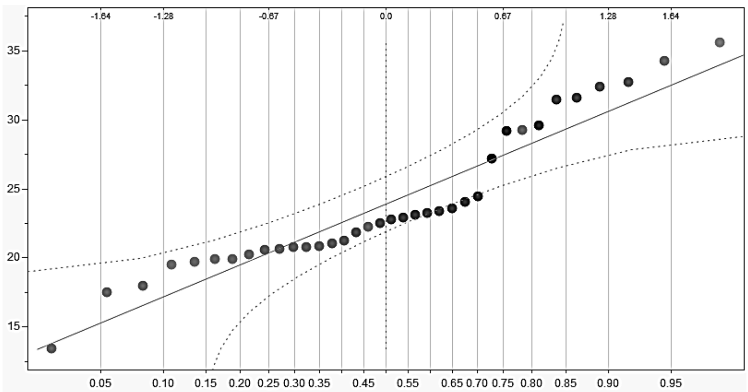

Fig. 1. Distribution of data.

Moreover, the distribution plot was generated as illustrated in Fig. 1. From the graph, one observation was the experiments ran at %DO of 30 seemed to show higher responses than others. This might be that Pichia pastoris generally requires a lot of oxygen for growing. The more oxygen fed in, the more cells grow. In overall, the data distribution was proper for further statistical analysis.

Statistical analysis was performed and listed in Table 3. The R^2 of 0.67 as well as R^2_{adj} of 0.59 were obtained suggesting that the predicted model was in range of acceptable as several literatures reported the value > 0.75 being considered as good model and < 0.25 being poor [16]-[18]. Moreover, p-value from Table 4 and lack of fit value from Table 5 also indicated that the predicted model was suitable for pursuing further response surface analysis.

Table 3. Summary of Fit

R^2	0.668528
R^2_{adj}	0.585659
Root Mean Square Error	3.367795
Mean of Response	23.91083
Observations	36

Table 4. The Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
Model	7	640.50276	91.5004	8.0674	<0.0001
Error	28	317.57712	11.3420	-	-
C. Total	35	958.07988	-	-	-

Table 5. Lack of Fit Analysis

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F	Max R Sq
Lack Of Fit	5	83.10479	16.6210	1.6304	0.1919	0.7553
Pure Error	23	234.47233	10.1944	-	-	-
Total Error	28	317.57712	-	-	-	-

Table 6. Response Surface Statistical Analysis

Parameter Estimates				
Term	**Estimate**	**Std Error**	**t Ratio**	**Prob>ItI**
Intercept	25.4975	1.683897	15.14	<.0001
T(28, 32)	-3.566972	0.756508	-4.72	<.0001
pH(5.5, 6.5)	1.394724	0.721302	1.93	0.0633
DO(10, 30)	1.330092	0.724634	1.84	0.0771
T*T	-1.602824	1.231878	-1.30	0.2038
pH*pH	-3.354676	1.231878	-2.72	0.0110
pH*DO	3.595833	0.972199	3.70	0.0009
DO*DO	2.336342	1.231878	1.90	0.0682

Table 6 provides the regression coefficients and the associated probability values (p-value) for OD_{600} response. The linear terms of temperature and intercept were statistically significant parameters affecting the OD_{600}, since their p-values were <0.05. Only the quadratic term of pH was significant meaning that pH was probable affecting the response. The only interaction term between pH and DO was also statistically significant with a p-value of 0.0009. These results indicate that the three parameters are important parameters for the growth of Pichia pastoris KM71H. Consequently, the predicted equation was generated as in equation (1).

$$OD_{600} = 25.45 - 3.16\left[\frac{(T-30)}{2}\right] + 1.12\left[\frac{(pH+6)}{0.5}\right] + 1.20\left[\frac{(DO-20)}{10}\right] - 2\left[\frac{(T-30)}{2}\right]^2 - 3.48\left[\frac{(pH+6)}{0.5}\right]^2 + 2.46\left[\frac{(DO-20)}{10}\right]^2 + 3.6\left[\frac{(pH+6)}{0.5}\right]\left[\frac{(DO-20)}{10}\right] \quad (1)$$

The response surface plots were shown in Fig. 2 in which Fig. 2A illustrates the relationship of temperature and pH where the optimal growth conditions were achieved at the highest point of the curve. While the plots of %DO either with pH in Fig. 2B or temperature in Fig. 2C demonstrates that higher %DO renders better yields which is consistent with other research groups. However, due to limitation of the high-throughput equipment, the control of higher %DO (> 30%) is quite difficult. For temperature plots, it was obviously seen that the lower temperature is preferred. Based on these predicted model, the highest response was calculated to be an OD_{600} around 32 and the prediction interval was 29.4 to 36.6 as listed in Fig. 2D.

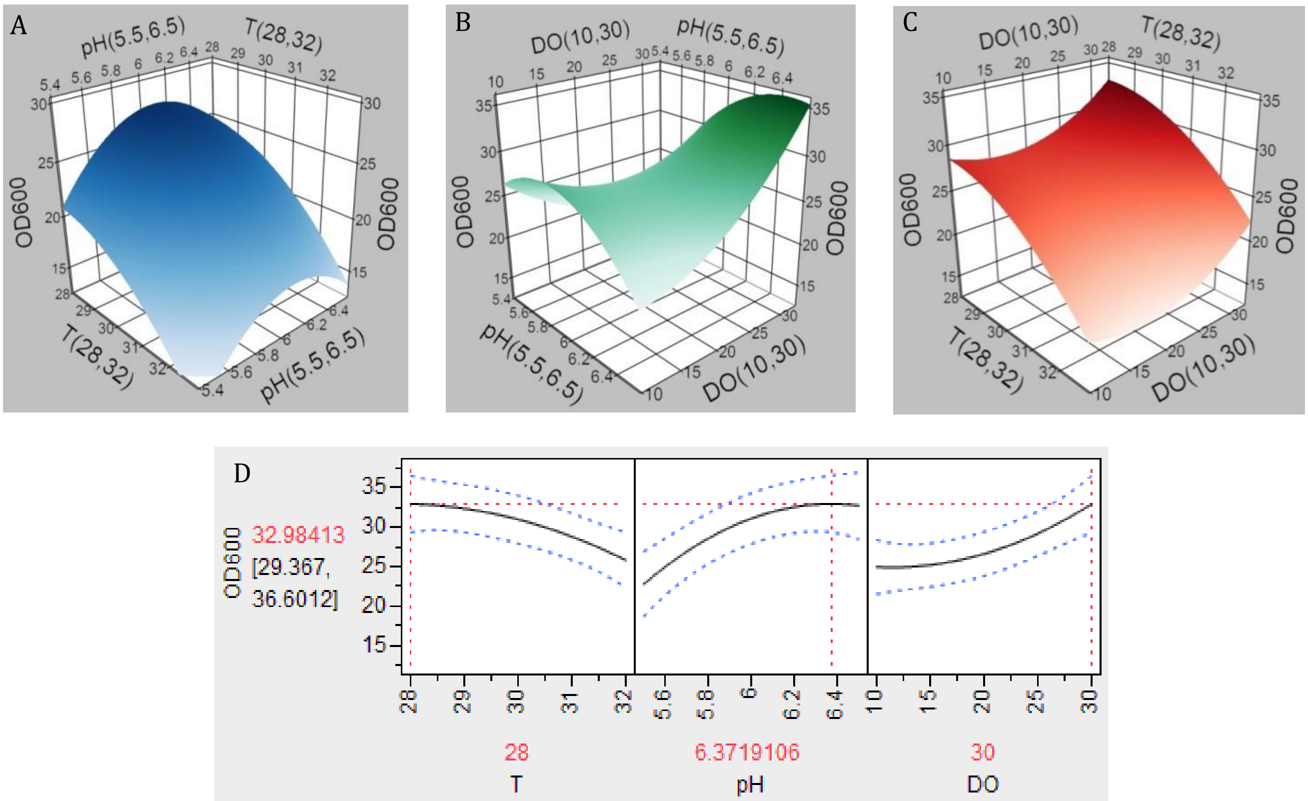

Fig. 2. Response optimization; response surface plot for A) Temperature and pH, B) pH and DO, C) Temperature and DO and D) Calculated optimized conditions.

Moreover, the prediction profilers shown in Fig. 3 and 4 were evaluated to obtain an initial indication of the robustness of the operating conditions. The information obtained from these plots was helpful in establishing operating ranges to confirm the confidence of the optimum parameters and predicted responses when the operating conditions have deviations. The operating conditions can deviate from optimal conditions for each parameters in order to achieve target. The slope was the criteria to choose the robust range of operating conditions. When the slope is closed to zero, it means that significant changes are not observed. Herein we consider the plateau region within 5% of OD_{600} loss. For example, considering temperature graph where the deviation of temperature was approximated -0.4 and +1 °C from optimal conditions (the deviation of temperature was shown in Fig. 3), then the response has a small change or loss lower than 5% and the slope is closed to zero. Therefore, it could be claimed that this temperature range is robust.

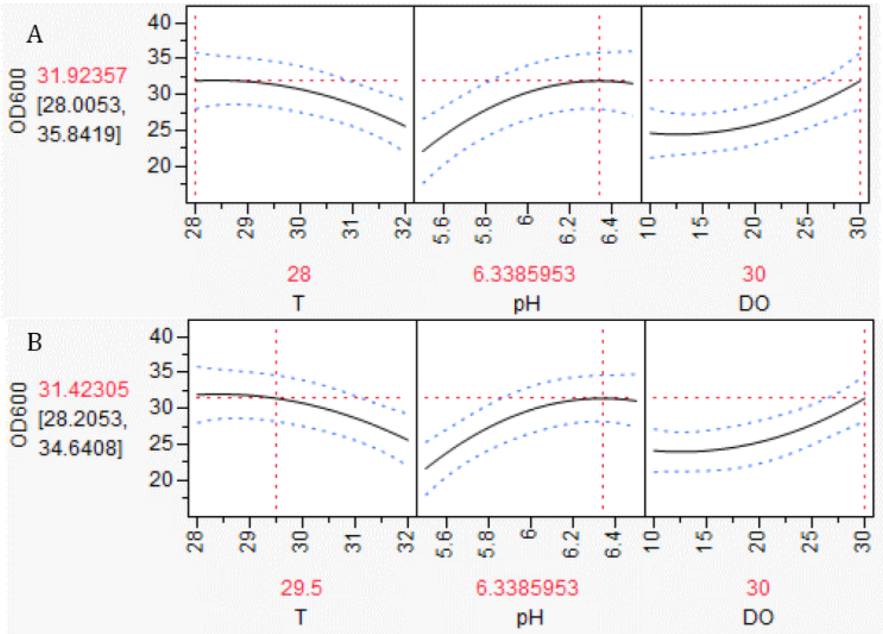

Fig. 3. Prediction profiler when temperature was changed from 28.0°C (A) to 29.5°C (B).

Fig. 4 illustrated that the robust range of temperature was 28^{0}C to 29.5°C and pH was 6.0 to 6.5 shown by the green lines. For DO, it was no robust range observed and this was possibly due to high oxygen consumption rate of yeast. However, higher DO such as 35% or more is very difficult to control and this is one of limitation of all fermenter.

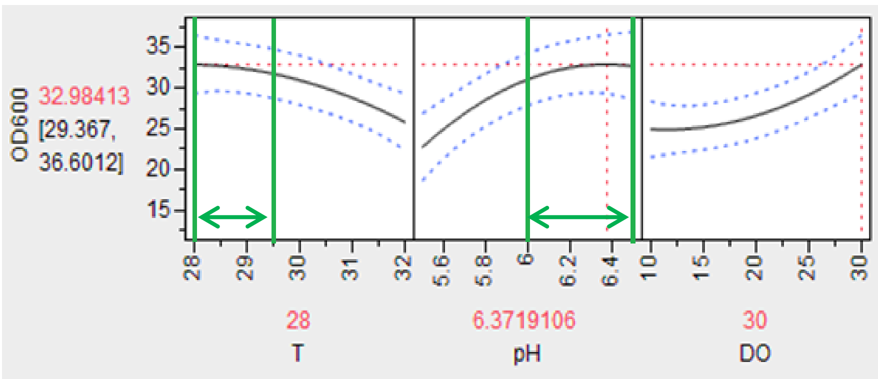

Fig. 4. Prediction profiler for ranges of operations.

4. Conclusion

Optimization and ranges of operations for growing *Pichia pastoris* KM71H were carried out in order to develop a robust process for a pilot-scale production where the process has to be well controlled and consistent. From the predicted model of growth conditions, it was found that the OD_{600}increases with decreasing temperature and increasing pH and DO.Optimal conditions where temperature was 28.0^{0}C, pH was 6.37 and %DO was 30 were achieved and the robust process predicted from the response surface plot ranges from 28.0^{0}C to 29.5^{0}C for temperature and 6.0 to 6.5 for pH. However, validation of these ranges of operations should be further proceeded by scaling-up study.

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the Correct Number of Variables.

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