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Optimization on Cell Disruption of *E. coli* BL21-AI Expressing Recombinant Bromelain

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Recent advancements in recombinant DNA technology proved to be a promising and effective approach for more sustainable large scale productions of many therapeutic proteins. Nevertheless, since this approach involves expression of proteins in a non-native host microorganism, the overall production processes are not straight-forward due to several common challenges, such as protein degradation, especially during cell disruption stage. As the process has been subjected to both protein and host-specific, a systematic process conditioning for maximal production of recombinant protein is therefore required. In this study, a simple approach to determine optimal conditions for cell disruption using ultrasonication to isolate recombinant bromelain from *E. coli* BL21-AI is reported. Suspension cells were lysed using ultrasonication which transmit sound wave in to break the cell wall. An optimized condition was obtained by response surface methodology (RSM). A three factor face-centered central composite design (FCCD) was applied to obtain the optimal process conditions consisting of amplitude, cycle and bursting period. The prediction model was further validated. Therefore, under the optimal conditions, having 20% amplitude, 0.5s cycle, and 1 minute bursting period in three times process, the specific enzyme activity of the recombinant bromelain was found to be functionally and reproducibly acceptable at 0.5270 U/mg.

Key words: Cell lysis, Downstream, Enzyme activity, Intracellular, Recombinant protein.

Cell lysis or cell disruption is one of the common methods in recovering intracellular products for downstream processing. There are many methods in cell lysis range from mild to harsh treatment techniques. These techniques contribute to cell disintegration which leads to the release of intracellular product of interest[1]. Cell lysis by sonication is one of known methods which are commonly used to break the cell wall then releasing the intracellular product.

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Disruption of cell wall can be achieved either by mechanical and non-mechanical methods. The non-mechanical methods such as chemical or enzymatic lysis are commonly used in the laboratory scale. However, these methods are expensive for large-scale intracellular protein recovery. Mechanical methods which include the techniques based on liquid shear and solid shear forces are rather easy to scale up and cost effective. Thus, these methods are preferred for large-scale preparations and commercialization. Ultrasonication works by disrupting the cells using liquid shear created by high frequency sound (ultrasound) that is produced electronically and

transported through a metallic tip to an appropriately concentrated cellular suspension[2]. This method provides fast and simple recovering and isolating technique for medium sized quantity to large scale by using appropriate needle tip probe size[3].

This unit operation is also simple and economically more viable compared to other cell disruption methods such as high pressure homogenization, chemical and enzymatic lysis methods[4,5].Ultrasonication procedure is also suitable to be applied on E.*coli*[6,7,8] in order to isolate the intracellular recombinant protein. Recombinant bromelain was expressed by E.*coli* BL21-AI[4] and this soluble recombinant bromelain was found in the intracellular component of the host. The growth condition in a shake flask of this recombinant enzyme been studied[9] before but the study on the downstream processing is to be improved. Therefore, the downstream processing in recovering the recombinant bromelain, through mechanical cell disruption by ultrasonication is needed. The aim of this study was to obtain the optimal conditions for the cell lysis using ultrasonication to get high recombinant bromelain protein recovery. The response surface methodology (RSM) was employed to optimize the ultrasonication process parameters and maximizing the specific activity of recombinant bromelain.

MATERIALS AND METHODS

Bacterial Strain and Plasmid

Recombinant bromelain clone was obtained with the courtesy of Assoc. Dr. Amid, A.

Cultivation of Recombinant Bromelain

Five colonies of recombinant *E. coli* BL21-AI harboring bromelain gene from fresh MDAG plate were inoculated into a 10ml non-inducing media for preparation of the starter culture. The starter culture was grown overnight for 12 hours at 37°C with 300 rpm in an incubator shaker (Stuart, Germany). Then the overnight starter culture was inoculated to 1L fresh medium in 2L working volume bioreactor (Infors, Germany) containing 1L of ZYM-5052 auto-induction media[10] with same fermentation condition of the starter culture. In preparation of 1L medium for auto-induction ZYM-5052 medium was used. This medium contains complex nitrogen source, ZY with 1% (w/v) of tryptone and 0.5% (w/v) of yeast extract, 20 ml of 50 X buffering salt, 20 ml of 50 X 5052 carbon source, 2 mM $MgSO_4.H_2O$, 0.2 ml of 1000 X trace element, 1ml of 100 mg/ml ampicillin and 0.02% of L-arabinose as inducer. Finally, RO water was used to top up until 1 L volume. Cells were then harvested by centrifugation at 5000 rpm for 30 minutes at 4°C using XIR centrifuge (Thermo Fisher, USA). Subsequently, the cell pellet was stored in -20ºC.

Ultrasonic cell disruption

The harvested cell pellet, 5g was re-suspended in extraction buffer[11] consisting of 100mM sodium phosphate buffer, 15mM of L-Cysteine and 2mM of EDTA and was chilled in ice before the sonication process. Disruption of cells was performed using lab scale ultrasonic homogenizer (Sartorius, Germany) operated at 30 kHz frequency with variation of 20 – 100% amplitude equipped with a 10mm diameter titanium needle probe. The disruption period was varied from 1 to 5 minutes with 60s intervals for three times with bursting cycle (pulse operation) from 0.2s to 60s on ice. Samples were kept in ice during the sonication process to prevent overheating and denaturation. Lysed cells were centrifuged at 12,000×g for 30 minutes at 4ÚC to remove cell debris.

Design of Experiment

To verify the influence of ultrasonication process consisting amplitude (X_1), cycle (X_2) and bursting period (X_3) towards the specific activity as the response, a face centered central composite (FCCD) design with three replicates at the center point were employed to optimize these parameters. The variation of the parameters was derived according to the design formulated by the statistical software,Stat-Ease DesignExpert® v8.0 (Minneapolis, USA). A total number of 20 experimental runs were summarized in Table 1 for obtaining optimal conditions for bromelain specific activity (Y).

Total Protein Quantification

The total protein content of the lysed samples was determined by using the Pierce 660nm Protein Assay (Pierce Biotechnology, IL) with bovine serum albumin (BSA) as standard. A 100µL of test sample was added into 1.5mL of the protein assay reagent and mixed. The mixture was incubated at 25°C for 5 minutes, transferred into

the semi-micro cuvette and absorbance was read at 660nm using MultiskanGo (Thermo Fisher, USA) spectrophotometer. All sample assays were carried out in triplicate.

Enzymatic Assay

Recombinant bromelain activity in the crude extract was determined by using protease assay as outlined by Sigma-Aldrich protocol (St. Louis, USA). Here, the activity was measured by the rate of substrate reaction towards bromelain using spectrophotometric measurement at 340 nm. About 100µl of enzyme solution was mixed with buffer A (30mM sodium acetate buffer with 100mM potassium chloride and 1mM L-Cysteine). The mixture was inverted few times in a cuvette and equilibrated at room temperature before adding Ná-CBZ-L-Lysine ester (LNPE) as the substrate. Absorbance measurement of enzymatic reaction was measured spectrophotometrically for 5 minutes. One unit of bromelain activity releases 1.0 µmoles p-nitrophenol per minute at pH 4.6 at 25°C.

Statistical Analysis

The specific bromelain activity is the dependent variable in this study. The quadratic model for obtaining the optimal point was expressed according to the equation below:

$$Y = \beta_0 + \beta X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + X_1^2 + X_2^2 + X_3^2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

where Y is the response variable, X_1, X_2 and X_3 are coded level of independent variables, β_0 is the intercept term, β_1, β_2 and β_3 are linear coefficients, β_{11}, β_{22} and β_{33} are quadratic coefficients, β_{12}, β_{13} and β_{23} are the interactive coefficients. The statistical significance of the second-order model equation was determined by the F-value and the proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R^2.

RESULTS AND DISCUSSION

The effect of cell disruption by ultrasonication for the release of intracellular product, particularly the recombinant bromelain from E.*coli* BL21-AI was studied by varying three parameters which are amplitude, bursting time and sonication cycle. These parameters which have high impact towards the efficiency of the cells disruption were varied to obtain the highest activity of recombinant bromelain[6]. About 1:5 ratio of wet cell (g) to buffer (ml) was used to maximize the possibility of cells being lysed and thus releasing the recombinant bromelain.

A total of 20 experimental runs shown in Table 2 were carried out to obtain the optimal conditions of ultrasonication with the recombinant bromelain specific activity (U/mg) as the response. Based on the result obtained, lowest specific activity value 0.0839 U/mg was shown in run 8 having 20% amplitude, 1s bursting cycle and 5 minutes sonication period. Having low amplitude indicates on low duty cycle which is insufficient to break the cell wall or incomplete cell disruption[2]. Harsh treatment also observed by applying maximal range for each parameter in run 9 having 100% amplitude, 1s bursting cycle and 5 minutes of sonication period yields in 0.1257 U/mg of specific activity. Higher amplitude and bursting cycle promoted higher chances of cell disruption. As period of sonication increased, total protein content also increase due to harsh treatment which disrupt most of the cells extra and intracellular components. However, Table 2 shows that run 6 with the lowest amplitude (20%), lowest cycle (0.5s) with one minute bursting period operated in three times with 60s interval gave the highest recombinant bromelain specific activity (0.5270Units/mg). Thus, suggesting that harsh treatment is not necessarily going to give high recombinant bromelain specific activity. This can be explain because the generation of heat created by the harsh cell disruption process (100% amplitude, 1s bursting cycle and 5 minutes of sonication period) contributes to protein denaturation consequently promoting the chances in getting low enzyme specific activity. Although samples were chilled in ice to maintain low

Table 1. Summary for the parameters involved to obtain high enzyme specific activity. Three paramaters involve encoded with amplitude (X1), Cycle (X2) and bursting time (X3) with three variation levels (-1, 0, 1)

Code	Parameter	Unit	Range		
			-1	0	+1
X1	Amplitude	%	20	60	100
X2	Cycle	sec	0.5	0.75	1
X3	Bursting time	min	1	3	5

Table 2. The tabulated optimization result for ultrasonication process. Showing three factors involved (amplitude, cycle and bursting time) with specific bromelain activity (U/mg) as the response

Run	Factor 1 A: Amplitude (%)	Factor 2 B: Cycle (sec)	Factor 3 C: Time (min)	Specific Activity (U/mg)
1	60	0.75	3	0.1327
2	60	1	3	0.1000
3	60	0.75	3	0.1687
4	60	0.75	1	0.2857
5	20	0.75	3	0.2273
6	20	0.5	1	0.5270
7	60	0.75	3	0.1645
8	20	1	5	0.0839
9	100	1	5	0.1257
10	20	1	1	0.2589
11	60	0.75	5	0.1371
12	100	0.75	3	0.1163
13	60	0.75	3	0.1097
14	60	0.75	3	0.1174
15	60	0.75	3	0.1606
16	20	0.5	5	0.2159
17	100	0.5	5	0.1285
18	100	0.5	1	0.2716
19	100	1	1	0.1182
20	60	0.5	3	0.1462

Table 3. Summary of the predicted model for response surface method for reduced quadratic model. Significant model was obtained with lack of fit insignificant

Source	F Value	p-value Prob> F	
Model	34.10	<0.0001	significant
X_1-Amplitude	39.53	<0.0001	
X_2-Cycle	47.01	<0.0001	
X_3-Time	76.83	<0.0001	
X_1X_2	9.63	0.0091	
X_1X_3	19.88	0.0008	
X_2X_3	13.31	0.0033	
X_1^2	32.54	<0.0001	
Lack of Fit	1.29	0.4025	not significant

temperature and prevent overheating during ultrasonication[2, 6, 11] but increased in the bursting cycle as well as the amplitude caused heat generation and needle tip erosion[11].

Analysis of Variance (ANOVA)

Based on the DesignExpert® software, after undergoing some model improvement by terms reduction of those insignificant, the best full second-order polynomial model for the optimization of ultrasonication was obtained from the regression analysis of results:

$$\text{Specific Activity} = +0.14 - 0.055X_1 - 0.060X_2 - 0.077X_3 + 0.030X_1X_2 + 0.0440X_1X_3 + 0.036X_2X_3 + 0.017X_3^2$$

The model adequacy was checked by the F test and the determination coefficient R^2. An acceptable F-value (34.10) implies that the model is significant and a low probability ($P > F = 0.0500$) indicated that the present model terms are significant. However, the goodness of fit of the model was expressed by the coefficient of determination R^2 (0.9521), indicating only 95.21% of the variability in the response could be explained by the model or about 40.25% of the total variation were not explained by the model. As a result, the lack of fit was not significant. Summary of terms included in the prediction model is shown in Table 3.

For specific enzyme activity (Y) in Figure 1, it can be observed that the contour tendency showed an increment in specific enzyme activity which is related to the lower value of amplitude

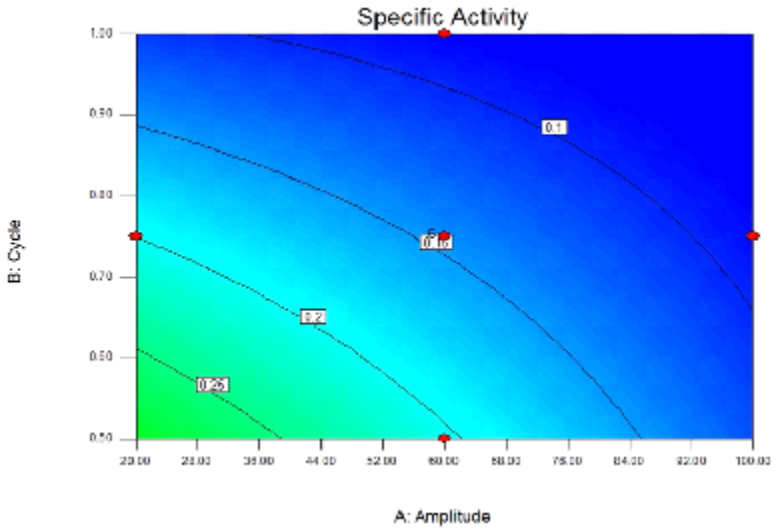

Fig. 1. The contour plot of the interaction of amplitude (%) and cycle (s) with specific activity (U/mg-protein) of bromelain as response.

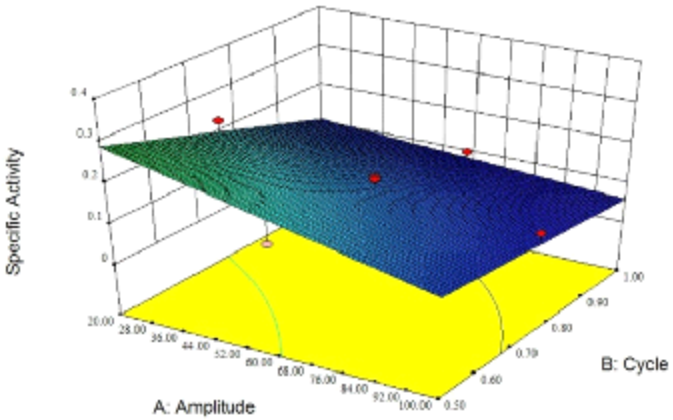

Fig. 2. The 3D contour plot of the interaction of amplitude and cycle with specific activity (U/mg-protein) of bromelain as response. Increment of the graph can be observed when low amplitude (%) and low cycle (s) was applied

applied and also the lower value of the cycle. Due to the equipment constraint, the value being used was the minimal range for this equipment that could be applied. The 3D plot in Figure 2, describes the second-order model response (Y), predicted that the maximum specific activity (0.5179 U/mg) could be attained at low amplitude (20%) and low cycle (0.5s). To confirm this model, a validation run was conducted under the optimized conditions and a specific activity of 0.5026 U/mg was obtained. The predicted R^2 of the model (0.8627) which is 86.27% is in reasonable agreement with the adjusted R^2 of (0.9242) which is 92.42%. This also indicated that the model has high percentage of reproducibility.

Further improvement can be made to increase the specific activity by combining the lysis methods such as the chemically is which is less in energy consumption[2] with ultrasonication. Even though, additional chemical use may enhance the activity, but it may also increase the cost of the process. Besides that, in the process cycle itself, improvements can be made by prolonging the

whole cycle, which in this experiment, three times of ultrasonication was used. Increasing the process cycle to more than three would decrease the intact cells and increase disruption efficiency resulting in higher specific activity value[2]. Also, a critical observation of heat generation whichis due to amplitude and cycle settings[12] must be handled in order to prevent protein denaturation at this addition process.

CONCLUSION

In conclusion, ultrasonication is a suitable method to disrupt *E. co*li BL21-AI cell to release recombinant bromelain. Optimized condition showed that with low amplitude at 20%, low cycle at 0.5s and short bursting time for 1 minute with three times sonication process produced the highest recombinant bromelain specific activity at 0.5270 U/mg.

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