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Optimization of Cellulase Production by *Aspergillus niger* ITBCC L74 with Bagasse as Substrate using Response Surface Methodology

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ABSTRACT

Cellulase is a very important enzyme for lignocelluloses based ethanol production. Bagasse contains mainly cellulose (57.76%), hemicellulose (12.44%), lignin (21.34%), and others (7.96%). Lignocellulosic material has been considered as the good option for cellulase production because it is cheap and already available in a huge amount. The objective of this research was to produce cellulase enzyme and to optimize it by using response surface methodology. The bagasse with water content of 80% was incubated with 2 ml inoculum of *Aspergillus niger* ITBCC L74 in a 250 ml Erlenmeyer flask. After reaching the specified time the enzyme was extracted and then determined for its activity. Effect of process parameters such as pH, urea and $MgCl_2$ addition were studied. The optimal cellulase activity was achieved at urea concentration of 4.5% (w/w), $MgCl_2$ concentration of 1 mM and pH of 3.5, with maximum enzyme activity was 0.630 U/gr.

1. Introduction

Commercial enzymes for industrial applications are mainly extracted from three main sources namely plants, animals, and microorganisms. Among three resources, microbial is more popular as enzyme sources (Abubakar and Oloyede 2013). The recent developments in bioconversion of agricultural and industrial wastes to chemical feedstock have led to extensive studies on cellulolytic enzymes produced by fungi and bacteria. Large quantities of lignocellulosic wastes are generated through forestry, agricultural practices, and industrial processes, particularly from agro-allied industries such as sugar cane, breweries, paper pulp, textile, and timber industries. These wastes generally accumulate in the environment thereby causing pollution problem. Lignocellulose is a major renewable natural resource of the world and represents a major source of renewable organic matter. The plant biomass regarded as "wastes" are biodegradable and can be converted into valuable products such as enzymes, biofuels, chemicals, cheap energy sources for fermentation, improved animal feeds and human nutrients (Acharya *et al.* 2008).

Lignocellulose or cellulose can be hydrolyzed become glucose, cellobiose and cello-oligosaccharides (Singhania *et al.* 2010). Due to that reason, cellulose or lignocellulose can be used for cellulase production via biological route utilizing bacterial or fungal (Sindhu *et al.* 2016). Aerobic and anaerobic bacteria, anaerobic fungi, soft rot fungi (SRF), white rot fungi (WRF), and brown rot fungi (BRF) are capable microorganisms widely used for producing cellulase (Lynd *et al.* 2002; Kuhad and Singh 2007; Chandel *et al.* 2012) which are able to produce a complete cellulase system such as endo-β-glucanase, exo-β-glucanase, and β- glucosidase (Knowles *et al.* 1987), compared to bacteria (Cen and Xia 1999).

Trichoderma reesei and *Aspergillus niger* are two strains of soft rot fungi most commonly used for commercial cellulase production via SF (Pandey *et al.* 2010) because of the ease of handling and greater control of environmental factors such as temperature and pH. But, cultivation of *T. reesei* or *A. niger* by SF process produced incomplete or deficiency in cellulase components although theoretically it is able to produce a complete cellulase system (Ahamed and Vermette 2008; Yoon *et al.* 2014). It also resulted low concentration of cellulase which can need further purification and affect to cost production (Rodriguez and Sanroman 2005). Due to the shortcomings

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mentioned, SSF can be used as one of alternative way to produce high concentration of cellulase and reduces the cost of enzyme production (Holker *et al.* 2004; Singhania *et al.* 2009). Moreover, the other advantages of SSF include superior productivity, simple technique, low capital investment, low energy requirement and less water content (Mrudula and Murugammal 2011).

Sugar industries that are located in Indonesia producing large quantity of solid waste namely bagasse (Daniyanto *et al.* 2015). The bagasse contains mainly cellulose (57.76%), hemicellulose (12.94%), lignin (21.34%), and others (7.96%) which is more less similar with lignocellulose contents in reference (Bahera and Ray 2016). For that reason, bagasse become a potential source for cellulase production by utilizing fungi, especially *A. niger*. The cellulase production using SSF by *A. niger* and *T. reesei* was carried out on bagasse with initial moisture content of 80% at 30°C. The result shows that *A. niger* was better than *T. reesei* after 72 hours incubation with the enzyme activity of CMCase for *A. niger* ITBCC L74, *A. niger* ITBCC L161 and *T. reesei* UGM 6131 reached the maximum of 0.5251, 0.3927, and 0.3264 U/g, respectively (Abdullah *et al.* 2016). In other experiment, Cunha *et al.* (2012) conducted cellulase production by *A. niger* by using sequential solid-state and submerged fermentation. While the behavior of *A. niger* growth on sugarcane bagasse had been reported by de Souza *et al.* (2011). Gottschalk *et al.* (2010) reported multi-enzymes production including cellulase by utilizing blend fungi between *Aspergillus* and *Trichoderma*. Amylase is also produced by SSF using *A. niger* utilizing sugarcane bagasse as source other than cellulase production, as reported by Rosés and Guerra (2009). Beside bagasse, other raw material which contain lignocellulose or cellulose can be used as substrate in cellulase production. For instance, wheat straw, orange waste, cassava waste, and banana waste have been investigated by several researchers for their potential to be used as substrates (Krishna 1999; Tabka *et al.* 2006; Omojasola and Jilani 2008; Olanbiwoninu and Odunfa 2016).

The response surface methodology (RSM) consists of a group of empirical techniques devoted to the evaluation of relations existing between a cluster of controlled experimental factors and are measure responses, according to one or more selected criteria. The maximum values were taken as the response of the design experiments. The optimal of the factors were obtained by solving the regression equation and also by analyzing the response surface contour plots (Sen 1997).

The first step in the experimental study of RSM is to decide a model from which expresses the response as a function on independent variable in the process. The different types of model have been used to predict the optimal response such as first and second-degree polynomial. However, many literatures have reported that by using the quadratic model, the optimal response can be obtained accurately (Murphy 1977; Vazquez and Martin 1998).

The aim of this study is to determine the optimum conditions in producing cellulase enzyme under solid state fermentation using bagasse as substrate with *A. niger* ITB CC L74. A statistical approach such as RMS and factorial experiment design is used to involve a minimum number of experiments for a large number of factors where these methods have also been demonstrated to improve the cellulase production. The present work describes the interaction and optimization among variables of pH, urea concentration, and $MgCl_2$ concentration in the culture medium which are successful to produce cellulase by *A. niger*.

2. Materials and Methods

2.1. Materials

Chemicals used for media and analysis in this study were purchased from Merck (Massachusetts, USA), Potato Dextrose Agar (PDA) was purchased from Oxoid (Hampshire, UK), while Ethanol was obtained from PT Brataco (Jakarta, Indonesia). Bagasse as substrate of fermentation process was obtained from Sugar Factory, Mojo Panggung, Tulungagung, Indonesia and was pre-treated by using 2% NaOH to remove the core and noncore lignin fractions (Doran *et al.* 1994), then dried in an oven at 80°C, crushed, and sieved to an average size of 40 mesh.

2.2. Inoculum Preparation

A. niger ITBCC L74, microorganism used in this study, was obtained from Bandung Institute of Technology and maintained at 4°C on Potato Dextrose Agar (PDA) slants.

2.3. Solid State Fermentation (SSF) Procedure

Cellulase is produced by *A niger* in a erlenmeyer flask with bagasse as substrate. 10 grams of bagasse with water content of 80% was incubated with 2 ml inoculum in a 250 ml Erlenmeyer flask. 5 ml of nutrients were added in accordance with Mandels nutrients (Omojasola *et al.* 2008; Raza *et al.* 2011) which is consisted of: 1 g peptone, 1.4 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, 0.3 g $CaCl_2$, 0.3 g $MgCl_2{\cdot}6H_2O$, 0.3 g Urea, with 1 ml of trace metal which consisted of 2.5 g $FeSO_4$, 0.98 g $MnSO_4{\cdot}H_2O$, 1.76 g $ZnSO_4{\cdot}H_2O$, 1.83 g $CoCl_2{\cdot}6H_2O$ in 495 ml De-ionized (DI) water and 5 ml HCl. The erlenmeyer flask was incubated at 35°C for at least 3 days (Vu *et al.* 2011). After reaching the specified time, the cellulase enzyme was extracted with a sodium citrate buffer solution pH 4.5 with the ratio against dry bagasse was 10:1 w/v. The enzyme activity was analyzed by the Ghose method (Ghose 1987). Composition of urea and $MgCl_2$ in the culture medium and pH were varied for optimization studies, while temperature and time of fermentation were fixed.

2.4. Response Surface Methodology (RSM) Determination

The statistical software, Minitab 17 – Trial Version (Pennsylvania, USA) was used for model equation determination and plotting the response surface while ANOVA also was used to analyze the statistical parameter as well.

3. Results

3.1. Bagasse Characterization

The treated bagasse with 2% NaOH (w/v) was analyzed for its cellulose, hemicellulose, and lignin content by Chesson-Datta method (Chesson 1981). The content of cellulose, hemicellulose, and lignin after pretreatment is 57.76 ± 0.49%, 12.44 ± 0.35%, and 21.34 ± 0.18% respectively. This is compatible with the results of research that has been done by Sarkar and Aikat (2012).

3.2. Determination Equation Models Using RMS

It is important to optimize the solid fermentation process by utilizing *A. niger* ITBCC L74 with duration of fermentation is three days and water content is 80%. The levels of variables investigated in this study are given in Table 1. The central values (zero level) chosen for experimental design were: urea concentration of 3% (w/w), magnesium chloride of 2.5 mM and pH of 4.5. For three variables using Box-Behnken Design using Design Expert 6 – Trial Version (Minnesota, USA), there are 14 experiments with 2 center point (Haaland 1989), the result was shown in Table 2.

The optimization process based on experimental design that states the relationship between the three variables to enzyme activity (E_a). Model equation is determined by response surface methods (RSM) and the mathematical model was presented in equation 1.

Table 1. Variables and levels for the Box-Behnken Design method experimental design

Independent variables	Symbols	Coded level		
		-1	0	+1
Urea (% w/w)	A	1.5	3.0	4.5
$MgCl_2$ (mM)	B	1.0	2.5	4.0
pH	C	3.5	4.5	5.5

$$E_a = 0.137 + 0.247A - 0.0917B + 0.00749C - 0.0158A2 + 0.00741B2 - 0.0123C2 - 0.0587AB + 0.000967AC + 0.0454BC \quad (1)$$

From the equation (1), it can be seen that there are three effects that affect in the values of Ea, namely the linear effects, quadratic effects and interaction effects.

In the linear effects, coefficient urea has the highest coefficient (+0.247) followed by $MgCl_2$ (-0.0197) and pH (+0.00749) while statistical result can be shown in Table 3. Therefore, effect of urea addition has high effect in increasing enzyme activity followed by $MgCl_2$. The negative sign indicates that addition of Magnesium Chloride has high effect in decreasing enzyme activity. In quadratic effect, urea addition has the highest effect (+0.0158) followed by pH (+0.0123) and Magnesium Chloride addition (+0.00741) while statistical result can be shown in Table 4. In interaction effects between urea concentration and pH shows that the coefficient has very low, therefore the equation (1) becomes equation (2):

$$E_a = 0.124 + 0.251A - 0.0917B + 0.0104C - 0.0158A2 + 0.00741B2 - 0.0123C2 - 0.0587AB + 0.0454BC \quad (2)$$

Table 2. Experimental design and enzyme activity results using Box-Behnken Design method

Run	Urea (w/w)	$MgCl_2$ (mM)	pH	Enzyme activity (U/g)
1	1.5	1.0	4.5	0.2901
2	4.5	1.0	4.5	0.5744
3	1.5	4.0	4.5	0.4931
4	4.5	4.0	4.5	0.2495
5	1.5	2.5	3.5	0.3539
6	4.5	2.5	3.5	0.3887
7	1.5	2.5	5.5	0.3539
8	4.5	2.5	5.5	0.3945
9	3.0	1.0	3.5	0.5163
10	3.0	4.0	3.5	0.2843
11	3.0	1.0	5.5	0.4293
12	3.0	4.0	5.5	0.4699
13	3.0	2.5	4.5	0.4235
14	3.0	2.5	4.5	0.4177

Table 3. Results of the linear effect of Box-Behnken Design

Source	Sum of squares	DF	Mean square	F value	F-stat with P=0,05	Prob > F	Note
Model	0.10981	9	0.012201	26.3616	2.48	0.00327	S
A	0.00168	1	0.001683	3.63636	2.48	0.129	NS
B	0.01227	1	0.012269	26.5091	2.48	0.00675	S
C	0.00136	1	0.001363	2.94546	2.48	0.161	NS
A^2	0.00404	1	0.004041	8.73091	2.48	0.0418	S
B^2	0.00089	1	0.00089	1.92364	2.48	0.238	NS
C^2	0.00049	1	0.000486	1.05091	2.48	0.363	NS
AB	0.06968	1	0.069684	150.564	2.48	0.000253	S
AC	8.41E-06	1	8.41E-06	0.01818	2.48	0.899	NS
BC	0.01859	1	0.018588	40.1636	2.48	0.00317	S
Residual	0.00185	4	0.000463				
Lack of Fit	0.00183	3	0.000611	36.3333	2.48	0.121	
Pure Error	1.68E-05	1	1.68E-05				
Corr. total	0.112	13					

DF = Degree of freedom; F = F ratio; S = Significant; NS = Not significant

Table 4. Results of the quadratic effect of Box-Behnken Design

Source	Sum of squares	DF	Mean square	F value	F-stat with P=0.05	Prob > F	Note
Model	0.10980	8	0.0137254	36.9025	2.48	0.000499	S
A	0.00168	1	0.0016830	4.52489	2.48	0.0867	S
B	0.01227	1	0.0122689	32.9864	2.48	0.00224	S
C	0.00136	1	0.0013632	3.66516	2.48	0.114	NS
A^2	0.00404	1	0.0040408	10.8643	2.48	0.0216	S
B^2	0.00089	1	0.0008903	2.39367	2.48	0.183	NS
C^2	0.00049	1	0.0004863	1.30769	2.48	0.305	NS
AB	0.06968	1	0.0696835	187.353	2.48	<0.0001	S
BC	0.01859	1	0.0185884	49.9774	2.48	0.000876	S
Residual	0.00186	5	0.0003719				
Lack of Fit	0.00184	4	0.0004607	27.375	2.48	0.142	
Pure Error	1.68E-05	1	1.683E-05				

DF = Degree of freedom; F = F ratio; S = Significant; NS = Not significant

3.3. Variance Analysis

Analysis of variance was used to evaluate the accuracy and significance of the models were obtained. The goodness of fit of the model can be checked by several criteria. Table 5 shows the coefficient of R^2 = 0.98, this indicates that only 2% of total variation not explained by the model. To test the adequacy of the fitted model using static F. The value of F is compared to the Table value F(p-1,N-p,α), which is the upper 100 α percent point of the F distribution with p-1 and N-p degrees of freedom, respectively. Since the value of F in linear and quadratic model are 26.36 and 36.90 respectively, exceed the Table value of F = 2.48 (Table 6), this indicates that by the Fisher F test also demonstrates a high significant for the fitted regressions model.

The value of F quadratic effect is higher than linear effect, but the value of Prob>F is smaller (Prob>F value of linear and quadratic effect are 0.00327 to 0.000499 respectively), this indicated that quadratic effect model is more accurate than linear effect model. Each of the observed values (Ea)o is compared with predicted value of (Ea)p calculated from the Equation (2) can be seen in shown in Figure 1. From that figure, the observed enzyme activity is directly proportional to the predicted enzyme activity. The residual value obtained is 0.0000168 and the significant level (α) is 95%, means the value "Prob> F" below 0.05.

3.4. Optimization by Analyzing the Response Surface

3.4.1. Effect of Urea and pH to the Enzyme Activity

It is important to investigate about urea and pH effect during fermentation process. For doing that, the urea concentration and pH were studied in the range 1.5-4.5% w/w and 3.5-5.5. From the analysis of the response surface shown in Figure 2a, it can be seen that the enzyme activity increase with increasing of urea concentration and will decrease with decreasing of pH. Optimal condition was achieved at urea concentration of 4.50% w/w, pH of 3.50 and $MgCl_2$ concentration of 1.00 mM with activity of 0.630 unit/gram. While Figure 2b shows that the enzyme activity increase with increasing of urea concentration and pH. Optimal condition was achieved at urea concentration of 3.00-3.75% w/w, pH of 5.00-5.50 and $MgCl_2$ concentration of 2.50 mM with activity was 0.420 U/g. Figure 2c shows that the enzyme activity decrease with increasing of pH and increase with decreasing of urea concentration. Optimal condition was achieved at urea concentration of 1.50 w/w, pH of 5.50 and $MgCl_2$ concentration of 4.00 mM with activity of 0.548 U/g.

3.4.2. Effect of $MgCl_2$ and pH to the Enzyme Activity

Beside correlation between urea concentration and pH, correlation between $MgCl_2$ concentration and pH also were studied in the range of 1.00-4.00 mM and 3.5-5.5. From the analysis of the response surface shown in Figure 3a, it can be seen that the enzyme activity increase with increasing of $MgCl_2$ concentration and will decrease with increasing of pH. Optimal condition was achieved when $MgCl_2$ concentration was 4.00 mM, pH 5.5, and urea concentration was 1.50% w/w, with the maximum activity is 0.549 U/g. While Figure 3b shows that the enzyme activity increase with decreasing of $MgCl_2$ concentration and pH. Optimal

Table 5. Parameter statistic for two models

Parameter	Linear	Quadratic
Std. Dev.	0.02151	0.01929
Mean	0.40280	0.40280
C.V.	5.34088	4.78788
PRESS	0.02942	0.02208
R-Squared	0.98342	0.98335
Adj. R-Squared	0.94612	0.95670
Pred. R-Squared	0.73654	0.80226
Adeq. Precision	18.82629	22.13674

C.V. = the coefficient of variation

Table 6. Analysis of variance activity values

Source	SS	DF	MS	F	F (005)	Prob > F
Linear	0.1098	9	0.0122	26.36	2.48	0.00327
Quadratic	0.1098	8	0.0137	36.90	2.48	0.000499

SS = sum of squares; DF = Degree of freedom; MS = Mean squares; F = F ratio

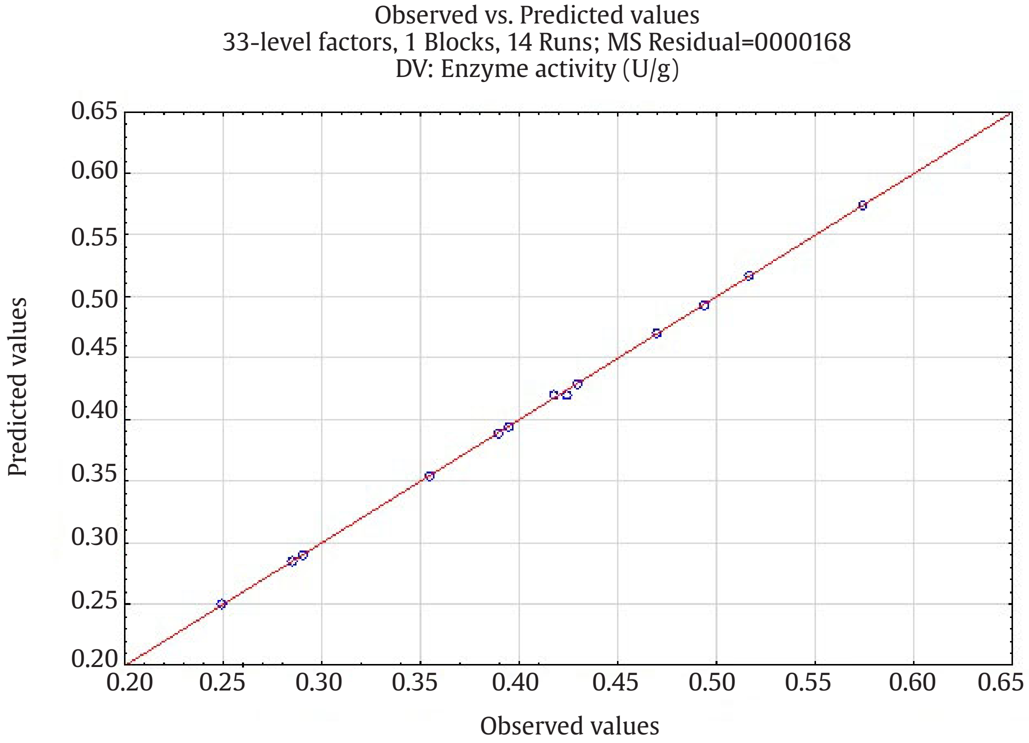

Figure 1. Correlation between observed and predicted value of cellulase enzyme activity

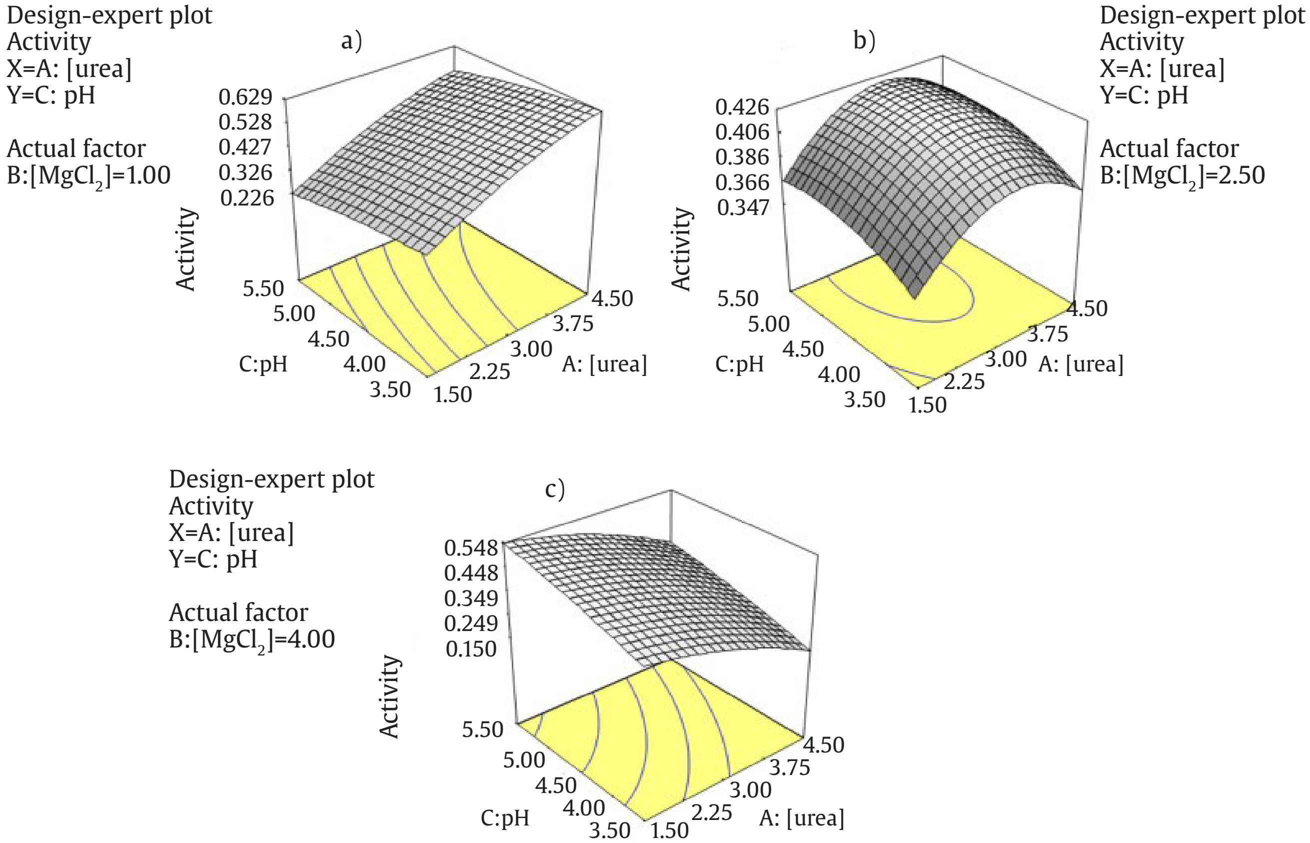

Figure 2. Predicted response as a function of urea concentration and pH at concentration of $MgCl_2$ is a) 1.00 mM, b) 2.50 mM, and c) 4.00 mM

condition was achieved at $MgCl_2$ concentration of 1.00 mM, pH of 3.50, and urea concentration was 3.00% w/w with enzyme activity was 0.515 U/g. Figure 3c shows that the enzyme activity stagnant with increasing of pH and increase with decreasing of $MgCl_2$ concentration. Optimal condition was achieved at $MgCl_2$ concentration of 1.00 mM, pH was between 3.50 - 5.50 and urea concentration of 4.00% w/w with activity of 0.569 U/g.

3.4.3. Effect of $MgCl_2$ and pH to the Enzyme Activity

It is also important to know the correlation between $MgCl_2$ and urea concentration. For that, The $MgCl_2$ and urea concentration were observed in the range of 1.00-4.00 mM and 1.5-4.5% w/w. From the analysis of the response surface in Figure 4a, it can be seen that the enzyme activity increase with increasing of $MgCl_2$ and urea concentration, but by using maximum $MgCl_2$ and urea concentration (4.00 mM and 4.50 w/w respectively), the activity will decrease up to 0.350 U/g. Optimal condition was achieved when $MgCl_2$ concentration was 1.00 mM, pH 3.5, and urea concentration was 4.50 w/w, with the maximum activity is 0.630 U/g. While similar phenomena also were found in Figure 3b, which shows that the enzyme activity increase with increasing of $MgCl_2$ and urea concentration. Similar with Figure 4a, activity will decrease up to 0.410 U/g if fermentation system used maximum $MgCl_2$ and urea concentration together with. Optimal condition was achieved at $MgCl_2$ concentration of 1.00 mM, pH of 4.50, while urea concentration was 4.50% w/w with enzyme activity was 0.587 U/g. Figure 4c shows similar phenomena with previous figure (Figure 4a and b) which enzyme activity increase with increasing $MgCl_2$ and urea concentration. Optimal condition was achieved at $MgCl_2$ concentration of 4.00 mM, pH was between 5.50 and urea concentration of 1.50% w/w with activity of 0.548 U/g. The enzyme activity will decrease up to 0.350 if 4.50 w/w urea and 4.00 mM $MgCl_2$ were used.

3.4.4. Interaction Between Factors

It is important to know the interaction between all parameter which affect to fermentation process. For doing that, interaction graph between pH, urea concentration, and $MgCl_2$ concentration were made. The urea factor and the pH factor do not interact each other. It can be shown by the two-factor interaction statistical curve as shown in Figure 5a. It is also shown by the very small value of urea-pH coefficient, and F value which almost reach insignificant value as shown Table 3 in previous section. This is also supported by the RSM SmF study on fungi by Mohan *et al.* (2013) that showed no interaction between pH

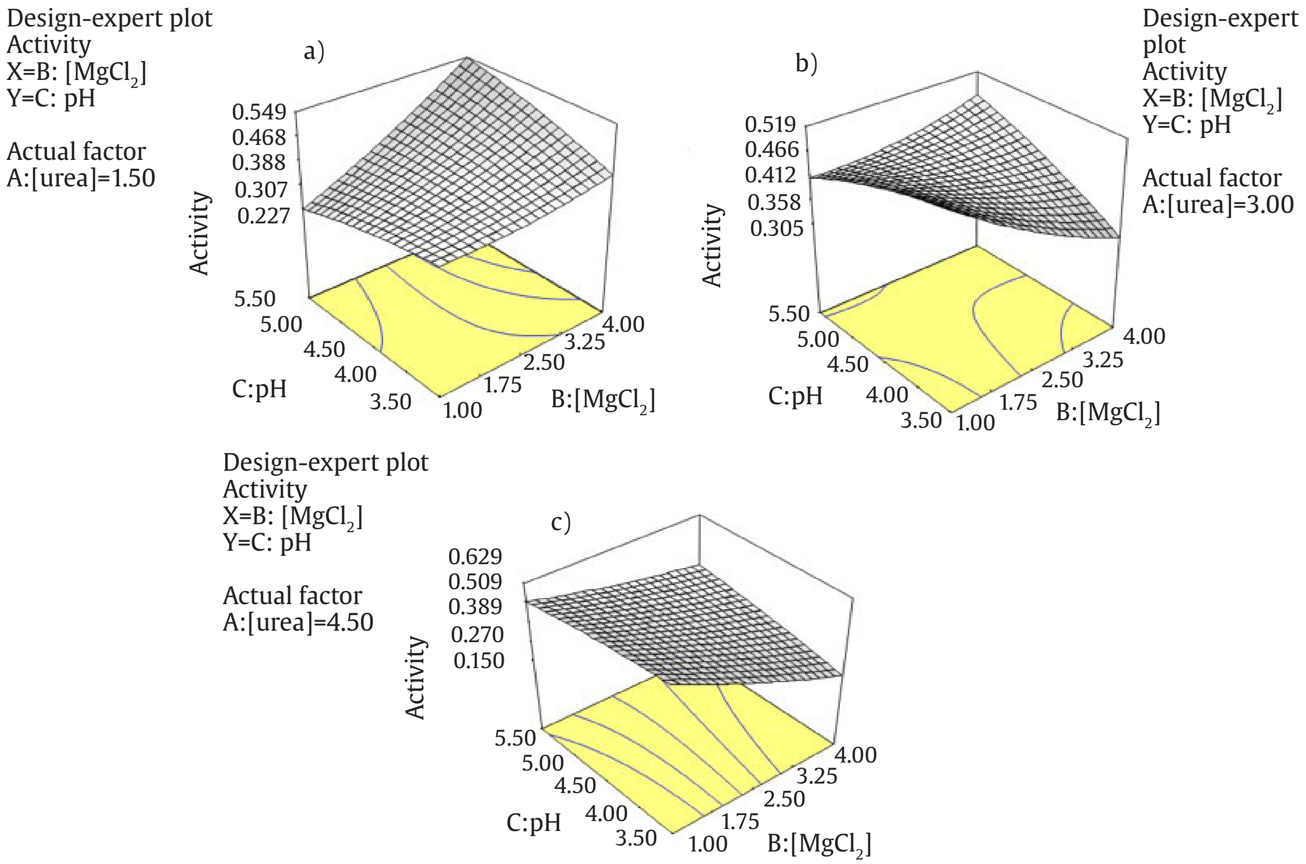

Figure 3. Predicted response as a function of $MgCl_2$ concentration and pH concentration at Urea concentration is a) 1.50% w/w, b) 3.50% w/w, and c) 4.00% w/w

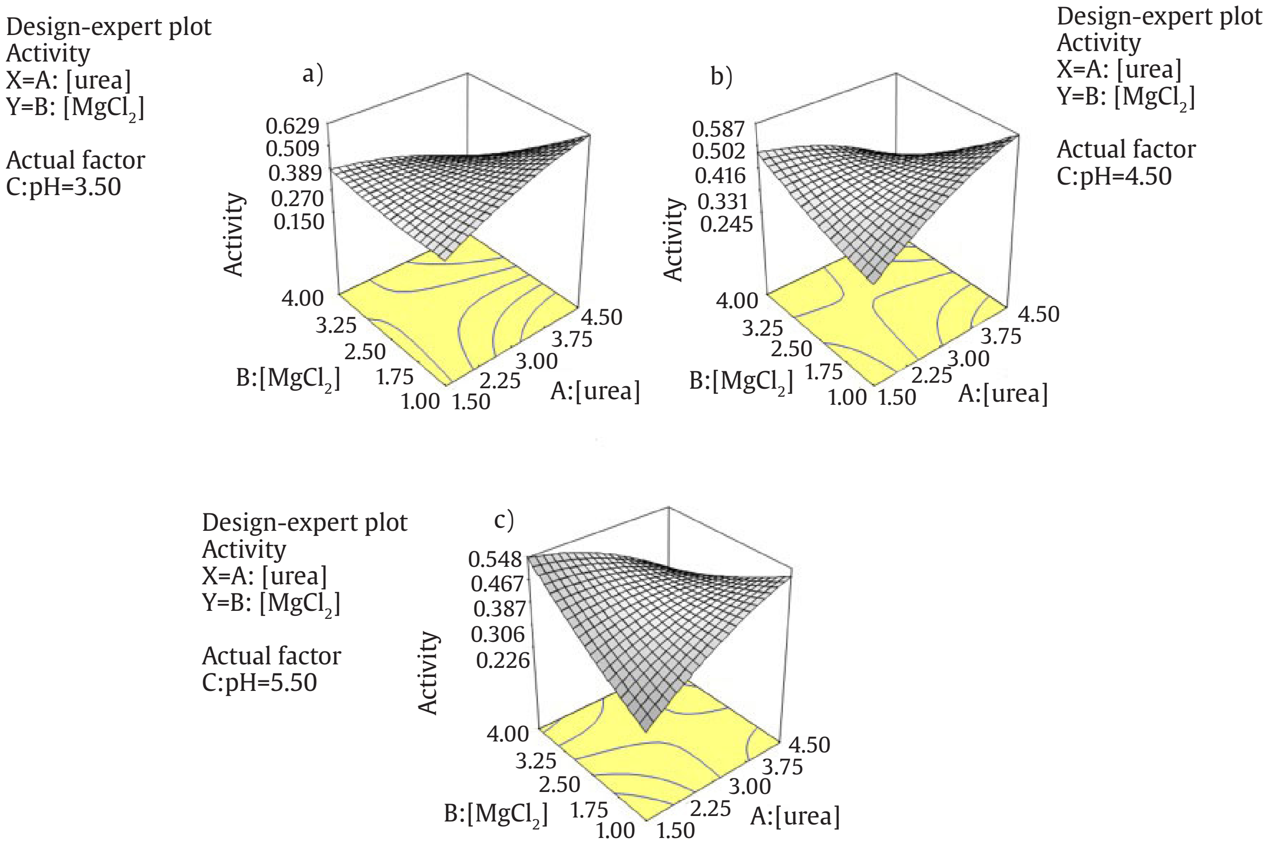

Figure 4. Predicted response as a function of urea and $MgCl_2$ concentration at pH a) 3.50, b) 4.50, and c) 5.50

and urea. Figure 5b shows about interaction between $MgCl_2$ factor and pH factor with an equilibrium point between two curves where the $MgCl_2$ concentration is 2.25 mM with pH between 3.50–5.50. High value of $MgCl_2$-pH coefficient and F value which reached significant value as shown Table 3 and 4 are another evidence strong relationship between pH and $MgCl_2$ concentration. This phenomena about interaction between magnesium and pH is compatible with studies have been performed by Myers and Campbell (1985). Interaction between urea factor and $MgCl_2$ factor can be seen in Figure 5c where there is an equilibrium point between two curves with the urea concentration is around 2.50% w/w and $MgCl_2$ concentration around 2.50. $MgCl_2$-urea coefficient and F value are very high, higher than $MgCl_2$-pH value, which reached significant value.

3.5. Enzyme Activity Under Optimal Condition

The activity model equation as a function of three factors: urea, $MgCl_2$ and pH was used to determine the optimum point of the SF process. From the equation (2), the optimal process condition was obtained with numerical method to produce cellulase enzyme using *A. niger* ITBCC L74. The result of the experiment was observed as bell-saddle shape or a turning optimum point (Figure 2). The optimum value of cellulase activity was achieved at urea concentration of 4.5% w/w, $MgCl_2$ concentration of 1mM and pH of 3.50, with maximum activity was 0.630 U/g.

It is important to compare enzyme activity between model and result from real fermentation under optimum condition. The comparison of results between model prediction and real experimental tests under optimum conditions is given in Table 7.

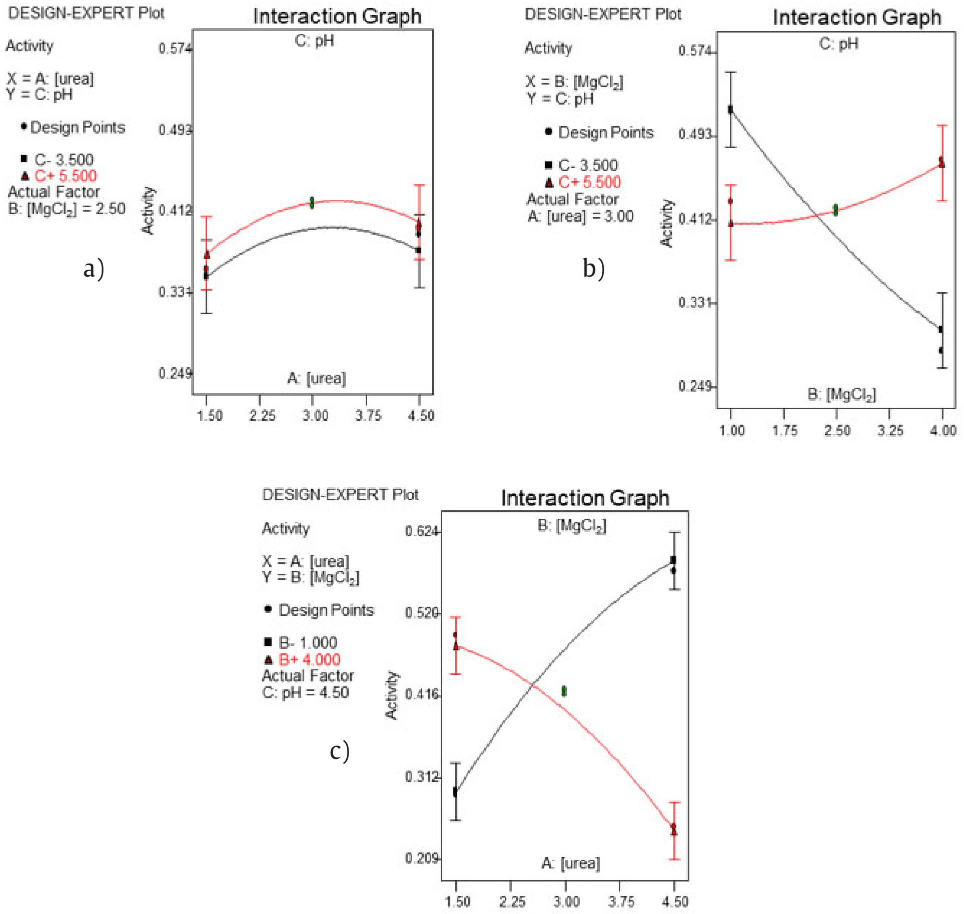

Figure 5. Interaction between factors: a) pH and urea concentration, b) pH and $MgCl_2$ concentration, and c) urea and $MgCl_2$ concentrations

Table 7. Enzyme activity comparison between model and real experiment under optimum condition

Water (% w/w)	Urea (% w/w)	$MgCl_2$ (mM)	pH	Activity (experiment) (unit/g)	Activity (predicted) (unit/g)	Error (%)
80	4.50	1.00	3.5	0.511	0.630	18.95
80	4.50	1.00	5.3	0.414	0.535	22.63
80	1.50	4.00	4.5	0.318	0.480	33.80

4. Discussion

In this experiment, relative high lignin content due to the reaction between bagasse and NaOH in pretreatment, where pretreatment process is expected will affect the lignin structure by changing its porosity (Pihlajaniemi *et al.* 2016). According to Galetti and Antonetti (2011), the use of alkali in lignocelluloses pretreatment can affect to degradation of ester and glycosidic chain which resulted in the degradation of lignin structure, cellulose swelling, partial hemicellulose solvation, decreased degree of cellulose crystallinity, increased internal surface area, lignin structure destruction, and bond separation structure between carbohydrates and lignin. The content of cellulose, hemicellulose, and lignin after pretreatment in this study is comparable with the experiment conducted by Rezende *et al.* (2011) which have done the pretreatment at 120°C. Although the content of lignin in this study is relatively high, but the content of cellulose in the treated bagasse in this study still has the potential to be utilized as a substrate for the production of cellulase enzyme in solid state fermentation.

Nitrogen is one of the nutrients needed for microbial growth, such as *A. niger* (Karray *et al.* 2016). In the process of fermentation, nitrogen obtained from urea is decomposed by *A. niger* for growth process. The increasing of urea concentration makes the growth of *A. niger* also increase (Jasani *et al.* 2016), but in certain or excesses concentrations, urea can affect to decreasing in enzyme activity due to an imbalance of composition in nutrients. This imbalance of the composition may cause the metabolism of *A. niger* became disturbed and affected to its growth rate.

The interaction between magnesium and pH levels in this study has a surface response similar to surface response which have been done by Myers and Campbells (1985). There are some noticeable things: the increasing pH (above 5) and the increasing concentration of $MgCl_2$ result in decrease of enzyme activity, whereas the decreasing $MgCl_2$ concentration and decreasing of pH (below 5) cause enzyme activity increase up to a certain point. Similar with urea, magnesium is needed for *A. niger* growth and cellulase production, but it is also inhibitory at high concentrations (Mandels and Reese 1999) The same phenomenon is also shown by RSM research which have been conducted by Mohan *et al.* (2013).

On the other side, interaction between urea and $MgCl_2$ is very unique. Each component can affect to increasing in enzyme activity, but on the contrary, increasing both components (urea and $MgCl_2$) makes the decreasing in activity decrease. This shows that urea and $MgCl_2$ have the same role, equally affecting the increase of activity, but raising the both concentration to the maximum level will disturb the metabolism of *A. niger* then the activity decreases. Lowering and raising one component (urea or $MgCl_2$ concentration) becomes the best alternative way to result optimal enzyme activity.

Enzyme activity on the model is slightly higher than real experiment. Differences in enzyme activity are due to fermentation temperature that can affect growth of *A. niger* and result in decreased enzyme activity (Tucker *et al.* 2003; Acharya *et al.* 2008; Sohail *et al.* 2009). According to Raghuwanshi *et al.* (2014), 25°C is the best temperature in fermentation process. The difference in 5°C of temperature made the logarithmic phase of *A. niger* was low then may cause the lowering enzyme activity result.

Bagasse is one of good substrate for the production of cellulase under solid state fermentation by *A. niger* ITBCC L74. Statistical analysis demonstrated the useful way to develop optimum fermentation condition. Box-Behnkem design exhibited that urea concentration of 4.50% w/w, $MgCl_2$ concentration of 1 mM, at pH 3.50 are the best condition for cellulase production with *A. niger* ITBCC L74 with enzyme activity is 0.630 U/g. The error resulted enzyme activity between model and real experiment is 18.95%. There is strong interaction between $MgCl_2$ and pH factor as well as $MgCl_2$ and urea factor, but not with urea and pH factor.

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