ORIGINAL ARTICLE

Improvement of halophilic cellulase production from locally isolated fungal strain

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Abstract Halophilic cellulases from the newly isolated fungus, *Aspergillus terreus* UniMAP AA-6 were found to be useful for *in situ* saccharification of ionic liquids treated lignocelluloses. Efforts have been taken to improve the enzyme production through statistical optimization approach namely Plackett–Burman design and the Face Centered Central Composite Design (FCCCD). Plackett–Burman experimental design was used to screen the medium components and process conditions. It was found that carboxymethylcellulose (CMC), $FeSO_4 \cdot 7H_2O$, NaCl, $MgSO_4 \cdot 7H_2O$, peptone, agitation speed and inoculum size significantly influence the production of halophilic cellulase. On the other hand, KH_2PO_4, KOH, yeast extract and temperature had a negative effect on enzyme production. Further optimization through FCCCD revealed that the optimization approach improved halophilic cellulase production from 0.029 U/ml to 0.0625 U/ml, which was approximately 2.2-times greater than before optimization.

1. Introduction

Lignocellulose is one of the most abundant bio-resources available on the surface of the earth (Wei et al., 2009). It could be hydrolyzed into simple sugar to be used by appropriate microbes for the production of various fermented products including bioethanol, biobutanol, enzymes, organic acids etc. The hydrolytic enzyme is commonly used for the conversion of lignocellulose into simple sugars belonging to the cellulase group. The use of cellulases requires a pretreatment step to disrupt the lignin and waxy materials which protect the cellulose within the lignocellulose structure. This pretreatment process renders cellulose more accessible to the enzyme to complete the lignocellulose hydrolysis process. Thus, the efficiency of overall enzymatic hydrolysis is affected by the type of media and conditions employed during the pretreatment process. In other words, effective conversion of lignocelluloses into simple sugar requires enzymes which are compatible with

the pretreatment step (de Diego et al., 2014). In the pretreatment process, Ionic liquids (ILs) are gaining interest as green and effective solvents for the pretreatment of lignocellulose biomass (Gunny and Arbain, 2013; Wang et al., 2012; Mäki-Arvela et al., 2010). However, since ILs are salt in the liquid form, they might not be compatible with the enzyme in the subsequent hydrolysis (saccharification) process. Hence, it is necessary to produce cellulase enzymes which are tolerant to the presence of salt. These kind of enzymes, generally known as halophilic enzymes are normally produced by halophiles. Previously, it was shown that halophilic cellulases are produced by the newly isolated halophiles, *Aspergillus terreus* Uni-MAP AA-6 has a great potential application for the *in situ* enzymatic saccharification of ILs pretreated lignocelluloses (Gunny et al., 2014). In *in situ* enzymatic saccharification, the pretreatment and saccharification steps can be performed in the same vessel, skipping the expensive washing steps (in between the two steps) (Gunny et al., 2014; Engel et al., 2010; Kamiya et al., 2008). From an industrial perspective, this *in situ* process would prove cost-effective. Thus, it is important to improve the efficacy of halophilic cellulase production by the new isolated strain.

The optimization of medium and process condition is suggested as one approach to improve cellulase production (Sukumaran et al., 2005) and it is regarded as one of the most important steps in the development of cost-effective fermentation processes (Vaidya et al., 2009). Hence, it is important to consider the optimization of fermentation medium and process condition in order to improve both production efficiency and profits.

Response surface methodology (RSM) was found to be an effective tool in medium and process optimization for the industrial productions of enzymes. Accordingly, the screening of significant parameters prior to optimization is important so as to reduce the number of experiments and neglect the less important ones. In the present study, Plackett–Burman experimental design was used to screen the significant medium and process conditions for the production of halophilic cellulases by *A. terreus* UniMAP AA-6. This was followed by the optimization of halophilic cellulases by *A. terreus* UniMAP AA-6 using Face Centered Central Composite Design (FCCCD) under RSM.

2. Materials and methods

2.1. Microorganism

A. terreus UniMAP AA-6 is a newly isolated and identified strain (the GenBank accession number is KF364668), maintained at the culture collection of the School of Bioprocess Engineering, University Malaysia Perlis. The culture is maintained on Malt Extract Agar (MEA) enriched with NaCl at 4 °C.

2.2. Growth media and conditions

All growth experiments were carried out in 250-ml Erlenmeyer baffled flasks with a 100-ml working volume.

The culture medium compositions consist of: KH_2PO_4, 1.36% (w/v); KOH, 0.42% (w/v); Yeast extract, 0.198% (w/v); $MgSO_4 \cdot 7H_2O$, 0.025% (w/v); $FeSO_4 \cdot 7H_2O$, 0.00017%

(w/v); NaCl, 3.0% (w/v); Peptone, 0.198% (w/v) and carboxymethylcellulose (CMC), 0.5% (w/v). The inoculum of 1–3% (1.0×10^7 spores/ml) was incubated in a rotary shaker operating at 50–150 rpm and 30–50 °C for 96 h. A crude enzyme was prepared by removing the cell by centrifugation at 10,000 rpm for 10 min at 4 °C. The harvested supernatant was assayed for cellulase activity.

2.3. Assay of cellulase activity

The total cellulase activity were determined by filter paper assay (FPase) using Whatman No. 1 filter paper strip with a dimension of 1.0×6.0 cm equivalent to 50 mg as a substrate according to standard International Union of Pure and Applied Chemistry (IUPAC) procedures recommended by Ghose (1987).

2.4. Production profile

The production profile of the halophilic cellulases was determined based on the measurement of total cell dry weight and halophilic cellulase production after every 12 h.

2.5. Screening of significant medium and process conditions using Plackett–Burman design

Plackett–Burman design from the statistical software package Design-Expert (version 6.0.8, Stat-Ease, Minneapolis, USA) was used to determine the necessary nutrient and suitable process conditions for the production of the halophilic cellulases from the newly isolated halophilic strain. A total of eleven (11) variables consisting of chemical factors (KH_2PO_4, KOH, yeast extract, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, NaCl, peptone and CMC), physical factors (growth temperature and agitation speed) and biological factors (inoculum size) were studied in 12 experiments (Table 1).

Each variable was evaluated at two levels, high and low, denoted by (+) and (−) signs, respectively. The main effect of each variable factor was calculated as the difference between the average of the factor setting at the high level (+) and the average of factor setting at the low level (−). The design was based on the first-order model as represented by the following equation:

$$Y = \beta_0 + \sum \beta_i x_i \qquad (1)$$

where Y is the response (cellulase activity), β_0 is the model intercept, β is the linear coefficient and X_i is the level of the independent variable (Plackett and Burman, 1946).

2.6. Optimum levels of parameters determined by one-factor-at-a-time (OFAT) design

The influences of inoculum size and growth temperature selected from Plackett–Burman design were investigated with the one-factor- at-a-time (OFAT) method. The parameters were investigated for different levels at a time, while leaving other factors constant. This method is useful in determining the optimum level of the parameters on the production of halophilic cellulases which can be useful in further optimization studies. The inoculum concentrations were tested at 2.0%,

Table 1 Plackett–Burman design of 11 variables with coded value along with the observed results.

Run	A	B	C	D	E	F	G	H	J	K	L	Halophilic cellulase activity (U/ml)
1	(−)	(+)	(+)	(+)	(−)	(+)	(+)	(−)	(+)	(−)	(−)	0.006
2	(−)	(−)	(−)	(+)	(+)	(+)	(−)	(+)	(+)	(−)	(+)	0.058
3	(+)	(−)	(+)	(+)	(−)	(+)	(−)	(−)	(−)	(+)	(+)	0.009
4	(+)	(+)	(−)	(+)	(+)	(−)	(+)	(−)	(−)	(−)	(+)	0.004
5	(−)	(+)	(+)	(−)	(+)	(+)	(−)	(−)	(+)	(+)	(+)	0.001
6	(−)	(+)	(−)	(−)	(−)	(+)	(+)	(+)	(−)	(+)	(+)	0.042
7	(+)	(−)	(−)	(−)	(+)	(−)	(+)	(−)	(+)	(+)	(−)	0.008
8	(+)	(−)	(+)	(−)	(−)	(−)	(+)	(+)	(+)	(−)	(−)	0.003
9	(−)	(−)	(+)	(+)	(+)	(−)	(+)	(+)	(−)	(+)	(+)	0.051
10	(+)	(+)	(−)	(+)	(−)	(−)	(−)	(+)	(+)	(+)	(−)	0.001
11	(+)	(+)	(+)	(−)	(+)	(+)	(−)	(+)	(−)	(−)	(−)	0.014
12	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	0.002

Variables are listed in alphabetical order and their levels are given in (% w/v), (A) KH_2PO_4, (low level: 0.5%, high level: 2%), (B) KOH, (low level: 0%, high level: 0.5%), (C) Yeast extract, (low level: 0%, high level: 0.2%), (D) $MgSO_4 \cdot 7H_2O$, (low level: 0%, high level: 0.03%), (E) $FeSO_4 \cdot 7H_2O$, (low level: 0%, high level: 0.0003%), (F) NaCl, (low level: 2%, high level: 6%), (G) Peptone, (low level: 0%, high level: 0.3%) and (H) CMC, (low level: 0.2%, high level: 1%) and (J) Temperature, (low level: 30 °C, high level: 50 °C), (K) Agitation speed (low level: 50 rpm, high level: 150 rpm), (L) Inoculum size (low level: 1%, (v/v), high level: 3%, (v/v)).

3.0%, 4.0%, 5.0% and 6.0% (w/v); at 30 °C; and the growth temperature was varied from 30 °C to 50 °C at the optimum inoculum size concentration.

2.7. Optimization process and medium condition using Face Centered Central Composite Design (FCCCD)

The FCCCD under the RSM (Box and Wilson, 1951) was generated by using the statistical analysis package Design-Expert Software (Stat-Ease Inc., Statistic made easy, Minneapolis, MN, USA, version 6.0.8) and was applied to determine the optimum level of each selected variable along with the interactions within the variables and their effect on response. The second-order empirical model was developed from the experimental data about the relationship between the response value and the variables through polynomial regression analysis. The form of the second-order polynomial model is:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_\circ X_i^2 \beta_2 + \sum \beta_{ij} x_i x_j \quad (2)$$

where Y is the predicted response (enzyme activity U/ml), β are the coefficients of the equation, and x_i and x_j are the coded levels of variables i and j, respectively. The generated model was analyzed using the values of regression coefficient, ANOVA (analysis of variance), p- and F-values.

3. Results and discussion

3.1. Halophilic cellulase production profile

Fig. 1 shows that an optimum growth time of 96 h is required for maximal halophilic cellulase production (0.029 U/ml). After 96 h, the enzyme production decreased gradually, most probably due to the depletion of nutrients in the medium or secretion of toxic substances that inhibit the production of
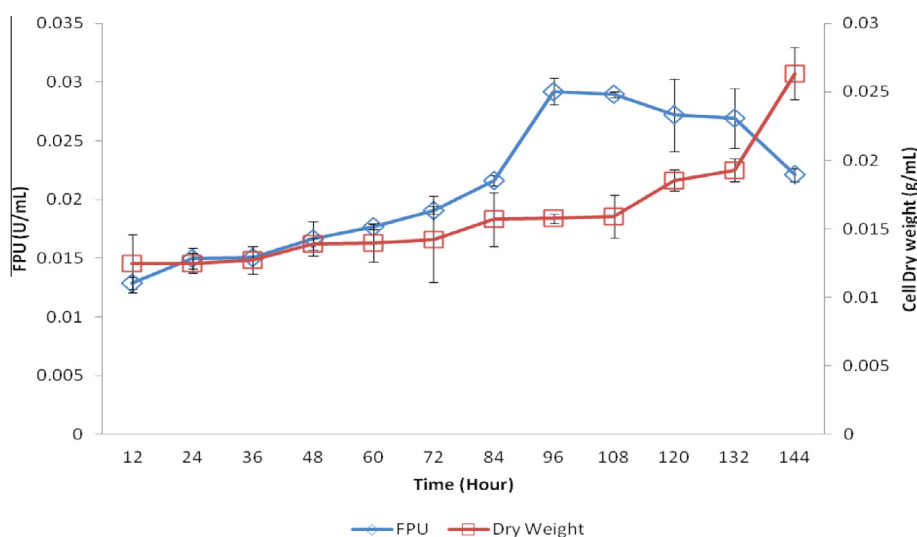

Figure 1 Production profile of halophilic cellulases by *Aspergillus terreus* UniMAP AA-6.
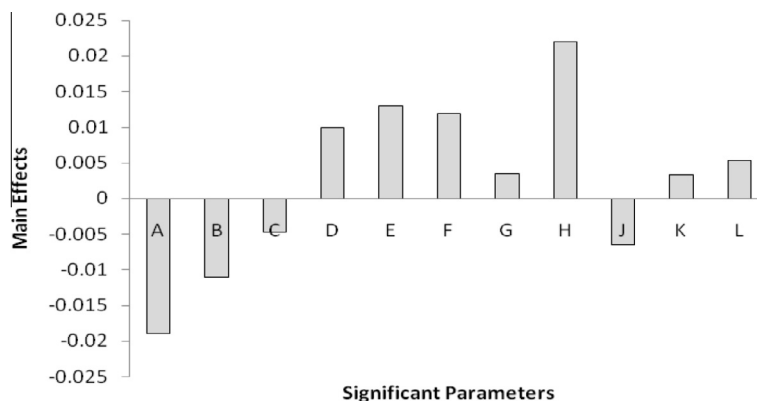
Figure 2 Main effects of medium and process conditions on the Plackett–Burman design experiment result.

enzymes by the microorganism (Stanbury et al., 1997). Alternatively, it could be due to degradation by protease in the medium (Sharada et al., 2013).

3.2. Screening of significant medium and process conditions using Plackett–Burman design

The main effect for each parameter was estimated and is shown in Fig. 2. The result revealed that CMC had the most positive effects on cellulase activity from *A. terreus* UniMAP AA-6 followed by $FeSO_4 \cdot 7H_2O$, NaCl, $MgSO_4 \cdot 7H_2O$, peptone, agitation speed and inoculum size. Here, the positive effect demonstrates the necessity of maintaining the parameters at a higher level to produce a higher cellulase activity in further optimization studies. It was reported that CMC as a

substrate induced the production of cellulases by activating cellulose regulatory protein called the cellulase activator molecule (CAM) (Shiang et al., 1991). NaCl had a positive effect indicating the characteristic of the halophilic fungus to grow and remain active in saline conditions. Similar observations were reported by Shivanand et al., 2012 where they found that their isolated halophilic bacteria strain reached the optimum growth at 6% (w/v) NaCl concentration for the production of cellulases. $FeSO_4 \cdot 7H_2O$ and $MgSO_4 \cdot 7H_2O$ had a positive effect on the production of halophilic cellulases due to the ions acting as an inducer for the production of cellulases. The positive influence of iron on the degradation of cellulose to produce simple sugar was reported by Xu and Goodell (2001). Subsequently the simple sugar could be utilized by the fungus to produce cellulases. The findings are in line with Rashid et al.
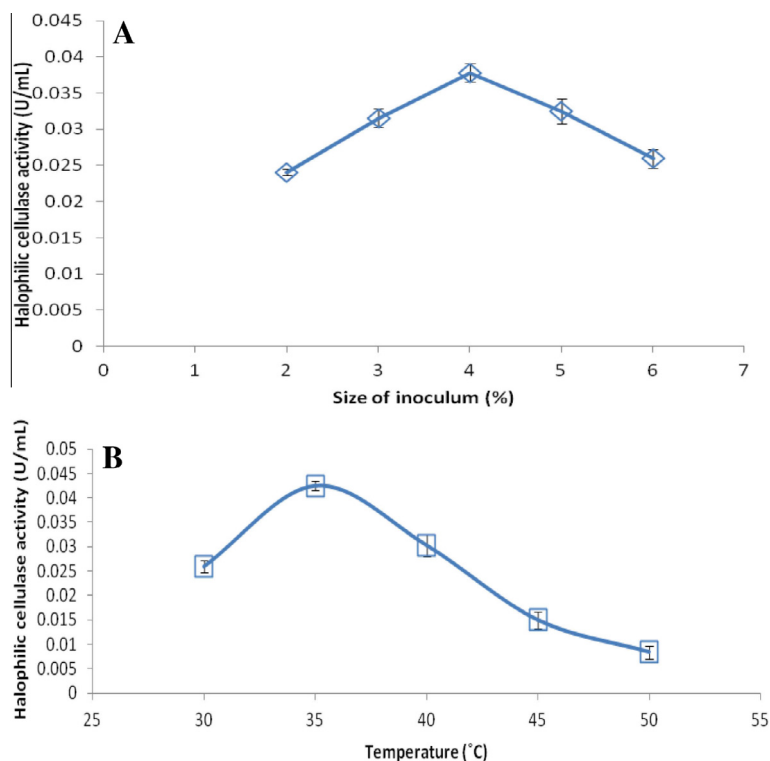

Figure 3 Effect of (A) inoculum size and (B) temperature on halophilic cellulase production by *Aspergillus terreus* UniMAP AA-6.

Table 2 Experimental design using FCCCD showing actual values along with the experimental data.

Standard run	Parameter			Response	
	NaCl	CMC	FeSO$_4$·7H$_2$O	Halophilic cellulase activity	
Unit	(% w/v)	(% w/v)	(C × 10^{-4}%, w/v)	(U/ml)	
Label	(A)	(B)	(C)	Experimental	Predicted
1	2.00	0.50	1.00	0.005	0.007
2	10.00	0.50	1.00	0.016	0.015
3	2.00	1.50	1.00	0.038	0.040
4	10.00	1.50	1.00	0.062	0.062
5	2.00	0.50	5.00	0.020	0.019
6	10.00	0.50	5.00	0.014	0.011
7	2.00	1.50	5.00	0.042	0.043
8	10.00	1.50	5.00	0.052	0.049
9	2.00	1.00	3.00	0.039	0.036
10	10.00	1.00	3.00	0.036	0.043
11	6.00	0.50	3.00	0.022	0.025
12	6.00	1.50	3.00	0.061	0.061
13	6.00	1.00	1.00	0.046	0.043
14	6.00	1.00	5.00	0.036	0.042
15	6.00	1.00	3.00	0.051	0.047
16	6.00	1.00	3.00	0.048	0.047
17	6.00	1.00	3.00	0.046	0.047
18	6.00	1.00	3.00	0.046	0.047
19	6.00	1.00	3.00	0.050	0.047

Table 3 ANOVA for FCCCD.

Source	Sum of squares	F-value	p-Value
Model	4.486×10^{-3}	26.54	< 0.0001
NaCl (A)	1.273×10^{-4}	6.78	0.0286
CMC (B)	3.171×10^{-3}	168.79	< 0.0001
FeSO$_4$·7H$_2$O (C)	7.618×10^{-7}	0.041	0.8449
A^2	1.648×10^{-4}	8.77	0.0159
B^2	4.405×10^{-5}	2.35	0.1600
C^2	5.497×10^{-5}	2.93	0.1213
AB	1.083×10^{-4}	5.76	0.0398
AC	1.187×10^{-4}	6.32	0.0331
BC	4.366×10^{-5}	2.32	0.1617
Residual	1.691×10^{-4}	1.878×10^{-5}	
Lack of fit	1.483×10^{-4}	2.965×10^{-5}	0.0583
Pure error	2.080×10^{-5}	5.70	
Total	4.655×10^{-3}		

(2009) who also reported that MgSO$_4$·7H$_2$O had positive effects on fungal cellulase production. In addition, agitation speed and inoculum size had a positive effect on enzyme production. As a result, maintaining these two parameters at a higher level increased the production of halophilic cellulases.

On the other hand, the following parameters namely: KH$_2$PO$_4$, KOH, yeast extract and temperature had a negative effect on enzyme production indicating that high cellulases production could still be achieved even at the low level of the parameters. In the case of phosphate, it might be related to the fact that phosphate is a major component of microbial cell walls. Higher phosphate concentrations in a fermentation medium promote larger pellet formation causing poor mass transfer and thus decreasing enzyme production El-Enshasy (2007). Therefore, maintaining phosphate concentrations at a low level could reduce production costs. The results also showed

the absence of KOH and yeast extract improved cellulase production. Therefore, these two medium components were excluded in further optimization study. Apart from the medium components, incubation temperature also had a negative effect on enzyme production. This result is in agreement with Shivanand et al. (2012) who found that maintaining growth temperature at a low level could improve the production of halophilic cellulase. Among the 11 parameters studied in the Plackett–Burman design, three parameters namely CMC, FeSO$_4$·7H$_2$O and NaCl, had the most significant and positive effect on enzyme production. These three parameters were further optimized together with temperature and inoculum concentration using FCCCD. The center points for temperature and inoculum size were determined by the OFAT method prior to FCCCD experiments.

3.3. Optimum levels of parameters determined by one-factor-at-a-time (OFAT) method

In this study, the OFAT method was used to examine the possible optimum level of growth temperature and inoculum size for the production of halophilic cellulases from the isolated fungus. Different levels of inoculum with the concentrations of 1.0×10^7 spores/ml from 2.0 to 6.0% (v/v) were tested to determine the level of inoculum for the maximum production of halophilic cellulases. The highest cellulase activity was observed at 4% (v/v), while the lowest activity was observed at 2% (v/v). Fig. 3A shows halophilic cellulase production was increased as the inoculum size increased up to 4.0% (v/v). However, it started to decrease gradually when the concentration was increased from 4.0% (v/v) to 6.0% (v/v) thus indicating that the optimum inoculum size was at 4.0% (v/v).

Finally, the effect of growth temperature on halophilic cellulase production was examined at the optimum inoculum size.
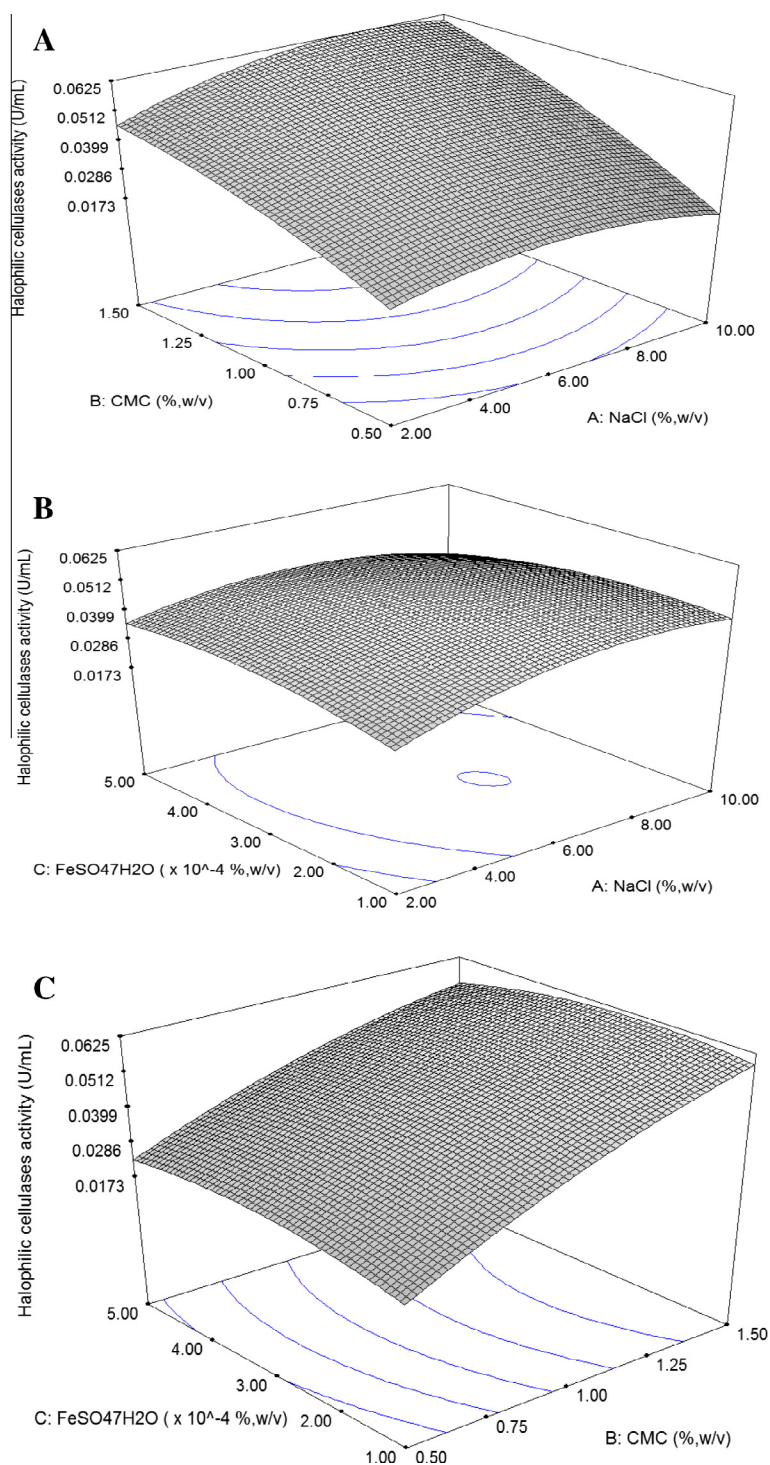
Figure 4 3D surface plot showing the effect of the interaction between; (A) CMC and NaCl; (B) FeSO$_4$ 7H$_2$O and NaCl; (C) FeSO$_4$·7H$_2$O and CMC on the production of halophilic cellulases.

The result in Fig. 3B shows that *A. terreus* UniMAP AA-6 produced the highest halophilic cellulases at 35 °C growth temperature. Thus, from the OFAT results, the optimum level of growth temperature was 35 °C and an inoculum size of 4% (v/v) were selected as fixed parameter values for the following FCCCD studies.

3.4. Optimization of process and medium condition using FCCCD

To find out the optimum concentration of the most effective variables (NaCl, CMC and FeSO$_4$·7H$_2$O), identified by the Plackett–Burman design and to study their interactions,

Table 4 Model validation.

Run	NaCl	CMC	FeSO$_4$·7H$_2$O	Halophilic cellulases activity		
Unit	(% w/v)	(% w/v)	(C × 10^{-4}%, w/v)	U/ml		
Label	A	B	C	Predicted	Observed	Error,%
1	7.33	1.50	2.61	0.0629	0.0594	5.56
2	7.77	1.46	2.27	0.0626	0.0606	3.19
3	7.70	1.48	2.43	0.0629	0.0625	0.64

RSM using the FCCCD was applied. Each variable in the design was studied at three different levels (Table 2). Other fixed parameters in the studies were determined based on the Plackett–Burman design and the OFAT results. KH$_2$PO$_4$ was set at a low level while MgSO$_4$·7H$_2$O, peptone and agitation speed were set at a higher level. The growth temperature and inoculum size were set based on the optimum level obtained from the OFAT results.

Table 2 shows the results of halophilic cellulase production for the experimental data and predicted data of the each parameter. The optimal values for the production of halophilic cellulases were predicted by fitting a polynomial model to the experimental results using the Design Expert software as follows:

$$Y = +0.047 + 3.568E - 003A + 0.018B - 2.760E$$
$$- 004C - 7.765E - 003A^2 - 4.015E - 003B^2$$
$$- 4.485E - 003C^2 + 3.679E - 003AB - 3.851E$$
$$- 003AC - 2.336E - 003BC \qquad (3)$$

where, halophilic cellulase production as a Respond (Y) is a function of NaCl (A), CMC (B) and FeSO$_4$·7H$_2$O (C). The highest values of halophilic cellulase production for both experimental and predicted were attained at run 4 which had the conditions: 10% (w/v) NaCl, 1.5% (w/v) CMC and 1.00 × 10^{-4}% (w/v) FeSO$_4$·7H$_2$O, while the lowest cellulase activity for both the experimental and predicted run was found at run 1 when the concentrations of NaCl and CMC in the fermentation medium were reduced to 2% (w/v) and 0.5% (w/v) respectively. These results showed that the fungus prefer a high level of NaCl and CMC concentrations.

A summary of the variance analysis (ANOVA) is presented in Table 3. The fit of the model was checked by the coefficient of determination R^2, which was calculated to be 0.9637, indicating that data from each parameters fitted the polynomial model and also explains 96.37% of the variables (NaCl, CMC and FeSO$_4$·7H$_2$O) were supported by the response. Moreover, the value of adequate precision (signal to noise ratio) of 17.531 was very high compared to the desirable value (greater than 4), which indicates adequate signal and that this model can be used to navigate the design space. In addition, the p-values for the model (<0.0001) and the lack of fit is 0.0583 which demonstrates that the experimental data are good fit with the model.

The results reveal that the linear effect of NaCl (A) and CMC (B) and the quadratic terms of NaCl (A^2) and also the interactive terms between AB and AC were a significant at a level of $p < 0.05$. These significant variables indicate that they can act as limiting media components and small variations in

their concentrations will affect either the growth rate or the product formation rate (Imandi et al., 2008). The results indicate that the linearly-formed NaCl and CMC variables have the highest F-value and lowest p-value. This indicates that the fungus prefers a high level of salinity and substrate concentration in order to maximize halophilic cellulase production.

It is apparent from Fig. 4A and C that halophilic cellulases production increased as the level of CMC concentration increased. This might be due to the proposition that cellulose within CMC acts as an inducer for the production of cellulases (Sukumaran et al., 2005). However, in Fig. 4A and B, the increment of NaCl, increased the cellulase production, which reflects the necessity for high saline conditions to induce the performance of halophilic microorganisms (Delgado-Garćia et al., 2012; Mesbah and Wiegel, 2005). Nevertheless, the halophilic fungus can only tolerate up to 8.0% (w/v) NaCl. This may be due to the saturated saline conditions of the fermentation medium which inhibit enzyme production. It could also be suggested that the fungus required FeSO$_4$·7H$_2$O around 2.50 × 10^{-4}% (w/v) to reach the optimum production of the enzyme (Fig. 4B and C). However, no significant change was observed in enzyme activity within the range of 1.00 × 10^{-4}% (w/v) to 5.00 × 10^{-4}% (w/v) FeSO$_4$·7H$_2$O concentrations.

To verify the generated polynomial model, a set of experiments was performed according to conditions suggested by the software as presented in Table 4. A slight deviation between the predicted and the observed results might be due to the condition of the fermentation culture, and the nature of the microorganism behavior which would appear to be varied as compared to the fixed chemical reaction. From the optimization study, it was clearly observed that the production of halophilic cellulases of 0.0625 U/ml obtained in the optimum conditions was 2.2-times higher than what had been obtained before optimized conditions which were only 0.029 U/ml.

4. Conclusion

The present study showed that the production of halophilic cellulases by *A. terreus* UniMAP AA-6 doubled from 0.029 U/ml to 0.0625 U/ml using the statistical optimization approach. It was also observed that the screening of significant medium and process conditions reduced the number of parameters and experiments required for the production of the enzyme. As a result, this eventually minimized the use of chemicals, nutrients and other additives which resulted in a considerable reduction in operating costs. This can be translated into significant savings in terms of production costs on an industrial scale.

Acknowledgements

References

Box, G., Wilson, K., 1951. On the experimental attainment of optimum conditions. J. R. Stat. Soc. Ser. B 13, 1–45.

Delgado-Garćıa, M., Valdivia-Urdiales, B., Aguilar-Gonźalez, C.N., Contreras-Esquivel, J.C., Rodŕıguez-Herrera, R., 2012. Halophilic hydrolases as a new tool for the biotechnological industries. J. Sci. Food Agric. 92, 2575–2580.

de Diego, T., Manjón, A., José, L.I., 2014. Biocompatibility of ionic liquids with enzymes for biofuel production. In: Fang, Z., Smith, R.L., Jr.Jr., Qi, X. (Eds.), . In: Production of Biofuels and Chemicals with Ionic Liquids, vol. 1. Springer, New York, pp. 284–290.

El-Enshasy, H.A., 2007. Filamentous fungal cultures – process characteristics, products, and applications. In: Yang, S.T. (Ed.), Bioprocessing for Value-Added Products from Renewable Resources – New Technologies and Applications. Elsevier, pp. 225–261.

Engel, P., Mladenov, R., Wulfhorst, H., Jäger, G., Spiess, A.C., 2010. Point by point analysis: how ionic liquid affects the enzymatic hydrolysis of native and modified cellulose. Green Chem. 12, 1959–1966. http://dx.doi.org/10.1039/C0GC00135J.

Ghose, V.K., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59, 257–268.

Gunny, A.A.N., Arbain, D., 2013. Ionic liquids: green solvent for pretreatment of lingnocellulosic biomass. Adv. Mater. Res. 701, 399–402.

Gunny, A.A.N., Arbain, D., Gumba, R.E., Jong, B.C., Jamal, P., 2014. Potential halophilic cellulases for in situ enzymatic saccharification of ionic liquids pretreated lignocelluloses. Bioresour. Technol. 155, 177–181. http://dx.doi.org/10.1016/j.biortech.2013.12.101.

Imandi, S.B., Bandaru, V.V., Somalanka, S.R., Bandaru, S.R., Garapati, H.R., 2008. Application of statistical experimental designs for the optimization of medium constituents for the production of citric acid from pineapple waste. Bioresour. Technol. 99 (10), 4445–4450.

Kamiya, N., Matsushita, Y., Hanaki, M., Nakashima, K., Narita, M., Goto, M., Takahashi, H., 2008. Enzymatic in situ saccharification of cellulose in aqueous-ionic liquid media. Biotechnol. Lett. 30, 1037–1040. http://dx.doi.org/10.1007/s10529-008-9638-0.

Mäki-Arvela, P., Anugwom, I., Virtanen, P., Sjöholm, R., Mikkola, J.P., 2010. Dissolution of lignocellulosic materials and its constituents using ionic liquids – a review. Ind. Crop. Prod. 32, 175–201. http://dx.doi.org/10.1016/j.indcrop.2010.04.005.

Mesbah, N.M., Wiegel, J., 2005. Halophilic thermophiles: a novel group of extremophiles. In: Satyanarayana, T., Johri, B.N. (Eds.), Microbial Diversity: Current Perspectives and Potential Applications. I.K. Publishing House, New Delhi, pp. 91–118.

Plackett, R.L., Burman, J.P., 1946. The design of optimum multifactorial experiments. Biometrika 33, 305–325.

Rashid, S.S., Alam, M.Z., Karim, M.I.A., Salleh, M.H., 2009. Optimization of the nutrient supplements for cellulase production with the basal medium palm oil mill effluent. World Acad. Sci. Eng. Technol. 60, 809–815.

Sharada, R., Venkateswarlu, G., Venkateshwar, S., Anand Rao, M., 2013. Production of cellulase – a review. IJPCBS 3 (4), 1070–1090.

Shiang, M., Linden, J.C., Mohagheghi, A., Grohmam, K., Himmel, M.E., 1991. Characterization of eng F, a gene for a non-cellulosomal clostridium cellulovoras endoglucanase. Gene 182, 163–167.

Shivanand, P., Mugeraya, G., Kumar, A., 2012. Utilization of renewable agricultural residues for the production of extracellular halostable cellulase from newly isolated Halomonas sp. strain PS47. Ann. Microbiol., doi: 10.1007/s13213-012-0583-8..

Stanbury, P.F., Whitaker, A., Hall, S.J., 1997. Principles of Fermentation Technology. Butterworth-Heinemann, Oxford.

Sukumaran, R.K., Singhania, R.R., Pandey, A., 2005. Microbial cellulases-production, application and challenges. J. Sci. Ind. Res. 64, 832–844.

Vaidya, B.K., Mutalik, S.R., Joshi, R.M., Nene, S.N., Kulkarni, B.D., 2009. Enhanced production of amidase from Rhodococcus erythropolis MTCC 1526 by medium optimisation using a statistical experimental design. J. Ind. Microbiol. Biotechnol. 36, 671–678. http://dx.doi.org/10.1007/s10295-009-0536-9.

Wang, H., Gurau, G., Rogers, R.D., 2012. Ionic liquid processing of cellulose. Chem. Soc. Rev. 41, 1519–1537. http://dx.doi.org/10.1039/C2CS15311D.

Wei, H., Xu, Q., Taylor 2nd, L.E., Baker, J.O., Tucker, M.P., Ding, S.Y., 2009. Natural paradigms of plant cell wall degradation. Curr. Opin. Biotechnol. 20, 330–338. http://dx.doi.org/10.1016/j.copbio.2009.05.008.

Xu, G., Goodell, B., 2001. Mechanisms of wood degradation by brown-rot fungi: chelator-mediated cellulose degradation and binding of iron by cellulose. J. Biotechnol. 87, 43–57.