Optimization of Culture Conditions for Cellulase Production in *Acanthophysium* Sp. KMF001 Using Response Surface Methodology

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There are differences in the extracellular enzymes produced from species of wood-rotting fungi and their activity due to variation in nutritional conditions such as carbon sources and nitrogen sources, as well as environmental conditions such as incubation temperature and pH. In this study, to determine the methods to promote the secretion of especially cellulase with high activity among the extracellular enzymes of wood-damaging fungi, the optimal nutrient sources and incubation conditions for the production of cellulase with high activity were investigated using response surface methodology based on a broth culture from *Acanthophysium* sp. KMF001, a novel strain of wood-damaging fungus. The nutrient sources that were optimal for the cellulase production with high activity from *Acanthophysium* sp. KMF001 were cellulose as a carbon source and tryptone:yeast extract (7:3) as a nitrogen source. The optimal incubation conditions were a temperature of 30 °C and a pH of 6. The optimal concentrations of carbon and nitrogen sources were cellulose at 31.1 g·L^{-1} and tryptone:yeast extract (7:3) at 15 g·L^{-1}, with pH of 5.9.

Keywords: Cellulase activity; Novel wood-damaging fungus; Nutrient sources; Optimal incubation

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INTRODUCTION

In the context of climate change due to the overuse of fossil fuels as well as constraints imposed by the high price of oil, there has been a global focus on developing novel means of energy production as alternatives to petroleum (Börjesson *et al.* 2007; Mansouri *et al.* 2016). In recent decades, biofuel production has expanded globally as a means of mitigating greenhouse gas emissions with the support of policies to diversify energy sources (Jonker *et al.* 2015). As part of the development of such alternative energy, technologies for producing ethanol from plant biomass are being used (Kim 2009; Kim 2011; Jonker *et al.* 2015; Mansouri *et al.* 2016).

However, there are problems associated with using starch-based materials such as sugarcane and corn as the main materials in current commercial bioethanol production, such as price and supply issues in food markets (Kim 2009, 2011). This background has enabled lignocellulosic biomass to emerge as a next-generation biofuel material because of its eco-friendliness and adequate supply and reproduction to produce biofuels including bioethanol (Kim 2009; Valchev *et al.* 2009).

Lignocellulosic biomass, which is mainly composed of cellulose, hemicellulose, and lignin, has disadvantages including its high cost, difficulty of degradation, and complex processes required to isolate monomers due to the hardness and strength of the material (Wright 1988; Mosier *et al.* 2005; Datar *et al.* 2007; Deswal *et al.* 2011). There is a need for a pretreatment process to achieve efficient saccharification by reducing the cellulose crystallinity and increasing the biomass surface area after the effective removal of lignin. The saccharification process should also release available monomers from cellulose and hemicellulose via hydrolysis. The acid saccharification process that is currently used

commercially based on concentrated or diluted acid causes environmental contamination and metal corrosion as well as the formation of fermentation inhibitors, such as furfural and 5-hydroxymethylfurfural (HMF), *via* its performance at high temperature. Thus, saccharification using enzymes has recently become popular (Howard *et al.* 2003; Mishima *et al.* 2006; Fang *et al.* 2008; Sukumaran *et al.* 2009; Salvi *et al.* 2010). However, enzyme-based saccharification has the disadvantage of taking a long time, although it has the advantages of reducing the amount of energy used and protecting the environment because it does not use lots of chemicals or require a chemical recovery or neutralization process (Howard *et al.* 2003; Mishima *et al.* 2006; Fang *et al.* 2008; Sukumaran *et al.* 2009; Salvi *et al.* 2010).

Enzymatic hydrolysis of cellulosic materials is accomplished by the hydrolysis reactions catalyzed by cellulase complex enzymes including endo-1,4-β-glucanase (EG), β-glucosidase (BGL), and cellobiohydrolase (CBH) (Wyman 1996; Kim 2010; Shahriarinour *et al.* 2011). The cellulase complex is generally obtained from organisms that use lignocellulosic biomass as a nutrient source. Many studies have focused on the development of cellulase derived from microorganisms, enabling the effective hydrolysis of cellulose in particular (Acebal *et al.* 1986; Akila and Chandra 2003; Kurabi *et al.* 2005; Wen *et al.* 2005; Rosgaard *et al.* 2006; Kim 2009; da Silva Delabona *et al.* 2012).

The cellulases derived from these microorganisms exhibit differences in their activity mainly depending on the incubation conditions. Carbon and nitrogen sources play important roles in the production of lignin-degrading enzymes (Galhaup *et al.* 2002; Mikiashvili *et al.* 2005; Kachlishvili *et al.* 2006; Mikiashvili *et al.* 2006; Stajić *et al.* 2006; Ali *et al.* 2013). The incubation conditions, such as the available nutrients, temperature, and pH, also influence the enzyme activity (Immanuel *et al.* 2006; Abou-Taleb *et al.* 2009; Kim 2009; Deka *et al.* 2011).

Because enzyme activity is affected by various factors such as nutrients and environmental conditions, it is very important to design experiments appropriately to achieve effective enzyme production (Wang and Wan 2009). Various statistical methods have been used in the search for cellulase derived from microorganisms, including the Plackett–Burman design (PBD), Box–Behnken design (BBD), and central composite design (CCD) (Francis *et al.* 2003; Soni *et al.* 2010; Elsanhoty *et al.* 2012; Ali *et al.* 2013).

There are many studies of the optimization of media components for microorganism based on stochastic optimization methods (Plackett and Burman 1946; Coello *et al.* 2000; Ergun and Mutlu 2000; Dey *et al.* 2001; Kim *et al.* 2005; Senthilkumar *et al.* 2005; Jatinder *et al.* 2006; Sannigrahi *et al.* 2010; Deka *et al.* 2011; Dong *et al.* 2011; Pal 2011; Zhang and Sang 2012; Zhang *et al.* 2012; Bagewadi *et al.* 2017;). Zhang and Sang (2012) reported that the EG, FPU (filter paper unit), and BGL activities of *Penicillium chrysogenum* QML-2 increased by 3.34, 5.12, and 3.75 times, respectively, compared with the initial levels under conditions optimized using the stochastic optimization method. Bagewadi *et al.* (2017) confirmed this result, with EG production being improved 2.31 times through a model built using CCD. However, there is a lack of more effective cellulosic saccharification enzymes to substitute for current commercial enzymes.

In the previous research, *Acanthophysium* sp. KMF001 was screened as a novel strain with high cellulase activity among the wood-rotting fungi of 54 strains (Kim *et al.* 2019), and this novel strain showed similar or higher levels of cellulase activity than other strains of the *Acanthophysium* genus which showed strong cellulase activity (Yoon *et al*. 2019). The research data stated the novelty of *Acanthophysium* sp. KMF001 with high cellulase activity was reported in the previous paper (Yoon *et al*. 2019).

The objective of this study was to maximize the production of EG in particular from extracellular enzymes of wood-rotting fungi, as well as to determine the optimal nutrient sources and incubation conditions for EG production with high activity from the

novel strain *Acanthophysium* sp. KMF001 using response surface methodology (RSM). The statistical medium optimization method used in this study was applied to search for the effect of various medium components on enzyme productivity by the one-factor-at-a-time (OFAT) method, which is an experimental design method in which all factors except the primary one are fixed. To determine the influence of each nutrient selected by the OFAT experiment results on enzyme production, a Plackett–Burman design (PBD) was performed (Plackett and Burman 1946), and the RSM designed with addition of the axis and point to the full factorial design (FFD) method, which is a statistical method to investigate the effect of interaction between factors, was used. The interacting effects of each component of the medium in the obtained incubation results were also analyzed from the regression equation elicited through statistical analysis, and the optimal concentration of each medium component for maximizing EG production was determined based on the results of the described tests (Jeong *et al*. 2010).

EXPERIMENTAL

Test Strain and Basic Incubation Conditions

Acanthophysium sp. KMF001 (KCTC 18282P), which was deposited at the Korea Collection for Type Culture (Jeongeup-si, South Korea) in 2016, was used as a test strain. This strain was activated on potato dextrose agar for 5 to 7 days and then used as an inoculum for a pre-culture. The activated strain was inoculated in potato dextrose broth, and this pre-culture was incubated at 25 °C and 150 rpm for 4 to 5 days. In the main culture, a broth including 2% w/v carbon source, 1% w/v nitrogen source, 5 $g \cdot L^{-1}$ KH_2PO_4, 5 $g \cdot L^{-1}$ K_2HPO_4, and 3 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$ at pH 5.5 was used, and the main culture was inoculated at a 5% (v/v) ratio with the pre-culture. This inoculated broth was incubated at 25 °C and 150 rpm for 3 weeks, and then the crude enzyme was collected. The incubated culture broth as the crude enzyme was centrifuged at 4 °C and 13500 rpm for 10 min to separate the cells, and the cell-free supernatant as the enzyme solution was used to analyze enzyme activity.

Screening Test of Carbon Source using OFAT

To screen carbon sources using OFAT, the following carbon sources were used: cellulose (Sigma-Aldrich, approximately 20 μm, St. Louis, MO, USA), glucose (Sigma-Aldrich), lactose (Ducksan, Seoul, Korea), cellobiose (Alfa Aeasar, Ward Hill, MA, USA), carboxymethyl cellulose sodium salt (CMC, Fluka, Steinheim, Germany), xylose (Ducksan), microcrystalline cellulose (Avicel® PH-101, ~50 μm from Fluka), sawtooth oak (*Quercus acutissima*), and pitch pine (*Pinus rigida*). Sawtooth oak and pitch pine were purchased from SINWOOIMSAN Co., Ltd. (Hongcheon-gun, Gangwon-do, Korea), and were tested as carbon sources after being ground into a powder. The sample for measurement of enzyme activity was collected at intervals of 3 days for 3 weeks after beginning the main culture.

Screening Test of Nitrogen Source using OFAT

To screen nitrogen sources using OFAT, the following nitrogen sources were used: yeast extract (BD), peptone (BD), tryptone (BD), corn steep (Sigma), urea (Ducksan), ammonium sulfate (Ducksan), potassium nitrate (Ducksan), sodium nitrate (Ducksan), yeast extract:peptone (1:4), and tryptone:yeast extract (7:3). The enzyme activity of each sample was measured in the same way as in the above carbon source test.

Test to Determine Optimal pH and Temperature using OFAT

To determine the optimal pH and temperature for incubating the test strain, the enzyme activities were measured in the pH range from 4.0 to 7.0 at 0.5 intervals every 3 days for 3 weeks after beginning the main culture and in the temperature range from 20 to 35 °C with 5 °C intervals.

To determine the optimal pH for the crude enzyme activity of the test strain, the response was tested throughout the pH range from 3.0 to 9.0. The pH buffers for each enzyme response were tested by determining the enzyme response at 30 °C using sodium citrate buffer (pH 3.0 to 6.0), sodium acetate buffer (pH 4.0 to 6.0), potassium phosphate buffer (pH 6.0 to 8.5), and Tris buffer (pH 8.0 to 9.0). The enzyme response was measured by absorbance at 405 nm after the reaction. To determine the optimal temperature for the crude enzyme activity of the test strain, the temperature range from 30 to 80 °C with 10 °C intervals was tested. The reaction was performed with 100 mM sodium acetate buffer (pH 4.5) at each temperature condition for 15 min, after which the absorbance was measured at 405 nm.

Temperature Stability Test of Cellulase using OFAT

To determine the temperature stability of the crude enzyme activity of the test strain, the remaining enzyme activity was measured by reaction of the enzyme excluding substrates for 72 h in a water bath in the temperature range from 30 to 60 °C with 10 °C intervals.

Screening for the Important Factors using Plackett–Burman Design

To develop the optimal incubation conditions for a high EG yield from the novel strain *Acanthophysium* sp. KMF001, the main experiments were conducted using the stochastic program (Minitab 17, State College, PA, USA). To screen the main factor affecting EG enzyme activity among the seven medium components, the Plackett–Burman design (PBD) was used. For detection of the main effects, seven factors were considered: carbon source of cellulose, nitrogen source of tryptone:yeast extract (7:3), minerals including K_2HPO_4, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$, temperature, and pH. The associated concentration ranges are shown in Table 1.

Table 1. Levels of the Variables for the Plackett–Burman Design

Factor		Levels of Factor		
		-	0	+
1	Cellulose (g/L)	10	20	30
2	Tryptone: Yeast extract (7:3) (g/L)	5	10	15
3	K_2HPO_4 (g/L)	2.5	5	7.5
4	KH_2PO_4 (g/L)	2.5	5	7.5
5	$MgSO_4 \cdot 7H_2O$ (g/L)	2	3	4
6	Temperature (°C)	27	30	33
7	pH	5.5	6.0	6.5

The experiment was conducted at three levels for each factor, referred to as a lower level of –1, middle level of 0, and higher level of +1. In the main experiment, there were a total of 26 experiments using the Plackett–Burman design (PBD), and the designed experiments are shown in Table 2 with coded units. The Plackett–Burman design (PBD) is based on a first-order polynomial model (Eq. 1),

$$Y = \beta_0 + \sum \beta_i X_i \quad (1)$$

where Y is the enzyme activity; $ß_O$ is the model intercept; $ß_i$ is the linear coefficient; and X_i is the factor level. This model was used to screen and evaluate the main factors influencing enzyme activity, but not to explain interactions between factors. In the regression analysis, factors were significant ($P < 0.005$) above the 95% confidence level made a major contribution to cellulase activity, which was then optimized by the RSM in further experiments.

Table 2. Plackett–Burman Design in Coded Units for Seven Variables Tested to Determine the Main Effect along with EG Activity

Run	Cellulose	Tryptone :Yeast Extract (7:3)	K_2HPO_4	KH_2PO_4	$MgSO_4 \cdot 7H_2O$	Temperature	pH	EG[1)] activity(U/mL)
1	-	-	-	+	+	+	-	57.00
2	-	-	-	-	-	-	-	42.76
3	-	+	+	+	-	+	+	36.70
4	-	+	-	-	-	+	+	30.16
5	0	0	0	0	0	0	0	55.02
6	+	+	-	+	-	-	-	56.64
7	+	-	+	+	-	+	-	54.90
8	-	-	+	+	+	-	+	55.84
9	-	-	-	-	-	-	-	56.06
10	-	+	-	-	-	+	+	30.08
11	-	-	-	+	+	+	-	40.80
12	-	+	+	+	-	+	+	32.90
13	+	-	-	-	+	+	+	80.10
14	+	+	-	+	-	-	-	40.66
15	+	-	+	+	-	+	-	39.44
16	+	-	+	-	-	-	+	55.12
17	+	-	-	-	+	+	+	107.20
18	+	+	+	-	+	+	-	33.58
19	+	+	-	+	+	-	+	43.68
20	-	+	+	-	+	-	-	32.18
21	0	0	0	0	0	0	0	36.60
22	-	-	+	+	+	-	+	43.76
23	+	+	-	+	+	-	+	70.98
24	-	+	+	-	+	-	-	32.00
25	+	-	+	-	-	-	+	96.46
26	+	+	+	-	+	+	-	33.80

1) EG: Endo-1,4-β-glucanase.

Determination of Optimal Production Medium Conditions using FFD

To determine the optimal production medium conditions for the high production of EG by *Acanthophysium* sp. KMF001, the experiments were performed using factors such as carbon source, nitrogen source, and pH. The experiments were conducted using a FFD, which finds optimal concentrations while considering the interaction among the three main effect factors.

In this study, the experimental design consisted of an experiment number of 19 (= $2^k + 2k + n_0$), where k is the number of independent variables and n_0 is the number of replicated runs at the center point. The relations and interrelations of variables were analyzed through the application of a second-order polynomial method with the data obtained from the 19 experiments. As the experimental design involved the creation of response surface graphs, a total of 25 experiments were conducted by adding six experiments within the center point, axis, and points at the FFD to presume curved surface

change of response (Eq. 2),

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j \quad (2)$$

where Y is the enzyme activity, k is the coefficient of a variable, $ß_O$ is the model constant, $ß_i$ is the linear coefficient, $ß_{ii}$ is the quadratic coefficient, and $ß_{ij}$ is the interaction coefficient. For statistical analysis, the data were expressed as a three-dimensional response surface graph shape from the multi-dimension equation using ANOVA to explain the effects and interaction effects of independent variables. As shown in Table 3, the concentration ranges of each variable were set at three levels: –1 (lower), 0 (middle), and +1 (higher). The designed experiments are shown in Table 4 with coded units. The regression equation was elicited through the statistical analysis using Design Expert Pro 7.0 (Stat-Ease Inc., Minneapolis, MN, USA).

Table 3. Levels of the Variables for Full Factorial Design

Factor		Levels of Factor		
		-	0	+
1	Cellulose (g/L)	20	30	40
2	Tryptone: Yeast extract (7:3) (g/L)	5	10	15
3	pH	5.5	6.0	6.5

Table 4. Full Factorial Design in Coded Units for Seven Variables Tested to Determine Optimal Concentration along with EG Activity

Run	Cellulose	Tryptone: Yeast Extract (7:3)	pH	EG[1)] activity (U/mL)
1	-	+	+	65.71
2	0	0	0	215.34
3	+	+	-	115.82
4	-	+	+	64.42
5	-	-	+	60.35
6	+	-	-	60.68
7	-	-	-	60.68
8	0	0	0	218.22
9	0	0	0	214.59
10	+	+	+	150.47
11	-	-	+	60.62
12	+	+	+	114.65
13	+	+	-	158.78
14	+	-	-	60.70
15	-	+	-	60.72
16	-	+	-	60.46
17	+	-	+	60.65
18	-	-	-	60.68
19	+	-	+	61.20
20	-	0	0	73.12
21	+	0	0	63.49
22	0	-	0	60.59
23	0	+	0	179.56
24	0	0	-	164.61
25	0	0	+	137.96
1) EG: Endo-1,4-β-glucanase.				

Measurement of Enzyme Activity

Measurement of endo-β-1,4-glucanase (EG)

The enzyme solution was reacted at 50 °C for 30 min after the addition of 5 μL of enzyme solution to 45 μL of 2% (w/v) carboxylmethylcellulose sodium salt (CMC; Sigma-Aldrich) in 0.1 M sodium acetate buffer (pH 5.0). After the reaction was terminated at 100 °C for 10 min with the addition of 50 μL of copper solution, the amount of generated reduced sugar was measured using the colorimetric assay developed by Nelson (1944) and Somogyi (1945). One unit of enzyme activity (U) was defined by the amount of enzyme required to release 1 μM p-nitrophenol for 30 min under the specified conditions of the assay method.

Measurement of β-glucosidase (BGL)

One milliliter of the reaction solution was reacted at 50 °C for 15 min after the addition of 0.1 mL of enzyme solution and 0.1 mL of 10 mM p-nitrophenyl-β-D-glycopyranoside (pNPG, Sigma) in 0.8 mL of 0.1 M sodium acetate buffer (pH 5.0). The reaction was terminated by the addition of 0.1 mL of 2 M Na_2CO_3 (sodium carbonate) solution; the amount of released p-nitrophenol was measured by the absorbance at 405 nm. One unit of enzyme activity (U) was defined by the amount of enzyme required to release 1 μM p-nitrophenol for 15 min under certain conditions (Riou *et al.* 1998).

Measurement of cellobiohydrolase (CBH)

One milliliter of the reaction solution was reacted at 50 °C for 15 min after the addition of 0.1 mL of enzyme solution and 0.1 mL of 4-nitrophenyl-β-D-cellobioside (pNPC, Sigma) in 0.8 mL of 0.1 M sodium acetate buffer (pH 5.0). The reaction was terminated by the addition of 0.1 mL of 2 M Na_2CO_3 (sodium carbonate) solution. The amount of released p-nitrophenol was measured by the absorbance at 405 nm. One unit of enzyme activity (U) was defined by the amount of enzyme required to release 1 μM p-nitrophenol for 15 min under certain conditions (Riou *et al.* 1998).

RESULTS AND DISCUSSION

Screening of Carbon Source using OFAT

To screen the optimal carbon source for the incubation of *Acanthophysium* sp. KMF001 using OFAT, the crude enzyme activity of *Acanthophysium* sp. KMF001 from various carbon sources was measured, as presented in Table 5. This table shows that, for the case of cellulose, EG production was 122.3 $U \cdot mL^{-1}$. With cellulose, the maximum activity levels of EG applied to BGL and CBH were 5.85 and 0.6 $U \cdot mL^{-1}$, respectively. The results showed that, in the case of *Acanthophysium* sp. KMF001, cellulose was the optimal carbon source because it showed the greatest effect on EG activity. When glucose was used as a carbon source, the enzyme activities of EG, BGL, and CBH were low at 0.1, 1.5, and 1.3 $U \cdot mL^{-1}$, respectively. Pandey *et al.* (2014) observed similar results of very low cellulase activities on glucose by *Trichoderma* species. These findings can be explained by the inhibition of cellulase biosynthesis for carbon sources such as glucose, which easily induce metabolism (Lee and Kang 1993; Kim *et al.* 2010). The medium containing lactose, cellobiose, and xylose exhibited low enzyme activities, and the carbon sources such as microcrystalline cellulose (MCC), CMC, and sawdust (*Quercus acutissima*) showed relatively higher enzyme activities. Especially, MCC showed a similar level of high enzyme activities as that achieved by cellulose. It has been reported that the MCC was the most effective carbon source for cellulase production by *Trichoderma reesei* strains (Dashtban *et al.* 2011). Thomas *et al.* (2018) also observed that the organism preferred to

utilize MCC rather than CMC to produce cellulases. Jiménez-Leyva *et al.* (2017) reported that the organisms showed good growth on either CMC or MCC, but their cellulase activities were better on MCC. In the literature, Chinedu *et al*. (2007) demonstrated that organisms have the tendency to grow rapidly on simple sugar such as glucose and synthesize enzymes to obtain simple sugars on polymers such as sawdust or cellulose. In this study, the enzyme activity of EG in the sawdust (*Pinus rigida*) containing medium was very low. This low cellulase activity in the sawdust (*Pinus rigida*) might be caused due to high lignin content of softwood biomass (Borand *et al*. 2020).

Table 5. Effect of Carbon Sources (2%, W/V) on Cellulase Activities of *Acanthophysium* Sp. KMF001

Carbon Sources	Enzyme Activity (U/mL)		
	EG[1)]	BGL[2)]	CBH[3)]
Cellulose	122.31±6.49	5.85±0.55	0.6±0.01
Glucose	1.30±0.0	0.4±0.00	0.1±0.00
Lactose	13.74±0.00	0.33±0.01	0.03±0.01
Cellobiose	38.77±0.21	0.24±0.00	0.02±0.00
CMC[4)]	75.95±2.09	0.96±0.02	0.17±0.01
Xylose	30.33±1.68	0.73±0.03	0.13±0.03
MCC (Avicel)	107.94±12.57	7.42±0.27	0.27±0.00
Sawdust (*Quercus acutissima)*	64.84±0.21	0.81±0.01	0.17±0.00
Sawdust (*Pinus rigida*)	0.63±0.04	0.27±0.01	0.02±0.00

1) EG: Endo-1,4-β-glucanase.
2) BGL: β-glucosidase.
3) CBH: Cellobiohydrolase
4) CMC: Carboxymethylcellulose sodium salt

Screening of Nitrogen Source using OFAT

To determine the optimal nitrogen source for the incubation of *Acanthophysium* sp. KMF001 using OFAT, the crude enzyme activity of *Acanthophysium* sp. KMF001 was measured, as indicated in Table 6.

Table 6. Effect of Nitrogen Sources (1%, W/V) on Cellulase Activities of *Acanthophysium* Sp. KMF001

Nitrogen Sources	Enzyme Activity (U/mL)		
	EG[1)]	BGL[2)]	CBH[3)]
Yeast extract	65.28±9.64	5.54±0.16	0.39±0.01
peptone	47.95±1.89	6.28±0.09	0.30±0.01
Tryptone	45.44±3.35	3.27±0.07	0.20±0.01
corn steep	3.20±0.07	0.03±0.00	0.03±0.00
Urea	0.54±0.02	0.02±0.00	0.01±0.00
Ammonium sulfate	2.49±0.14	0.01±0.00	0.01±0.00
Potassium nitrate	3.72±0.04	0.01±0.00	0.01±0.00
Sodium nitrate	1.21±0.07	0.12±0.00	0.02±0.00
Yeast extract:Peptone (1:4)	66.17±4.19	7.64±0.08	0.44±0.02
Tryptone: Yeast extract (7:3)	107.49±9.84	12.89±0.18	1.15±0.05

1) EG: Endo-1,4-β-glucanase.
2) BGL: β-glucosidase.
3) CBH: Cellobiohydrolase

The EG had the highest enzyme activity of 107.5 $U \cdot mL^{-1}$ with the tryptone:yeast extract (7:3) mixture. When this mixture was used, BGL and CBH showed the highest

enzyme activities of 12.9 and 1.15 U·mL^{-1}, respectively. However, when corn steep, urea, ammonium sulfate, potassium nitrate, and sodium nitrate were used, there was low enzyme activity. As a result, tryptone:yeast extract (7:3) mixture was determined as the optimal nitrogen source for *Acanthophysium* sp. KMF001. In the literature, it has been reported that organic nitrogen sources such as yeast extract stimulated cell growth and cellulase production because yeast extract provides important growth factors such as carbon, sulfur, trace nutrient as well as nitrogenous compounds as complex hydrolysate obtained from yeasts (Thomas *et al*. 2018). These researchers also mentioned that mixtures of the organic nitrogen sources such as tryptone or peptone caused low growth and cellulase production, and corn steep caused high cellulase production with maximum growth. Tong and Rajendra (1992) have reported that peptone was not suitable for both mycelial growth and cellulase production.

Determination of Optimal pH and Temperature for Incubation using OFAT

To determine the optimal pH for the incubation of *Acanthophysium* sp. KMF001 using OFAT, enzyme activities were measured in pH 4.0 to 7.0, as indicated in Fig. 1. Enzyme activity increased rapidly from pH 5.0 to 6.0, while there was decreased enzyme activity from pH 6.0 to 7.0. The results indicated that enzyme activity showed the highest EG value of 65.08 U·mL^{-1} at pH 6.0, while BGL and CBH also showed high enzyme activity of 11.73 and 3.51 U·mL^{-1} under the same conditions.

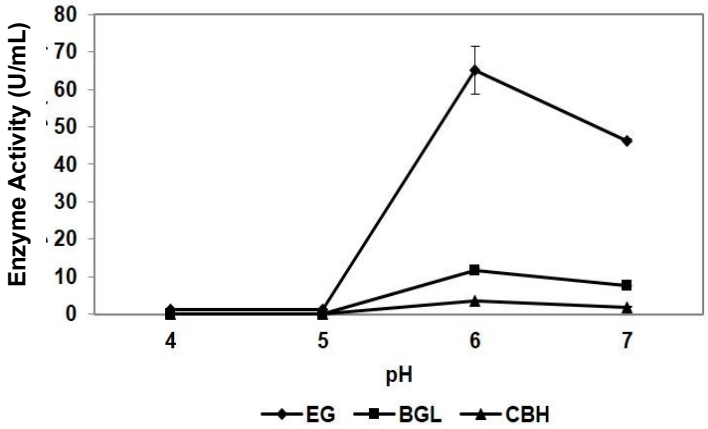

Fig. 1. Effect of medium pH on cellulase production of *Acanthophysium* sp. KMF001

To determine the optimal temperature for the incubation of *Acanthophysium* sp. KMF001 using OFAT, enzyme activity was measured through culture in the temperature range from 20 to 35 °C with 5 °C intervals, as shown in Fig. 2a–c. This figure indicates that an incubation temperature of 30 °C was associated with the highest enzyme activity of EG of 83.1 U·mL^{-1}, while BGL and CBH at the same temperature showed high enzyme activity of 59.9 and 8.8 U·mL^{-1}, respectively. These results show that the pH of 6.0 and incubation temperature of 30 °C were the optimal incubation conditions to produce crude enzyme with high EG activity from *Acanthophysium* sp. KMF001. Conditions such as temperature and pH are related to the shape of an enzyme's active site; thus a maximum activity can be achieved at optimal temperature and pH (Andreaus *et al*. 1999; Herlet *et al.* 2017). Silias *et al.* (2017) found that the enzyme released from *Trichoderma reesei* for hydrolysis of glucose showed the optimal enzyme activity at the experimental conditions of a temperature of 30 °C and pH of 4.5. The composite microbial system FH3, which was combined of cellulose-degrading strains, reached the maximum cellulase activity under reaction condition of 30 to 35 °C and pH of 6.0 to 6.5.

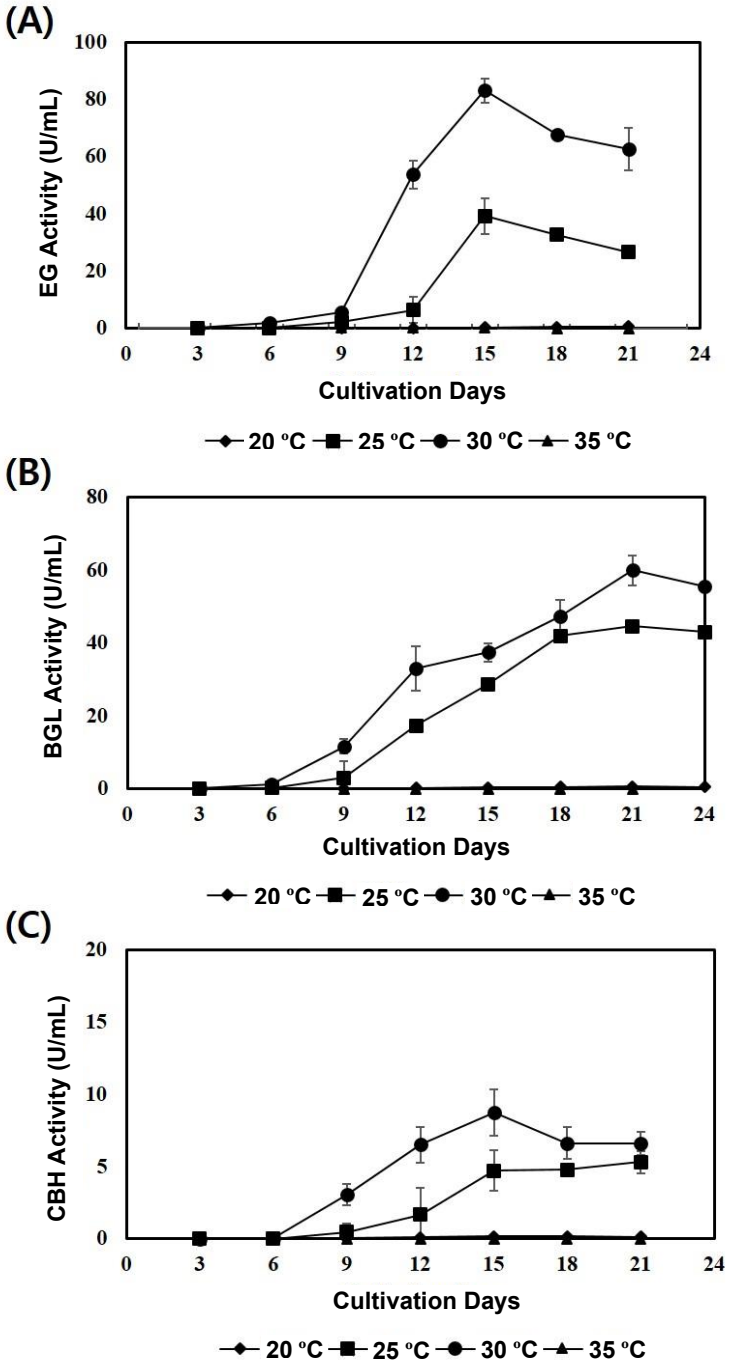

Fig. 2. Effect of medium temperature on cellulase production of *Acanthophysium* sp. KMF001

Determination of Optimal pH and Temperature for Enzyme Activity Response using OFAT

The optimal pH is one of the important factors that affect the accurate measurement of cellulase activity using reducing sugars (Criquet 2002). There is a need to optimize the related factors for measuring cellulase activity depending on each cellulase-producing microorganism. Figure 3a–c shows the results of the experiment conducted to determine the optimal pH of the enzyme response of crude enzyme generated from *Acanthophysium* sp. KMF001 using OFAT. As shown in this figure, the enzyme activities of EG, BGL, and CBH gradually increased above pH 3.0 but decreased from pH 5.0; no enzyme activity was shown in the pH range from 6.0 to 9.0. In particular, EG exhibited the highest activity at pH 3.5 to 4.0 (sodium citrate buffer) and pH 5.0 (sodium acetate buffer).

BGL and CBH exhibited the highest activity at pH 4.5 (sodium citrate buffer). These results showed that the optimal pH for the enzyme responses of EG, BGL, and CBH was pH 4.5, given their relatively high enzyme activity.

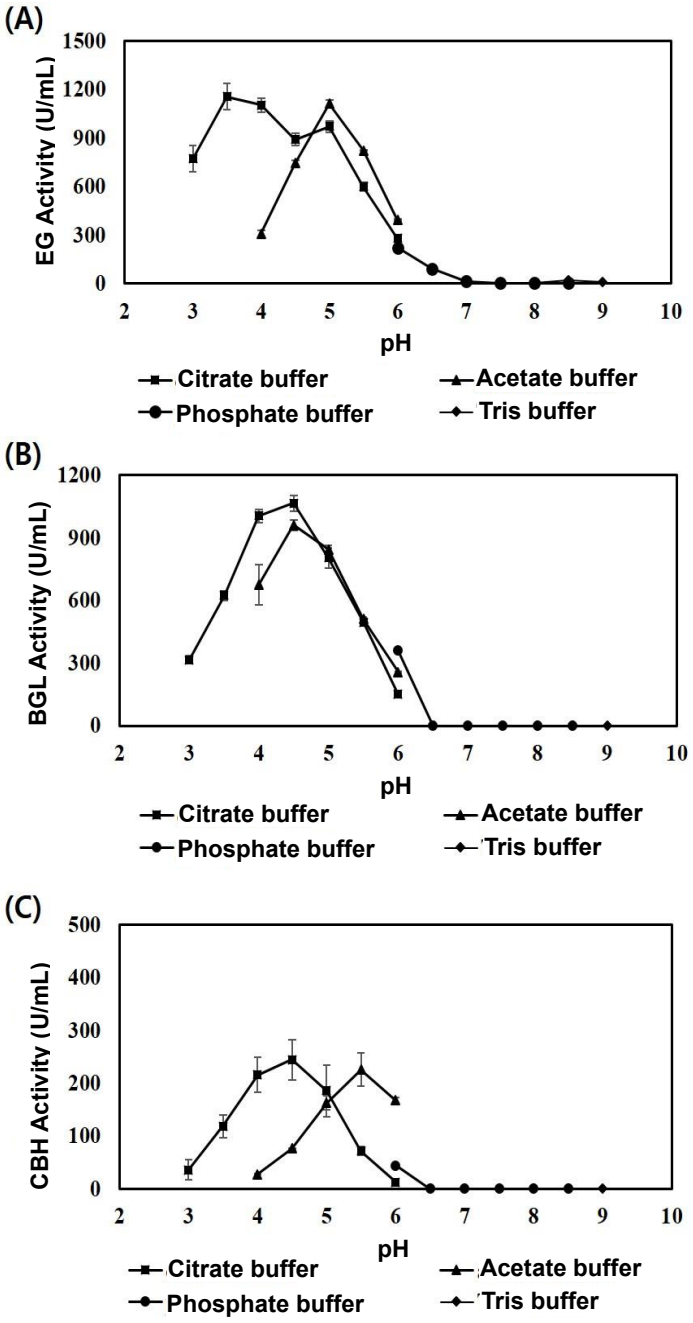

Fig. 3. Effect of pH on cellulase activity of *Acanthophysium* sp. KMF001

Results for the optimal temperature for the enzyme response of *Acanthophysium* sp. KMF001 crude enzyme using OFAT are shown in Fig. 4. This figure reveals the observed decreases of enzyme activity, with rapid decreases of enzyme activity of EG at 50 °C and BGL and CBH at 60 °C, and all of EG, BGL, and CBH above 60 °C. EG and BGL showed the maximum activity of 857 $U \cdot mL^{-1}$ at 50 °C and 1055 $U \cdot mL^{-1}$ at 50 °C, respectively. CBH had the maximum activity at 50 to 60 °C. The results show that the optimal temperature for the enzyme responses of EG, BGL, and CBH was 50 °C, given their relatively high enzyme activity.

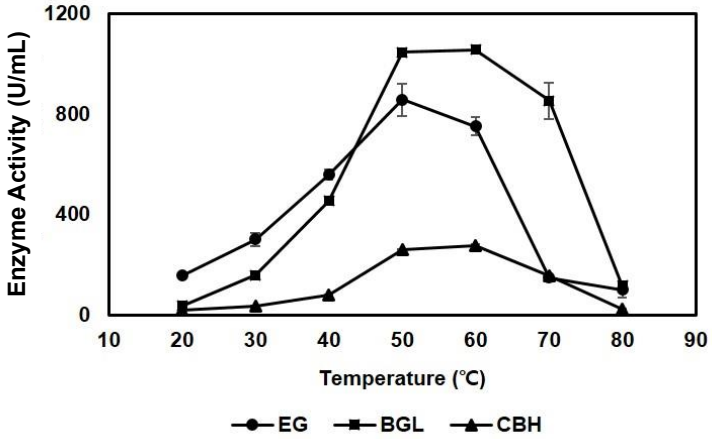

Fig. 4. Effect of temperature on cellulase activity of *Acanthophysium* sp. KMF001

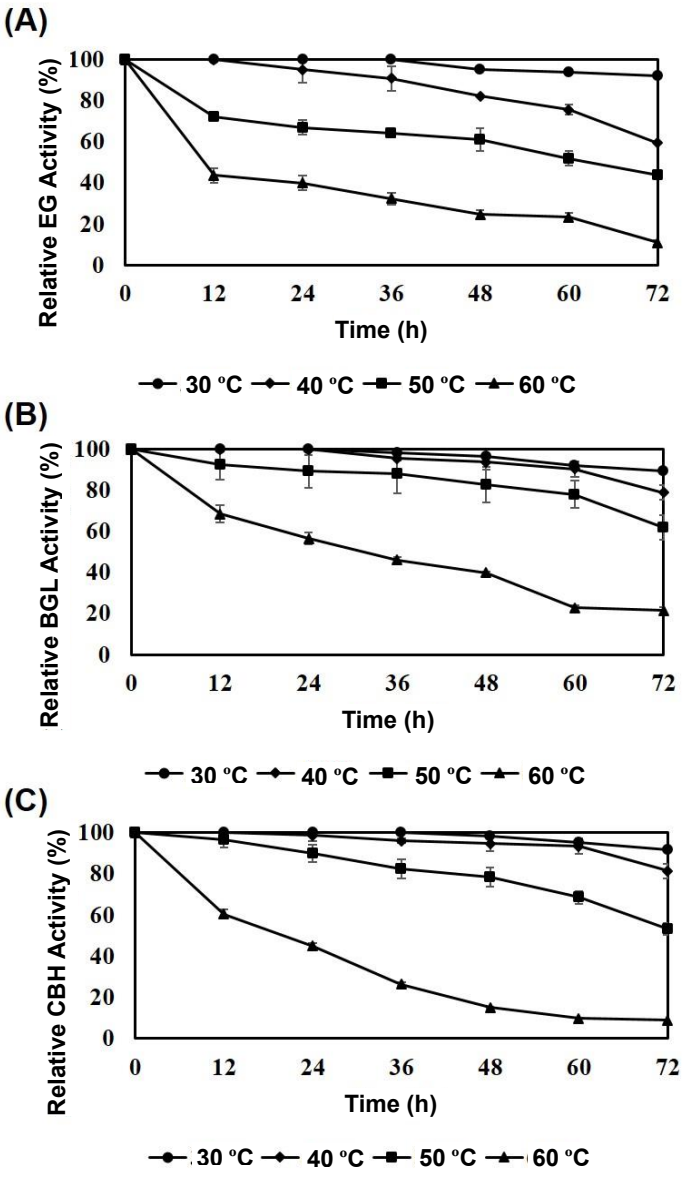

Fig. 5. Thermostability of cellulase from *Acanthophysium* sp. KMF001

Temperature Stability Results of Cellulase using OFAT

To determine the temperature stability of *Acanthophysium* sp. KMF001 crude enzyme, the temperature stability test results were determined at 30 to 60 °C for 0 to 72 h, as shown in Fig. 5. Enzyme activity of EG decreased over 60% with the passing of time upon incubation at 60 °C, while it showed a decrease of over 30% after 12 h at 50 °C. In addition, about 91% of enzyme activity remained after 72 h at 30 °C. Regarding BGL and CBH, their enzyme activities decreased 35% and 40%, respectively, after 72 h at 50 °C. They showed a large drop of enzyme activity of over 50% upon incubation for 24 h at 60 °C, while remaining enzyme activity was about 90% after 72 h at 30 °C. As a result, *Acanthophysium* sp. KMF001 crude enzyme showed the highest temperature stability at 30 °C, but more data on temperature stability over longer times are needed for further commercialization. The cellulase enzyme produced from *Bacillus* sp. has been reported to show moderate thermo-stability as its crude enzyme was retained around 68% after heating at 50 ºC for 30 min (Islam *et al*. 2019). Listyaningrum *et al.* (2018) reported that *Bacillus licheniformis* C55 crude cellulase showed a decrease of over 50% of its relative activity after incubation for 1.5 h at 50 °C. In another study, the cellulase produced by *Aspergillus flavus* have shown 50% reduction of enzyme activity at temperatures between 40 °C and 60 °C for 82-144 min (Okonkwo 2018).

Screening for the Important Factors using Plackett–Burman design

To detect the main effect factors through the Plackett–Burman design, EG activity depending on the designed experimental conditions was measured, as shown in Table 2. There was a wide variation in enzyme activity, with values ranging from 30 to 107 $U \cdot mL^{-1}$ from 26 experiments; this confirmed the importance of different variables for enzyme activity. As shown in Fig. 6, the main effect indicates that the greater slope along the concentration range of each nutrient had a substantial influence on enzyme activity. The slopes of the carbon source of cellulose, the nitrogen source of tryptone:yeast extract (7:3), and pH were significant among the seven factors; this means that these three factors had the largest effects on enzyme activity. The regression coefficient and t-value analysis of seven factors are shown in Table 7. In general, a high t-value related to a low P-value of factors indicates a significant effect on corresponding enzyme activity. The carbon source of cellulose, $MgSO_4 \cdot 7H_2O$, and pH had positive effects on EG enzyme production, but the nitrogen source of tryptone:yeast extract (7:3) and $MgSO_4 \cdot 7H_2O$ had negative effects.

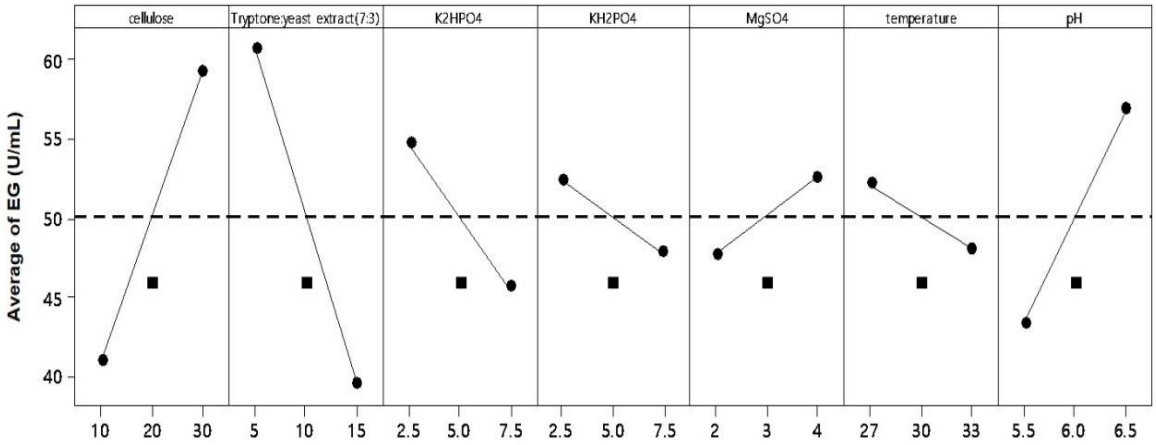

Fig. 6. Main effect plot for EG activity

As shown in Fig. 7, the main effect factors were confirmed through a Pareto chart. The factors with confidence ranges of above 95% based on the standard effect were

considered significant. The carbon source of cellulose, nitrogen source of tryptone:yeast extract (7:3), and pH were significant factors. The multi-dimension equation for the model is as follows,

$$Y \text{ (U/mL)} = -1.6 + 0.926X_1 - 2.134X_2 - 1.82X_3 - 0.94X_4 + 2.46X_5 - 0.687X_6 + 13.60X_7 - 4.3\text{Ct Pt} \quad (3)$$

where Y is EG enzyme activity, X_1 is cellulose, X_2 is tryptone:yeast extract (7:3), X_3 is K_2HPO_4, X_4 is KH_2PO_4, X_5 is $MgSO_4 \cdot 7H_2O$, X_6 is temperature, X_7 is pH, and Ct Pt is central points. Based on the *t*-value in Table 7, carbon source of cellulose, nitrogen source of tryptone:yeast extract (7:3), and pH had the most important influences on EG activity; thus, those factors were determined to be the main effect factors for the subsequent optimization experiment. The remaining factors were set as follows in the optimization experiment: 5 g/L KH_2PO_4, 5 g/L K_2HPO_4, 3 g/L $MgSO_4 \cdot 7H_2O$, and 30 °C.

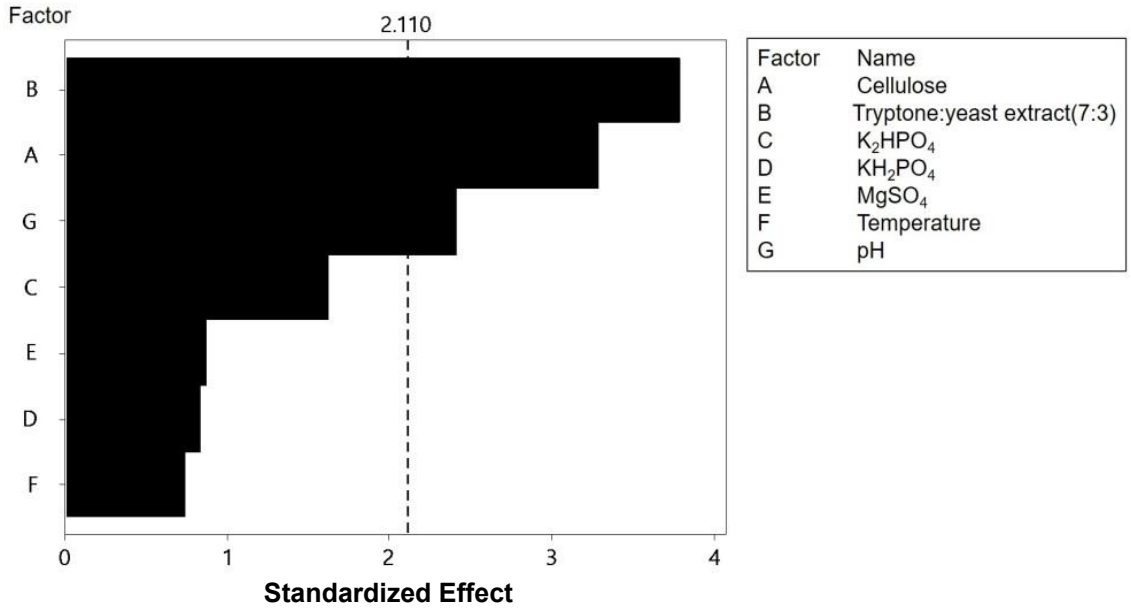

Fig. 7. Pareto chart of seven-factor standardized effects on EG activity

Table 7. Statistical Analysis of Plackett–Burman Design Showing Coefficient Value, *T*-Value, and *P*-Value for Each Variable

Variable	Coefficient	*t*-Stat	*P*-value
Intercept	50.12	17.81	0.000
cellulose	9.26	3.29	0.004
Tryptone: yeast extract (7:3)	−10.67	−3.79	0.001
K_2HPO_4	−4.56	−1.62	0.123
KH_2PO_4	−2.34	−0.83	0.417
$MgSO_4 \cdot 7H_2O$	2.46	0.87	0.394
Temperature	−2.06	−0.73	0.474
pH	6.80	2.42	0.027

Determination of Optimal Production Medium Conditions using FFD

Three factors played key roles in cellulase production from the experiment in determining the main effect factors by the Plackett–Burman design, as shown in Figs. 6 and 7 and Table 7. To determine the optimal production medium conditions through FFD, the EG activity along the designed experimental conditions was measured, as indicated in

Table 4, while the associated results of analysis of variance are shown in Table 8. As shown in Table 8, the coefficient of determination of R^2 based on regression analysis was evaluated in order to confirm the appropriateness of the model equation.

It was interpreted that the R^2 value close to 1 indicated the similarity between the experimental value and the predicted value (Purama and Goyal 2008). In this study, the high coefficient of determination ($R^2 = 0.9281$) indicated that the experimental value was very close to the predicted value, as shown in Table 8. The regression equation for the model is as follows,

$$Y\ (\text{U/mL}) = -1811 + 54.08X_1 + 13.2X_2 + 357X_3 - 0.903\ X_1{*}X_1 - 0.686X_2{*}X_2 - 29.4X_3{*}X_3 + 0.2396X_1{*}X_2 - 0.22X_1{*}X_3 - 0.01X_2{*}X_3 \quad (4)$$

where Y is EG enzyme activity, X_1 is cellulose, X_2 is tryptone:yeast extract (7:3), and X_3 is pH. As shown in Table 8, the statistical significance of the regression equation using Fisher's F-test was confirmed, with the P-value of <0.001 for the model and the P-value of 0.007 for the appropriateness scarcity indicating the accuracy of the obtained experimental data model. As shown in Table 9, the significance of each coefficient as assumed from the regression analysis of each variance was determined using t-value and P-value. The reliability of the coefficient was high, as shown by the large size of t-value and small P-value (Purama and Goyal 2008). In particular, tryptone:yeast extract (7:3) (X_2) had a more significant effect on cellulase production than the other variables, as confirmed by the P-value (<0.001) and t-value (23.58).

Table 8. ANOVA Table for Response Surface Model

Source	Degrees of freedom	Adj. sum of squares	Adj. mean squares	F-value	P-value
Model	10	74162.6	7416.3	18.08	0.000
Residual error	14	5743.0	410.2		
Lack of fit	4	4170.2	1042.6	6.63	0.007
Pure error	10	1572.7	157.3		
Total	24	79905.5			
R-Sq = 92.81%, R-Sq(adj) = 87.68%					

As shown in Figs. 8 through 10, in a three-dimensional response surface plot, EG activity for two random variables was shown on the Z-axis while maintaining other variables of the median value. These plots by using RSM are helpful to predict the optimal operating conditions from combinations of major variables influencing the cellulase activity among multiple variables. As shown in Fig. 8, the findings on the effects of cellulose and tryptone:yeast extract (7:3) on EG activity showed an increase of EC activity at a cellulose concentration of about 31 $g{\cdot}L^{-1}$ or a tryptone:yeast extract (7:3) concentration of 15 $g{\cdot}L^{-1}$.

Strong interaction between cellulose and tryptone:yeast extract (7:3) was observed, as confirmed by the P-value (0.003) and t-value (17.97) obtained by the t-test, as shown in Table 9. As shown in Fig. 9, regarding the effects of cellulose and pH on EG activity, there was an increase of enzyme activity at a cellulose concentration of about 31 $g{\cdot}L^{-1}$. However, no significant change of enzyme activity upon changing pH was observed; there was also no significant interaction between them. As shown in Fig. 10, regarding the effects of tryptone:yeast extract (7:3) and pH on EG activity, there was an increase of enzyme activity approaching a tryptone:yeast extract (7:3) concentration of 15 $g{\cdot}L^{-1}$, but there was no significant interaction between these variables because in the case of pH, no significant change in surface was observed.

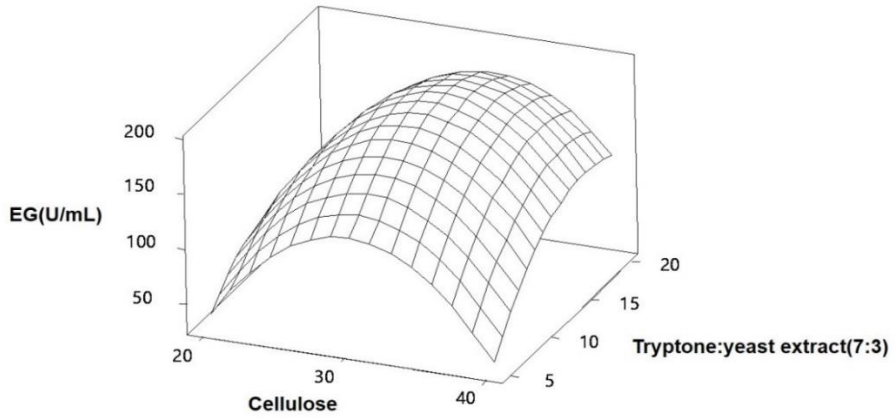

Fig. 8. Effect of cellulose and tryptone:yeast extract (7:3) on EG activity

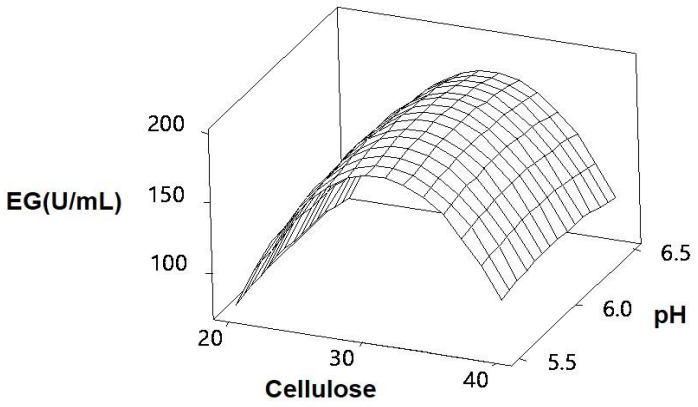

Fig. 9. Effect of cellulose and pH on EG activity

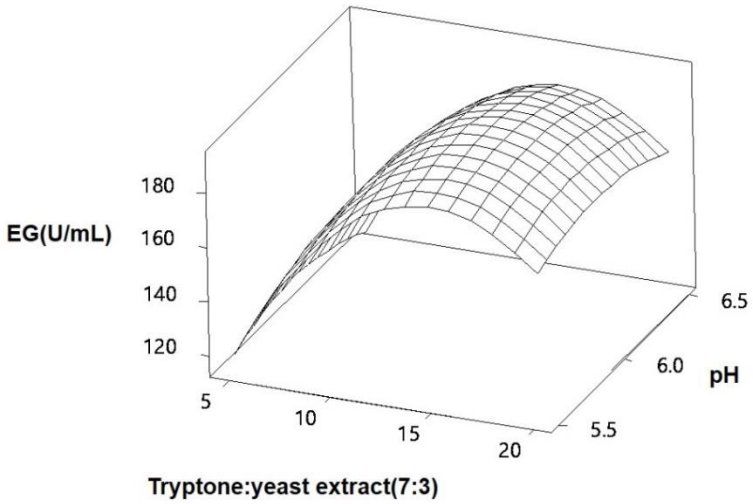

Fig. 10. Effect of tryptone:yeast extract (7:3) and pH on EG activity

The optimal levels of variables were determined through analysis of response surface graph. The optimal incubation conditions for high EG production of *Acanthophysium* sp. KMF001 were a carbon source of cellulose at 31.1 $g \cdot L^{-1}$, a nitrogen source of tryptone:yeast extract (7:3) at 15 $g \cdot L^{-1}$, and pH of 5.94; the predicted value of EG enzyme activity was 192.3 $U \cdot mL^{-1}$.

Table 9. Statistical Analysis of Full Factorial Design Showing Coefficient Value, *T*-Value, and *P*-Value for Each Variable

Variable	Coefficient	*t*-Stat	*P*-value
Intercept	187.35	21.13	0.000
X_1(Cellulose)	15.54	3.25	0.006
X_2(Tryptone: yeast extract (7:3))	23.58	4.94	0.000
X_3(pH)	−1.51	−0.32	0.757
X_1^2	−90.3	−7.26	0.000
X_2^2	−38.6	−3.10	0.008
X_3^2	−7.4	−0.59	0.564
X_1X_2	17.97	3.55	0.003
X_1X_3	−1.10	−0.22	0.831
$^{X}{}_{2}X_3$	−0.04	−0.01	0.994

CONCLUSIONS

1. In this work, the environmental conditions for maximizing cellulase production by novel strain *Achanthophysium* sp. KMF001 screened in the previous study. From the results of investigating the influence of various medium-related factors on the strain's production by the OFAT method as a statistical medium optimization approach, the optimal conditions were determined to be a carbon source of cellulose, nitrogen source of tryptone:yeast extract (7:3), temperature of 30 °C, and pH of 6.0. The response temperature and pH for the optimal enzyme activity were elicited to be 30 °C and 4.5 (sodium citrate), respectively.
2. From the results of the Plackett–Burman design for determining the influence of each selected nutrient component on enzyme activity from the experimental results of OFAT, it was observed that a carbon source of cellulose, nitrogen source of tryptone:yeast extract (7:3), and pH were key factors for enzyme production.
3. From the results confirming the levels of interaction between variables influencing the enzyme activity of crude enzyme using RSM as tested and designed by the addition of axis and point at FFD, it was determined that there was a significant interaction between the carbon source of cellulose and nitrogen source of tryptone:yeast extract (7:3).
4. From interinfluence analysis for each medium-related factor by regression obtained from the statistical analysis using Design Expert Pro 7.0 (Stat-Ease, USA) and the result to elicit the optimum concentration of each medium-related factor for the maximum EG production based on that analysis result, it was possible to obtain the most favorable results with a carbon source of cellulose at 31.1 $g \cdot L^{-1}$, nitrogen source of tryptone:yeast extract (7:3) at 15 $g \cdot L^{-1}$, and pH of 5.94.

ACKNOWLEDGMENTS

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