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Enhanced Production of Cellobiase by a Marine Bacterium, *Cellulophaga lytica* LBH-14, in Pilot-Scaled Bioreactor Using Rice Bran

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The aim of this work was to establish the optimal conditions for the production of cellobiase by a marine bacterium, *Cellulophaga lytica* LBH-14, using response-surface methodology (RSM). The optimal conditions of rice bran, ammonium chloride, and the initial pH of the medium for cell growth were 100.0 g/l, 5.00 g/l, and 7.0, respectively, whereas those for the production of cellobiase were 91.1 g/l, 9.02 g/l, and 6.6, respectively. The optimal concentrations of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ for cell growth were 6.25, 0.62, 0.28, and 0.42 g/l, respectively, whereas those for the production of cellobiase were 4.46, 0.36, 0.27, and 0.73 g/l, respectively. The optimal temperatures for cell growth and for the production of cellobiase by *C. lytica* LBH-14 were 35 and 25℃, respectively. The maximal production of cellobiase in a 100 L bioreactor under optimized conditions in this study was 92.3 U/ml, which was 5.4 times higher than that before optimization. In this study, rice bran and ammonium chloride were developed as carbon and nitrogen sources for the production of cellobiase by *C. lytica* LBH-14. The time for the production of cellobiase by the marine bacterium with submerged fermentations was reduced from 7 to 3 days, which resulted in enhanced productivity of cellobiase and a decrease in its production cost. This study found that the optimal conditions for the production of cellobiase were different from those of CMCase by *C. lytica* LBH-14.

Key words : *Celluophaga lytica*, cellobiase, marine microorganism, rice bran, optimization

Introduction

Conversion of cellulosic materials to fermentable sugars represents a major challenge in global efforts to utilize renewable resources [3]. Complete enzymatic hydrolysis of cellulose requires the synergistic action of three types of enzymes: endoglucanses (carboxymethylcellulase, EC 3.2.1.4), exoglucanases (avicelase, EC 3.2.1.91), and cellobiases (β-glucosidase, EC 3.2.1.21) [2]. Most commercial cellulases have been produced by *Aspergillus* and *Trichoderma* species with solid-state cultures [32]. However, the most widely used cellulases by *T. reesei* are poor in cellobiase, which thus restricts the conversion of cellulosic materials to glucose [6].

Poor activity of cellobiase in cellulases produced by *T. reesei* restricted the conversion of cellobiose to glucose, and the accumulated cellobiose caused severe feedback inhibition to the activities of β -1,4-endoglucanase and β-1,4-exoglucanase in cellulase system [26]. Cellobiases hydrolyzed β-glycosidic bonds between glucose and aryl or alkyl aglycone or olygosaccharides, which resulted in production of glucose and reduced inhibition of cellobiose and allowed enhanced functions of endoglucganase and exoglucanase [2, 27]. Supplementing cellobiase produced by *Aspergillus niger* greatly reduced the inhibitory effect caused by cellobiose, and the hydrolysis yield was improved to 83.9% with enhanced cellobiase activity [5, 9, 32].

Enzymes produced by marine microorganisms can provide numerous advantages over traditional enzymes due to the severe and wide range of environments [18, 20, 28]. Production of carboxymethylcellulase (CMCase) by a marine bacterium, *Cellulophaga lytica* had been reported [10]. In this study, the optimal conditions for the production of cellobiase by *C. lytica* were established using response surface methodology [16, 25]. The optimization of culture medium by the traditional "one-factor-at-a-time" method requires a considerable amount of work and time. An alternative strategy is a statistical approach, for example, RSM involving a mini-

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mum number of experiments for a large number of factors [24].

Materials and Methods

Production of cellobiase by *C. lytica* LBH-14

Starter cultures of *C. lytica* LBH-14 were prepared by transferring cells from agar slants to 50 ml of medium in 250 ml Erlenmeyer flasks [10]. The resulting cultures were incubated at 30℃ for 2 days under aerobic conditions. Each starter culture was used as an inoculum for 150 ml of medium in 500 ml Erlenmeyer flasks. The main culture was carried out in a medium containing 20 g/l carbon source, 2.5 g/l nitrogen source, 5.0 g/l K_2HPO_4, 1.0 g/l NaCl, 0.2 g/l $MgSO_4 \cdot 7H_2O$, and 0.6 g/l $(NH_4)_2SO_4$ at 30℃ for 3 days under aerobic conditions. Batch fermentations for production of cellobiase by *C. lytica* LBH-14 were performed in a 100 l bioreactor (Ko-Biotech Co., Korea). Working volumes of 100 l bioreactors were 70 l and inoculum sizes of batch were 5% (v/v). Agitation was provided by three six-flat-blade impellers. Samples were periodically withdrawn from the cultures to examine cell growth and production of cellobiase.

Experimental design for optimization for production of cellobiase

The rice bran (X_1), ammonium chloride (X_2), and initial pH of the medium (X_3) were chosen as the independent variables of the first experiment for optimization and cell growth (Y_1) and cellobiase (Y_2) were used as a dependent output variable. The interrelationships of the variables were determined by fitting the second degree polynomial equation to data obtained from 20 experiments using mean values of the triplicates of each experiment conducted trice at different occasions. The model constructed as a response function of the variables on cell growth and production of CMCase was a second-order polynomial as follows (Eq. 1).

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

Where, Y is the measured response (cell growth as measured dry cells weight or production of cellobiase), β_0, β_i, and β_{ij} are the regression coefficients, and X_i and X_j are the factors under study. For three variable systems, the model equation is given below (Eq. 2). Regression analysis and estimation of the coefficient were performed using the statistical software, Design-Expert (Version 7.1.6, Stat-Ease Inc., Minneapolis, USA). The contribution of individual parameters and their quadratic and interactive effects on cell growth and production of cellobiase were determined.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (2)$$

The K_2HPO_4 (X_1), NaCl (X_2), $MgSO4 \cdot 7H_2O$ (X_3), and $(NH_4)_2SO_4$ (X_4) were also chosen as the independent variables of the second experiment, and each variable was designated as −1, 0, and 1, respectively. Cell growth (Y_1') and cellobiase (Y_2') were used as dependent output variables. For four variable systems, the model equation is given below (Eq. 3).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 \quad (3)$$

Analytical methods

Dry cells weight was measured by directly weighing the biomass after drying to a constant weight at 100-105℃, after collection of cells by centrifugation at 12,000× *g* for 10 min. Activity of cellobiase produced by *C. lytica* LBH-14 was determined based on the release of reducing sugar from cellobiose using the 3,5-dinitrosalicylic acid (DNS) method, as described in the previous report [15]. Glucose (Sigma-Aldrich, UK) was used to prepare a calibration curve. One unit of each cellulase was defined as the amount of enzyme that released 1μmol of reducing sugar equivalent to glucose per minute under the assay conditions.

Results and Discussion

Effect of carbon and nitrogen sources on production of cellobiase

The effect of carbon and nitrogen sources on cell growth and the production of cellobiase by *C. lytica* LBH-14 was investigated. Carbon sources tested for the production of cellobiase were 20.0 g/l glucose, fructose, maltose, sucrose, rice bran, and rice hulls. Nitrogen sources were 2.5 g/l malt extract, peptone, tryptone, yeast extract, ammonium chloride, and ammonium nitrate. Initial pH of the medium and cultural temperature were 6.8 and 30℃. Production of cellobiase from 36 combinations with 6 carbon sources and 6 nitrogen sources ranged from 6.7 to 17.2 U/ml. Maltose and tryptone were found to be the best combination of carbon

and nitrogen sources for cell growth of *C. lytica* LBH-14, whereas glucose and yeast extract were the best combination for production of CMCase, as shown in Fig. 1. However, a combination of rice bran and ammonium chloride was chosen for next examination based on their cost and availability. Cell mass and production of cellobiase from 20.0 g/l glucose and 2.5 g/l yeast extract were 1.51 g/l and 17.2 U/ml, whereas those from 20.0 g/l rice bran and 5.0 g/l ammonium chloride were 0.69 g/l and 10.1 U/ml.

The best carbon source for the production of cellobiase produced by *Trichoderma reesei* ZU-02 was cellulose, whereas those by *Streptomyces sp. MDS* were carboxymethylcellulose (CMC) and xylan [26, 30]. Wheat straw was reported to be the best carbon source for the production of cellobiase by *Cellomons* sp. [7]. The composition of the rice bran used in this study was as follows: 48.0% carbohydrate, 6.9% fiber, 14.9% crude lipid, 13.1% crude protein, 7.6% ash, and 9.5% water. All strains investigated to date for production of cellulases are inducible by cellulose, lactose or sophorose, and repressible by glucose, which are reasons why the best carbon sources for the production of cellulases by bacterial and fungal strains are rice hulls, rice bran or wheat bran [7, 8, 17, 19]. Induction, synthesis, and secretion of the β-glucanase appear to be closely associated [29].

Optimization of rice bran, ammonium chloride, and initial pH for production of cellobiase

The effect of rice bran, ammonium chloride, and initial pH of the medium on cell growth and the production of cellobiase by *C. lytica* LBH-14 was investigated using one-factor-at-a-time experiment. Concentrations of rice bran and ammonium chloride ranged from 25 to 125 g/l and from 2.5 to 12.5 g/l. The initial pH of the medium ranged from 5.5 to 7.5. Composition of basic medium and culture conditions were 75.0 g/l rice bran, 7.5 g/l ammonium chloride, initial pH of 6.5, and temperature of 30℃. The optimal conditions of rice bran, ammonium chloride, and initial pH for cell growth of *C. lytica* LBH-14 were 125.0 g/l, 5.0 g/l, and 7.5, respectively, whereas those for production of cellobiase were 100.0 g/l, 5.0 g/l, and 7.0, as shown in Fig. 2. The optimal conditions for cell growth of *C. lytica* LBH-14 were found to be different from those for production of cellobiase. Based on results from one-factor-at-a-time experiment, the simultaneous effect of rice bran, ammonium chloride, and initial pH of the medium on cell growth and the production of cellobiase by *C. lytica* LBH-14 was investigated using response surface methodology (RSM). The coded values of minimum and maximum ranges of rice bran (X_1), ammonium chloride (X_2), and initial pH of the medium (X_3) were 50.0 and 100.0 g/l, 5.0 and 10.0 g/l, and 6.0 and 7.0, respectively. Cell mass, measured as dry cells weight (DCW), and production of cellobiase from 20 different conditions ranged from 2.36 to 3.16 g/l and from 45.6 to 62.2 U/ml, as shown in Table 1.

The model *F*-value of 23.45 from the analysis of variance (ANOVA) of cell growth implied that this model was significant, as shown in Table 2. There was only a 0.01% chance that a "Model *F*-value" could occur to die to noise. The *P* values were used as a tool to check the significance of each of the coefficients, which, in turn were necessary to under-

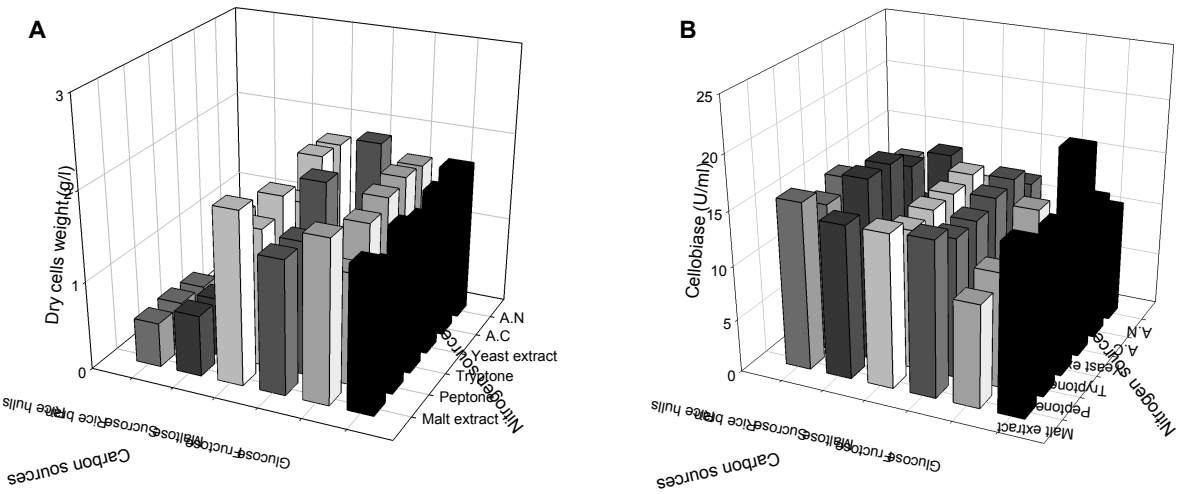

Fig. 1. Effect of carbon and nitrogen sources on cell growth (A) and production of cellobiase (B) by *C. lytica* LBH-14.

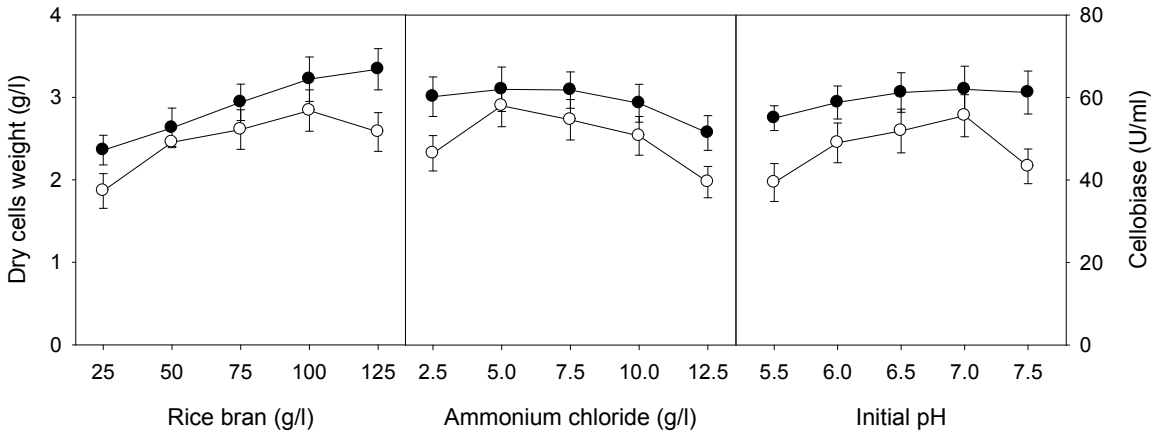

Fig. 2. Effect of rice bran, ammonium chloride, and initial pH of the medium on cell growth (●) and production of cellobiase (○) by *C. lytica* LBH-14.

Table 1. Central composite design and determined response values (Y_1 and Y_2 were DCW and cellobiase, respectively)

Run	X_1	X_2	X_3	Y_1 (g/l)	Y_2 (U/ml)
1	75.0	7.5	6.5	2.99	55.4
2	50.0	10.0	6.0	2.36	45.6
3	50.0	5.0	7.0	2.67	55.2
4	75.0	7.5	6.5	3.04	55.8
5	75.0	7.5	6.5	3.10	55.3
6	75.0	7.5	6.5	3.02	55.0
7	50.0	5.0	6.0	2.58	48.6
8	117.0	7.5	6.5	3.16	57.1
9	100.0	5.0	6.0	3.22	55.6
10	75.0	11.7	6.5	2.73	50.6
11	75.0	7.5	6.5	2.95	54.2
12	100.0	10.0	7.0	3.08	59.4
13	33.0	7.5	6.5	2.51	48.5
14	100.0	5.0	7.0	3.31	62.2
15	50.0	10.0	7.0	2.44	52.2
16	75.0	7.5	5.7	2.88	50.4
17	100.0	10.0	6.0	3.06	51.7
18	75.0	3.3	6.5	2.96	53.9
19	75.0	7.5	6.5	2.90	54.3
20	75.0	7.5	7.3	2.92	57.7

stand the pattern of the mutual interactions between the test variables. The smaller the magnitude of the *P* value, the more significant is the corresponding coefficient. The ANOVA indicated that this model and the model terms of X_1 and X_1^2 ("probe > F" less 0.0001) were highly significant and the model term of X_2 ("probe > F" less 0.0500) was significant for cell growth of *C. lytica* LBH-14. The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R^2 was 0.9511. The model can explain 95.11% variation in the response. The value of the adjusted determination coefficient (Adj. R^2=0.9070) was very high to advocate for a high significance of this model [18]. The predicted determination of coefficient of 0.8368 was in reasonable agreement with the Adj. R^2 of 0.9070. From the statistical results obtained, it was shown that the above models were adequate to predict the cell growth of *C. lytica* LBH-14 within the range of variables studied. Multiple regression analysis of the experimental data gave the following second-order polynomial equation in terms of coded factors (Eq. 4). The optimal conditions of rice bran, ammonium chloride, and initial pH of the medium for cell growth extracted by Design Expert Software were 100.0 g/l, 5.00 g/l,

Table 2. Parameter estimates and analysis of variance (ANOVA) of the design for cell growth production of cellobiase by *C. lytica* LBH-14

	Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*-value	Probe>F
Cell growth	Model	9	1025.960	114.000	23.45	<0.0001
	X_1	1	337.950	337.950	69.53	<0.0001
	X_2	1	29.200	29.200	6.01	0.0342
	X_3	1	10.100	10.100	2.08	0.1800
	X_1^2	1	511.300	511.300	105.20	<0.0001
	X_2^2	1	162.480	162.480	33.43	0.0002
	X_3^2	1	68.070	68.070	14.01	0.0038
	Error	5	4.930	0.990	-	-
	Total	19	1074.570	-	-	-
Cellobiase	Model	9	280.950	31.220	22.76	<0.0001
	X_1	1	127.710	127.710	93.12	<0.0001
	X_2	1	24.390	24.390	17.78	0.0018
	X_3	1	115.860	115.860	84.47	<0.0001
	X_1^2	1	4.990	4.990	3.64	0.0857
	X_2^2	1	8.830	8.830	6.44	0.0295
	X_3^2	1	0.310	0.310	0.22	0.6456
	Error	5	2.020	0.400	-	-
	Total	19	294.670	-	-	-

and 7.0, respectively. The maximum cell growth of 3.15 g/l was predicted by this model.

$$Y_1 = 3.05 + 0.09X_1 - 0.02X_2 + 0.02X_3 - 0.01X_1X_2 + 0.01X_1X_3 - 0.01X_2X_3 - 0.02X_1^2 - 0.01X_2^2 - 0.02X_3^2 \quad (4)$$

The model *F*-value of 22.76 from the ANOVA of production of cellobiase implied that this model was also significant. The ANOVA indicated that this model and the model terms of X_1 and X_3 were highly significant and those of X_2 and X_2^2 were significant for the production of cellobiase by *C. lytica* LBH-14. The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R^2 was 0.9698. The value of the adjusted determination coefficient (Adj. R^2=0.9426) was high to advocate for a high significance of this model. The predicted determination of coefficient of 0.8621 was also in reasonable agreement with the Adj. R^2 of 0.9426. Multiple regression analysis of the experimental data gave the following second-order polynomial equation in terms of coded factors (Eq. 5). The optimal conditions of rice bran, ammonium chloride, and initial pH of the medium for production of cellobiase were 91.1 g/l, 9.02 g/l, and 6.6, respectively. The maximum production of cellobiase of 55.6 U/ml was predicted by this model.

$$Y_2 = 54.61 + 0.88X_1 + 1.62X_2 + 0.93X_3 + 0.04X_1X_2 + 0.14X_1X_3 + 0.01X_2X_3 - 0.64X_1^2 - 1.22X_2^2 - 1.10X_3^2 \quad (5)$$

The three-dimensional response surface was generated to study the interaction among three factors tested and to visualize the combined effects of factors on the response of the production of cellobiase by *C. lytica* LBH-14, as shown Fig. 3. When the effect of two factors was plotted, the other two factors were set at the coded value zero, which were 75.0 g/l rice bran, 7.5 g/l ammonium chloride, and initial pH of 6.5. This kind of graphical visualization allows the relationships between the experimental levels of each factor and the response to be investigated, and the type of interactions between test variables to be determined, which is necessary to establish the optimal conditions for production of cellobiase. In contrast to the circular shapes, the elliptical nature of curves indicates more significant mutual interactions between variables. The most significant combination of variables on production of cellobiase was rice bran and initial pH ("Probe > F"=0.4946). *P* value of combined effect of rice bran and ammonium chloride was 0.8506 and that of ammonium chloride and initial pH was 0.9499.

Optimization of salts in medium for production of cellobiase

The optimal concentrations of salts in the medium for cell growth and the production of cellobiase by *C. lytica* LBH-14 were also investigated using one-factor-at-a-time experiments. Carbon and nitrogen sources and initial pH of the medium were 91.1 g/l rice bran, 9.02 g/l ammonium chloride and 6.6, which were previously optimized in this study.

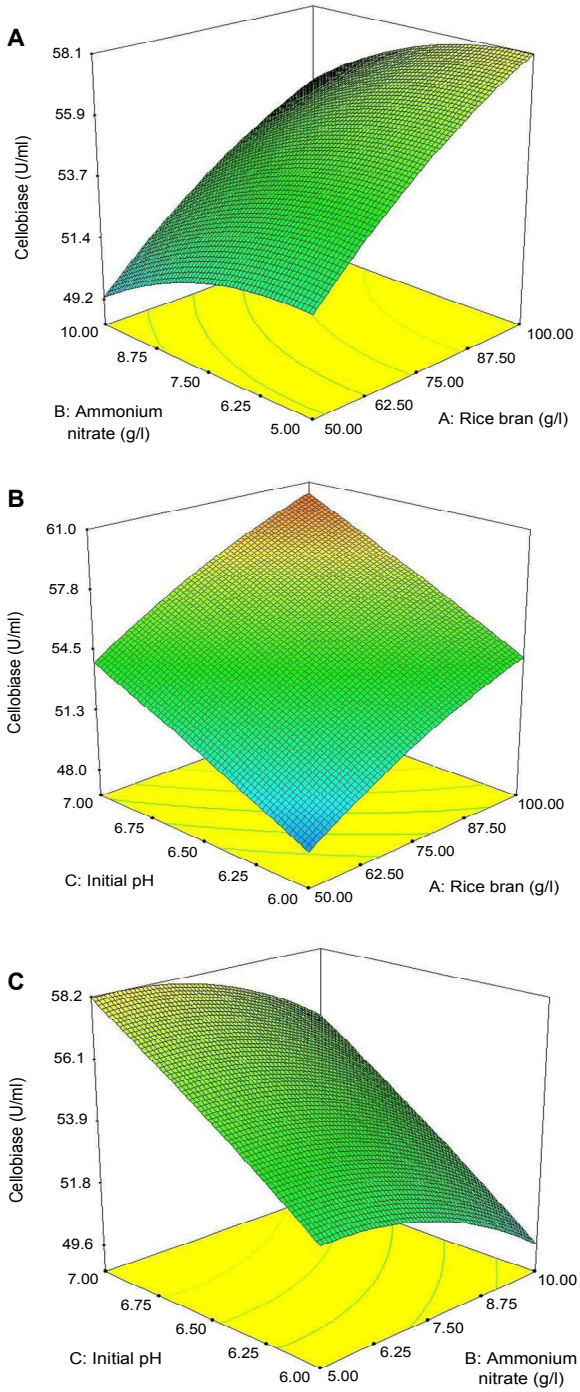

Fig. 3. Three-dimensional response surface of production of cellobiase by *C. lytica* LBH-14 as functions of rice bran and ammonium chloride (A), rice bran and initial pH (B), and ammonium chloride and initial pH (C).

Concentrations of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ ranged from 0.0 to 10.0 g/l, from 0.0 to 2.0 g/l, from 0.0 to 0.8 g/l, and from 0.0 to 1.2 g/l, respectively. Concentrations of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ in the basic medium were 5.0, 1.0, 0.4, and 0.6 g/l, respectively. The optimal concentrations of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ for cell growth of *C. lytica* LBH-14 were 7.5, 0.5, 0.2, and 0.3 g/l, respectively, whereas those for production of cellobiase were 2.5, 0.5, 0.2, and 0.6 g/l, as shown in Fig. 4. The optimal concentrations of salts in the medium for cell growth were also different from those for production of cellobiase. Based on results from one-factor-at-a-time experiment, the optimal concentrations of salts in the medium for cell growth and the production of cellobiase by *C. lytica* LBH-14 were also investigated using RSM. The coded values of minimum and maximum ranges of K_2HPO_4 (X_1), NaCl (X_2), $MgSO_4 \cdot {}_{7H2}O$ (X_3), and $(NH_4)_2SO_4$ (X_4) were 2.5 and 7.5 g/l, 0.25 and 0.75 g/l, 0.25 and 0.75 g/l, and 0.3 and 0.9 g/l, respectively. Cell mass and production of cellobiase from 30 different conditions ranged from 2.79 to 3.17 g/l, from 82.1 to 84.6 U/ml, as shown in Table 3.

The model *F*-value of 9.88 from the ANOVA of cell growth implied that this model was significant, as shown in Table 4. The ANOVA indicated that this model and the model terms of X_1^2 and X_3^2 were highly significant ("probe > F" less 0.0001) and those of X_1, X_2, X_3, X_4, X_2^2 and X_4^2 were significant ("probe > F" less 0.0500) for cell growth of *C. lytica* LBH-14. The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R^2 was 0.9021. The value of the adjusted determination coefficient (Adj. R^2=0.8108) was very high to advocate for a high significance of this model. The predicted determination of coefficient of 0.5141 was in reasonable agreement with the Adj. R^2 of 0.8108. Multiple regression analysis of the experimental data gave the following second-order polynomial equation in terms of coded factors (Eq. 6). The optimal concentrations of K_2HPO_4, NaCl, $MgSO_4 \cdot {}_{7H2}O$, and $(NH_4)_2SO_4$ for cell growth were 6.25, 0.62, 0.28, and 0.42 g/l, respectively. The maximum cell growth of 3.57 g/l was predicted by this model.

$$Y_1' = 3.56 + 0.01X_1 + 0.01X_2 - 0.01X_3 - 0.01X_4 - 0.01X_1^2 - 0.01X_2^2 - 0.01X_3^2 - 0.01X_4^2 \quad (6)$$

The model *F*-value of 13.87 from the ANOVA of production of cellobiase implied that this model was also significant. The ANOVA indicated that this model and the model terms of X_2, X_3, and X_1^2 were highly significant and that of X_4, X_2^2, X_3^2, and X_4^2 were significant for then production of cellobiase by *C. lytica* LBH-14. The regression equation obtained from ANOVA indicated that the multiple

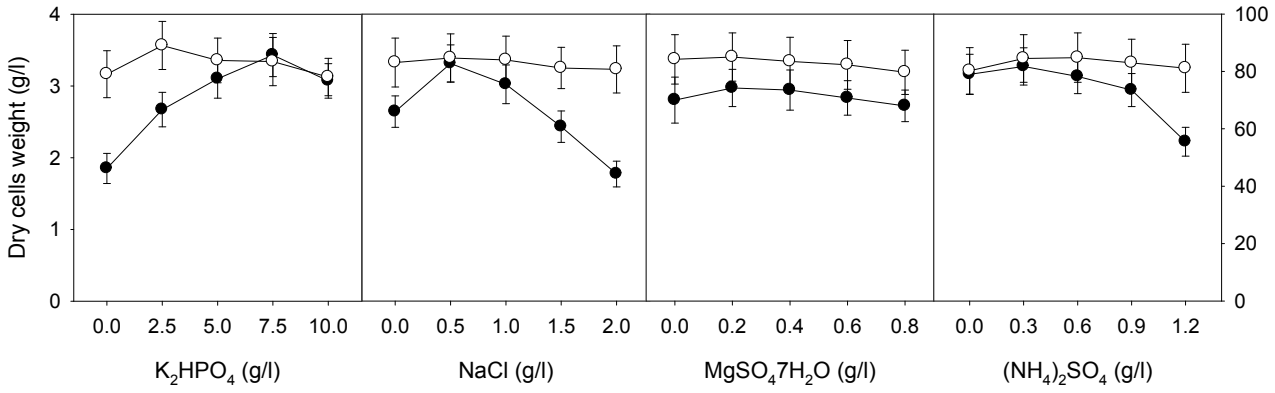

Fig. 4. Effect of K_2HPO_4, NaCl, $MgSO4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ on cell growth (●) and production of cellobiase (○) by *C. lytica* LBH-14.

Table 3. Central composite design and determined response values (Y_1 and Y_2 were DCW and cellobiase, respectively)

Run	X_1	X_2	X_3	X_4	Y_1 (g/l)	Y_2 (U/ml)
1	2.5	0.25	0.3	0.3	2.89	83.9
2	7.5	0.25	0.3	0.9	3.00	83.1
3	5.0	0.50	0.2	0.6	3.10	84.1
4	7.5	0.75	0.3	0.3	3.25	83.0
5	2.5	0.75	0.1	0.9	2.97	83.7
6	5.0	0.50	0.0	0.6	3.06	84.1
7	5.0	1.00	0.2	0.6	3.10	83.9
8	5.0	0.50	0.2	0.6	3.15	84.3
9	2.5	0.75	0.3	0.9	2.98	83.5
10	5.0	0.50	0.2	0.6	3.17	84.5
11	7.5	0.25	0.1	0.9	2.98	83.3
12	7.5	0.75	0.1	0.9	3.16	83.0
13	2.5	0.75	0.1	0.3	3.05	83.8
14	2.5	0.25	0.1	0.9	2.79	84.0
15	5.0	0.50	0.2	0.6	3.18	84.4
16	2.5	0.25	0.3	0.9	2.81	83.8
17	5.0	0.50	0.2	0.6	3.09	84.0
18	5.0	0.00	0.2	0.6	2.96	84.1
19	5.0	0.50	0.2	1.2	2.88	83.3
20	5.0	0.50	0.4	0.6	3.07	84.6
21	7.5	0.75	0.3	0.9	3.17	82.8
22	7.5	0.25	0.1	0.3	3.07	83.4
23	7.5	0.75	0.1	0.3	3.24	83.1
24	2.5	0.25	0.1	0.3	2.88	84.1
25	2.5	0.75	0.3	0.3	3.06	83.7
26	5.0	0.50	0.2	0.6	3.12	83.9
27	7.5	0.25	0.3	0.3	3.08	83.2
28	5.0	0.50	0.2	0.0	3.11	83.0
29	0.0	0.50	0.2	0.6	2.79	83.1
30	10.0	0.50	0.2	0.6	3.10	82.1

correlation coefficient of R^2 was 0.9021. The value of the adjusted determination coefficient (Adj. R^2=0.8108) was very high to advocate for a high significance of this model. The predicted determination of coefficient of 0.5141 was in reasonable agreement with the Adj. R^2 of 0.8108. Multiple regression analysis of the experimental data gave the following second-order polynomial equation in terms of coded factors (Eq. 7). The optimal concentrations of K_2HPO_4, NaCl,

Table 4. Parameter estimates and analysis of variance (ANOVA) of the design for cell growth and production of cellobiase by *C. lytica* LBH-14

	Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*-value	Probe>F
Cell growth	Model	14	0.016	0.001	9.88	<0.0001
	X_1	1	0.002	0.002	20.37	0.0004
	X_2	1	0.001	0.001	5.09	0.0394
	X_3	1	0.002	0.002	20.37	0.0004
	X_4	1	0.002	0.002	17.12	0.0009
	X_1^2	1	0.005	0.005	40.62	<0.0001
	X_2^2	1	0.002	0.002	20.83	0.0004
	X_3^2	1	0.004	0.004	33.30	<0.0001
	X_4^2	1	0.001	0.001	4.63	0.0482
	Error	5	0.006	0.001	-	-
	Total	29	0.450	-	-	-
Cellobiase	Model	14	6.29	0.45	13.87	<0.0001
	X_1	1	0.01	0.01	0.32	0.5790
	X_2	1	1.76	1.76	54.35	<0.0001
	X_3	1	1.35	1.35	41.80	<0.0001
	X_4	1	0.15	0.15	4.64	0.0478
	X_1^2	1	1.62	1.62	49.89	<0.0001
	X_2^2	1	0.13	0.13	4.12	0.0604
	X_3^2	1	0.24	0.24	7.28	0.0165
	X_4^2	1	0.77	0.77	23.82	0.0002
	Error	5	0.00	0.00	-	-
	Total	29	6.77	-	-	-

$MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ for production of cellobiase were 4.16, 0.36, 0.27, and 0.73 g/l, respectively. The maximum production of cellobiase of 84.4 U/ml was predicted by this model.

$$Y_2' = 82.8 + 0.45X_1 - 2.45X_2 + 1.08X_3 + 3.60X_4 + 0.05X_1 \cdot X_2 + 0.01X_1 \cdot X_3 - 0.13X_1 \cdot X_4 + 0.70X_2 \cdot X_3 - 0.580.01X_2 \cdot X_4 - 0.04X_1^2 + 1.12X_2^2 - 1.48X_3^2 - 1.86X_4^2 \quad (7)$$

Analysis using the statistical method indicated that the most significant factor for cell growth was K_2HPO_4. However, that for production of cellobiase was NaCl and $MgSO_4 \cdot 7H_2O$. Potassium phosphate is one of major salts in the medium for productions of microbial polysaccharides and enzymes as well as a well-known ingredient in buffer solutions [13, 23]. Sodium chloride was reported to be used as a physiological modulator of biosynthetic pathway of biopolymers [12, 24]. Magnesium sulfate added to media assists with spore germination and initial growth of *A. fisheri*, which results in 1.9 fold increased production of xylanase [31]. Sulphur starvation affected the level of proteins more than nitrogen deprivation, which was coupled with the accumulation of glutamine, asparagine and serine, additionally, a decrease in both glutathione and cysteine levels [4].

Effect of temperature on production of cellobiase

The effect of temperature on cell growth and the production of cellobiase by *C. lytica* LBH-14 was examined. Carbon and nitrogen sources and initial pH of the medium were 91.0 g/l rice bran, 9.02 g/l ammonium chloride and 6.6. And concentrations of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ were 4.16, 0.36, 0.27, and 0.73 g/l, respectively, which were optimized in this study. The temperature for cell growth and production of cellobiase ranged from 20 to 40℃. The optimal temperature for cell growth of *C. lytica* LBH-14 was found to be 35℃, whereas those for production of cellobiase were 25℃, as shown in Fig. 5. The cell growth and production of cellobiase at 35℃ were 3.18 g/l and 79.3 U/ml, whereas those at 25℃ were 2.45 g/l and 91.7 U/ml.

The optimal temperatures for the production of cellobiase by A. niger was 30℃ and those for the production of filter paperase (FPase) by *T. reesei* QM9414 and *T. reesei* MCG77 from rice bran in solid-state fermentation were 30 and 25℃, respectively [1, 21]. The optimal temperature for production of CMCase by *C. lytica* LBH-14 had been reported to be 25℃ [10]. The optimal temperature for production of cellobiase was the same as that of CMCase by *C. lytica* LBH-14. However, those of cellobiase and CMCase were different

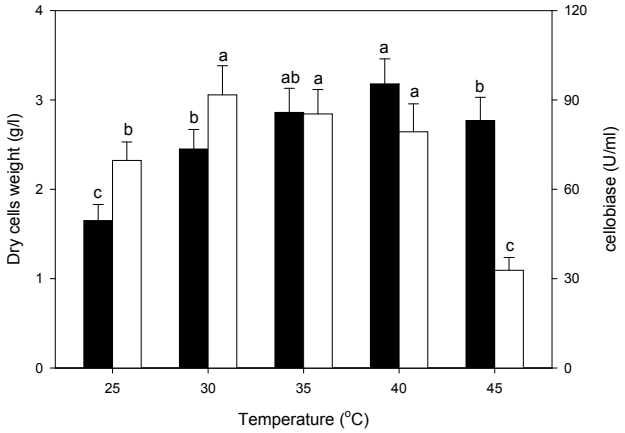

Fig. 5. Effect of temperature on cell growth and production of cellobiase by *C. lytica* LBH-14 (■, DCW and □, cellobiase).

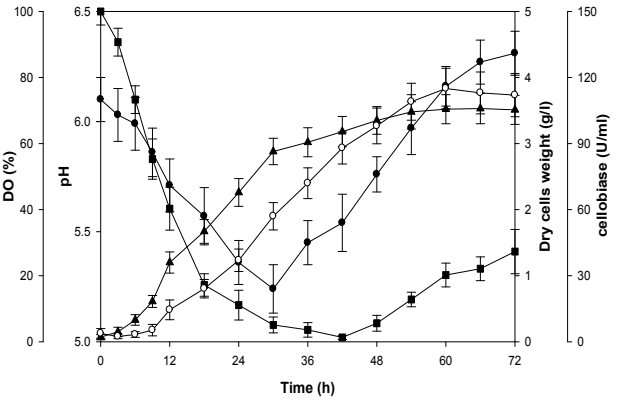

Fig. 6. Pilot-scaled production of cellobiase by *C. lytica* LBH-14 in a 100 l bioreactor (●, pH; ■, DO; ▲, DCW; and ○, cellobiase).

from that for cell growth of *C. lytica* LBH-14. The optimal temperatures for cell growth of *B. amyloliquefaciens* and *B. subtilis* subsp. *subtilis* were 32 and 35℃, respectively, whereas those for production of CMCase were 37 and 30℃, respectively [14, 22].

Mass production of cellobiase under optimized conditions

Batch culture for the production of cellobiase by *C. lytica* LBH-14 was performed in a 100 l bioreactor under optimized conditions in this study. Carbon and nitrogen source was 91.1 g/l rice bran and 9.02 g/l ammonium chloride. The initial pH and cultural temperature were 6.6 and 25℃. Agitation speed and aeration rate of a 100 l bioreactor were 200 rpm and 1.0 vvm. The pH in the medium dramatically decreased until 30 h of cultivation, and then gradually increased at approximately 6.3 thereafter, as shown in Fig. 6. Decrease in pH might result from accumulated production of organic acids from substrates in the medium, which were used as precursors for biosynthetic pathway for cell growth. Dissolved oxygen in the medium also dramatically decreased until 18 h and gradually increased after 42 h. Cell growth of *C. lytica* LBH-14 rapidly increased until 30 h. Production of cellobiase started after a dramatic decrease in the concentration of dissolved oxygen at 9 h. The production of cellobiase by *C. lytica* LBH-14 appeared to be paralleled with cell growth.

A major constrain in enzymatic saccharification of cellulosic materials for fermentable sugars is the cost of cellulases and low productivity [33]. Corncob had been used for production of cellobiase, but cost for pretreatment of corncob was too expensive to apply commercially [32]. Production of cellobiase from many forms of lignocellulo-

Table 5. Comparison of optimal conditions using two experimental methods for cell growth and production of cellobiase by *C. lytica* LBH-14

Scale	Optimal conditions	One factor at a time experiment		Response surface method	
		DCW	Cellobiase	DCW	Cellobiase
Flask scale 1	Rice bran (g/l)	125	100	100.0	91.1
	Ammonium nitrate (g/l)	5.0	5.0	5.00	9.02
	Initial pH	7.0	7.0	7.0	6.6
	Maximal production	3.84 g/l	58.0 U/ml	3.15 g/l	55.6 U/ml
Flask scale 2	K_2HPO_4 (g/l)	7.5	2.5	6.25	4.16
	NaCl (g/l)	0.5	0.5	0.62	0.36
	$MgSO4 \cdot 7H_2O$ (g/l)	0.2	0.2	0.28	0.27
	$(NH_4)_2SO_4$ (g/l)	0.3	0.6	0.42	0.73
	Maximal production	3.43 g/l	84.8 U/ml	3.57 g/L	84.4 U/ml
Flask scale 3	Temperature (℃)	35	25	-	-
	Maximal production	3.18 g/l	91.7 U/ml	-	-

Table 6. Comparison of optimal conditions for cell growth and production of cellobiase and CMCase by *C. lytica* LBH-14

		DCW	Cellobiase	CMCase[1)]
Flask scale 1	Rice bran (g/l)	100.0	91.1	79.9
	Ammonium nitrate (g/l)	5.00	9.02	8.52
	Initial pH	7.0	6.6	6.1
	Maximal production	3.15 g/l	55.6 U/ml	70.1 U/ml
Flask scale 2	K_2HPO_4 (g/l)	6.25	4.16	3.72
	NaCl (g/l)	0.62	0.36	0.54
	$MgSO4 \cdot 7H_2O$ (g/l)	0.28	0.27	0.70
	$(NH_4)_2SO_4$ (g/l)	0.42	0.73	0.34
	Maximal production	3.57 g/l	84.4 U/ml	105.4 U/ml
Flask scale 3	Temperature (℃)	35	25	25
	Maximal production	3.18 g/l	91.7 U/ml	110.8 U/ml

[1)]Reference 10

sic biomass can improve the economics of cellobiase production [11]. In this study, optimization conditions for the production of cellobiase by *C. lytica* LBH-14 from rice bran and ammonium chloride was established using response surface method (RSM), as shown in Table 5. Rice bran from the rice processing industry is produced in large amounts in Korea, as well as other rice producing countries. Low-cost ammonium nitrate as a nitrogen source is also available in large quantities. The production of cellobiase by *C. lytica* LBH-14 in a 100 l bioreactor under optimized conditions in the flask scale was almost the same as the maximal production of cellobiase obtained in the flask scale, which meant that the optimized conditions in this study would be directly applied for mass production of cellobiase in an industrial scale. Time to produce cellobiase by fungal species in solid-state fermentation normally takes 4 to 10 days [26]. The educed time of 3 days for production of cellobiase using a marine bacterium with submerged fermentations in this study also results in increase in productivity of cellobiase and decrease in its production cost. The optimal conditions for production of CMCase by *C. lytica* LBH-14 in the previous study had been reported [10]. In this study, the optimal conditions for production of cellobiase was found to be different from those of CMCase by *C. lytica* LBH-14, as shown in Table 6.

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초록 : 파이롯트 규모에서 미강을 이용한 해양미생물 *Cellulophaga lytica* LBH-14 유래의 cellobiase 생산

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본 연구의 목적은 통계학적 방법을 사용하여 해양미생물 *Cellulophaga lytica* LBH-14가 생산하는 cellobiase의 생산조건을 확립하는 것이었다. 이 균주의 생육에 최적인 미강, ammonium chloride 및 배지의 초기 pH는 100.0 g/l, 5.00 g/l 및 7.0이었으나, 이 균주가 생산하는 cellobiase의 생산에 최적인 조건은 각각 91.1 g/l, 9.02 g/l 및 6.6이었다. 이 균주의 생육에 최적인 K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$ 및 $(NH_4)_2SO_4$ 등과 같은 배지의 염농도는 각각 6.25, 0.62, 0.28 및 0.73 g/l이었으나, cellobiase 생산에 최적인 염들의 농도는 각각 4.46, 0.36, 0.27 및 0.73 g/l이었다. 또한, 균체의 생육 및 cellobiase의 생산에 최적인 온도는 각각 35 및 25℃이었다. 플라스크 규모에서 최적화한 조건으로 파이롯트 규모의 생물배양기에서 cellobiase를 생산한 결과, 이 균주가 생산하는 cellobiase의 생산성은 92.3 U/ml이었으며, 이는 최적화하기 전에 비하여 5.4배 향상된 것 이었다. 본 연구를 통하여 쌀 도정공정의 부산물인 미강 및 ammonium chloride를 cellobiase를 생산하는 기질로 개발하였으며 해양 미생물을 사용하여 cellobiase의 생산기간을 7일에서 3일로 단축시켰다. 또한, 본 연구를 통하여 *C. lytica* LBH-14가 생산하는 cellobiase의 최적 생산조건은 이 균주가 생산하는 CMCase의 최적 생산조건과 다르다는 사실을 확인하였다.