Korean J. Chem. Eng., **30**(5), 1105-1110 (2013)
DOI: 10.1007/s11814-012-0219-5

Enhanced production of carboxymethylcellulase by *Cellulophaga lytica* LBH-14 in pilot-scale bioreactor under optimized conditions involved in dissolved oxygen

Wa Cao*,, Sang-Un Lee*,**, Jianhong Li***, and Jin-Woo Lee**,****,†**

*Department of Medical Bioscience, Graduate School of Dong-A University, Busan 604-714, Korea
**BK21 Bio-Silver Program of Dong-A, Busan 604-714, Korea
***College of Plant Science & Technology, Huazhong Agricultural University, Wuhan, Hebei 430070, China
****Department of Biotechnology, College of Natural Resources and Life Science, Dong-A University, Busan 604-714, Korea
(*Received 17 August 2012 • accepted 16 December 2012*)

Abstract−The optimal conditions for the production of carboxymethylcellulase (CMCase) by *Cellulophaga lytica* LBH-14 at flask scale has been previously reported. In this study, we optimized the parameters involved in dissolved oxygen in 7 and 100 L bioreactors for pilot-scaled production of CMCase by *C. lytica* LBH-14. The optimal conditions of agitation speed and aeration rate for cell growth in 7 L bioreactors were 395 rpm and 0.98 vvm, whereas those for production of CMCase were 357 rpm and 0.55 vvm. The optimal inner pressures for cell growth and production of CMCase by *C. lytica* LBH-14 in 100 L bioreactors were 0.00 and 0.06 MPa, respectively. The production of CMCase under an optimized inner pressure was 1.38 times higher than that without an inner pressure. The maximal production of CMCase by *C. lytica* under optimized conditions at pilot scale using rice bran and ammonium chloride was 153.6 U/mL, which was 1.39 times higher than that at flask scale.

Key words: *Cellulophaga lytica*, Carboxymethylcellulase, Marine Microorganism, Pilot-scaled Production, Response Surface Method

INTRODUCTION

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) [1]. The enzymatic saccharification of lignocellulosic materials for the production of ethanol was performed by commercial cellulases, in which the major cellulase was carboxymethylcellulase [2,3]. Most commercial cellulases are produced by solid state fermentations of fungal species and their production normally takes 7 to 10 days [4,5]. However, the time for production of carboxymethylcellulase (CMCase) by marine bacteria with submerged cultures has been reported to be 3 to 5 days [6,7].

Enzymes are produced at large-scale mostly in batch fermentation in stirred tank bioreactors [8]. The factors affected by scale-up are the number of generations, the mutation probability, the quality of temperature, and pH controls, agitation, aeration, and inner pressure [9]. Oxygen transfer often can be most important upon scale-up due to its low solubility in medium [10]. The oxygen transfer rate into the medium can be influenced by agitation speed, aeration rate, and the inner pressure of bioreactors [11]. In this study, the optimal agitation speed and aeration rate for cell growth and the production of CMCase by *C. lytica* LBH-14 were established using response surface methodology (RSM). And the inner pressure of pilot-scaled bioreactors was optimized for industrial-scaled production of CMCase.

†To whom correspondence should be addressed.
E-mail: jwlee@dau.ac.kr

MATERIALS AND METHODS

1. Bacterial Strain Producing CMCase

Cellulophaga lytica LBH-14, which had been isolated from seawater in the previous study, was used for producing carboxymethylcellulase (CMCase) [12]. The medium used for the production of CMCase contained the following components: 80.0 g/L rice bran, 8.52 g/L ammonium chloride, 3.72 g/L K_2HPO_4, 0.54 g/L NaCl, 0.70 g/L $MgSO_4 \cdot 7H_2O$, and 0.34 g/L $(NH_4)_2SO_4$.

2. Production of CMCase by *C. lytica* LBH-14

Starter cultures for the production of CMCase by *C. lytica* LBH-14 were prepared by transferring cells from agar slants to 50 mL of medium in 250 mL Erlenmeyer flasks. The resulting cultures were incubated at 30 °C 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 in the above-mentioned medium for 3 days under aerobic conditions [12]. Batch fermentations for the production of CMCase by *C. lytica* LBH-14 were performed in 7 and 100 L bioreactors (Ko-Biotech Co., Incheon, Korea). Working volumes of the 7 and 100 L bioreactors were 5 and 70 L, respectively. Inoculum sizes of batch fermentations for production of cellulases were 5% (v/v). Agitation was provided by three six-flat-blade impellers in 7 and L bioreactors. Samples were periodically withdrawn from the cultures to examine cell growth and production of CMCase.

3. Experimental Design Using Response Surface Methodology

The agitation speed (X_1) and aeration rate (X_2) were also chosen as the independent variables and cell growth (Y_1) and CMCase (Y_2) were used as a dependent output variable. The model constructed as a response function of the variables on cell growth and produc-

tion 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 CMCase), 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)).

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{12}X_1X_2 \quad (2)$$

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). Regression analysis and estimation of the coefficient were performed with 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 CMCase were determined.

4. Analytical Methods

Dry cells weight was measured as described in the previous report [13]. Activity of CMCase was determined based on the release of reducing sugar from CMC using the 3,5-dinitrosalicylic acid (DNS) method, as described in the previous report [14]. Glucose (Sigma-Aldrich, St. Louis, USA) was used to prepare a calibration curve. One unit of each CMCase was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to glucose per minute under the assay condition.

RESULTS AND DISCUSSION

1. Effect of Agitation and Aeration on Production of CMCase

The effect of agitation speed on cell growth of CMCase by *C. lytica* LBH-14 in a 7 L bioreactor was investigated using the one-factor-at-a-time method. Carbon and nitrogen sources for production of CMCase were 80.0 g/L rice bran and 8.52 g/L ammonium chloride [12]. The initial pH of the medium and temperature were 6.1 and 25 °C. Agitation speed ranged from 200 to 500 rpm and aeration rate was 1.0 vvm. Higher agitation speeds and aeration rates, which resulted in an increase of dissolved oxygen in the medium, enhanced cell growth as well as production of CMCase, as shown in Fig. 1. The optimal agitation speeds for cell growth and production of CMCase were 400 rpm. The maximal cell growth, measured as dry cell weight (DCW) and production of CMCase were 3.23 g/L and 118.2 U/mL, respectively. The effect of aeration rate on cell growth, and production of CMCase also was investigated. Aeration rate ranged from 0.5 to 2.0 vvm and the agitation speed was fixed to 400 rpm. The optimal aeration rate for cell growth was 1.0 vvm, whereas that for production of CMCase was 0.5 vvm. The maximal cell growth and production of CMCase were 3.15 g/L and 121.9 U/mL, as shown in Fig. 2. The optimal agitation speed and aeration rate for cell growth have been reported to be higher than those for the production of CMCase [6,7]. It seems that a dissolved oxygen level higher than the optimal concentration for the production of CMCase by *C. lytica* LBH-14, due to higher agitation speeds and aeration rates, leads the biosynthetic pathway to cell growth, but not to production of CMCase.

2. Simultaneous Effect of Agitation and Aeration on Production of CMCase

Based on results from one-factor-at-a-time experiments as shown in Fig. 3, the simultaneous effect of agitation speed and aeration rate on cell growth and the production of CMCase by *C. lytica* LBH-14 was investigated using the response surface method (RSM). The minimum and maximum range of two variables, agitation speed and aeration rate were 300 and 500 and 0.5 and 1.5 vvm. The results of central composite design (CCD) experiments consisted of experimental data to investigate the effect of two independent variables, as shown in Table 1. Cell growth and production of CMCase

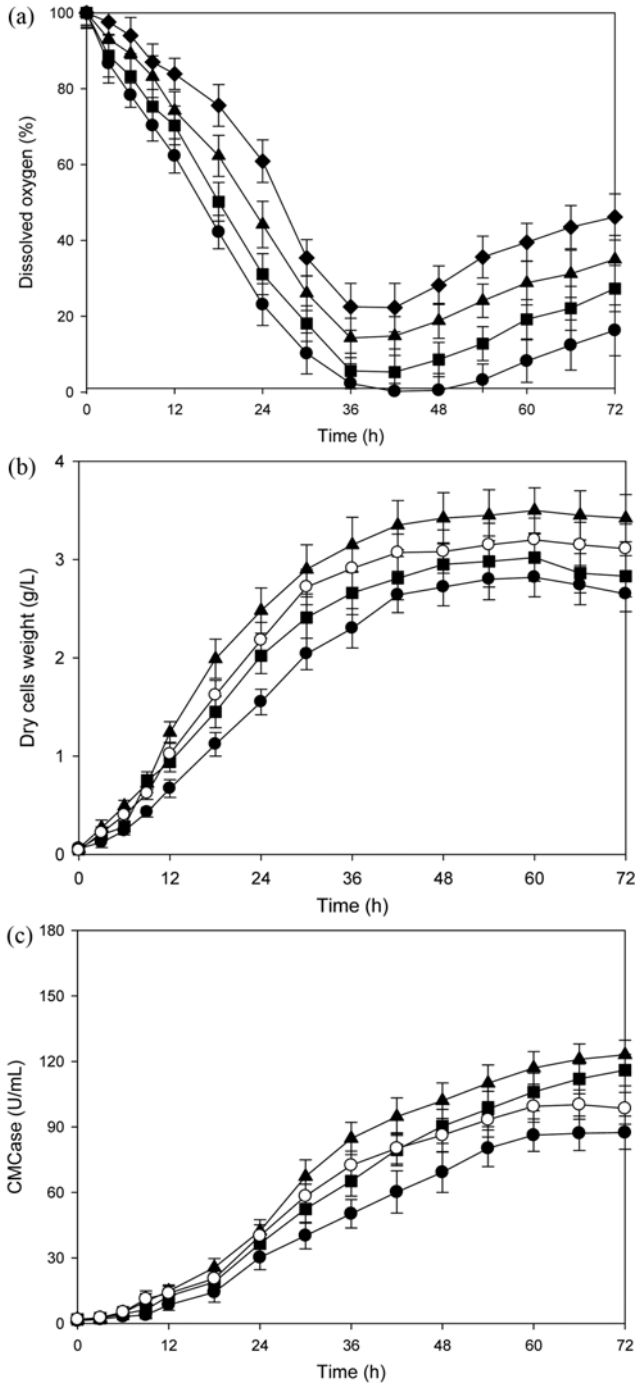

Fig. 1. Effect of agitation speed on dissolved oxygen (a), cell growth (b), and production of CMCase (c) by *C. lytica* LBH-14 in 7 L bioreactors (●, 200 rpm; ■, 300 rpm; ▲, 400 rpm; and △, 500 rpm).

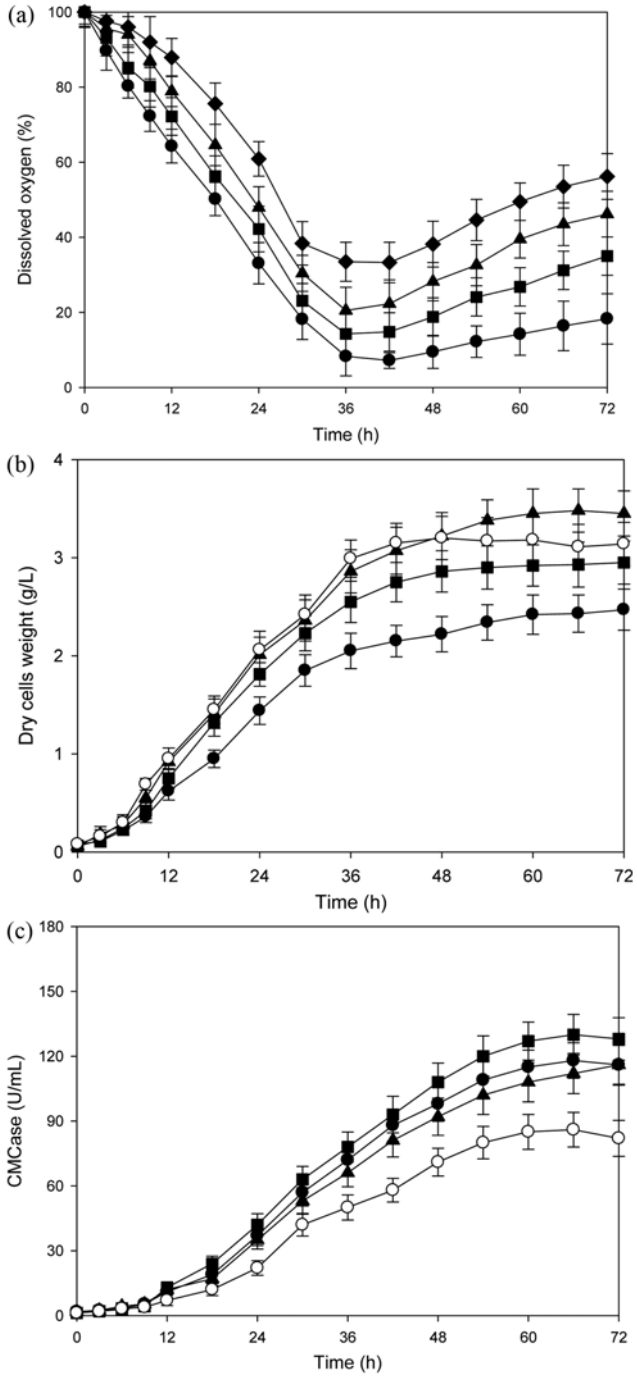

Fig. 2. Effect of agitation speed on dissolved oxygen (a), cell growth (b), and production of CMCase (c) by *C. lytica* LBH-14 in 7 L bioreactors (●, 0.5 vvm; ■, 1.0 vvm; ▲, 1.5 vvm; and △, 2.0 vvm).

from 13 different conditions ranged from 2.98 to 3.24 g/L and from 85.4 to 119.4 U/mL, respectively. The model *F*-value of 34.02 from the ANOVA of cell growth implied that this model was significant, as shown in Table 2. The *P* values were used as a tool to check the significance of each of the coefficients, which, in turn were necessary to understand 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

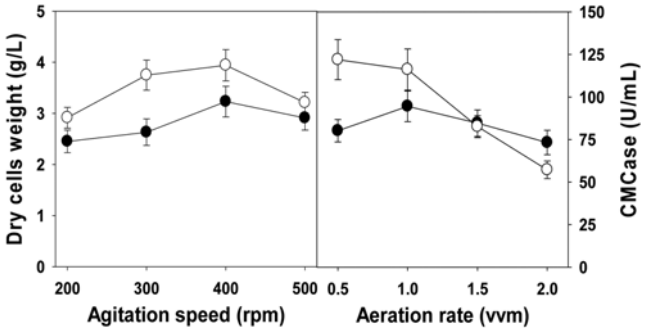

Fig. 3. Comparison of optimal agitation speed and aeration rate for cell growth and production of CMCase by *C. lytica* LBH-14 in 7 L bioreactors (●, DCW and ○, CMCase).

Table 1. Central composite design and determined response values

Run	X_1 (rpm)	X_2 (vvm)	Y_1 (g/L)	Y_2 (U/mL)
1	540	1.00	3.08	97.5
2	400	0.29	2.98	117.1
3	400	1.00	3.21	119.4
4	300	1.50	3.05	102.9
5	400	1.00	3.18	118.2
6	300	0.50	3.03	119.2
7	400	1.71	3.07	89.6
8	500	0.50	3.03	110.0
9	400	1.00	3.19	118.4
10	500	1.50	3.08	85.4
11	260	1.00	3.01	116.3
12	400	1.00	3.23	112.4
13	400	1.00	3.24	112.0

that the model and model terms of X_1^2 and X_2^2 were highly significant and that of X_2 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 0.9605. The value of the adjusted determination coefficient (Adj. R^2=0.9322) was high, which indicates high significance of this mode [15]. The predicted determination of coefficient of 0.8645 was in reasonable agreement with the Adj. R^2 of 0.9322. From the statistical results obtained, 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. (3)). The optimal conditions of agitation speed and aeration rate for cell growth were 398 rpm and 1.10 vvm, respectively. The maximum cell growth of 3.21 g/L was predicted by this model.

$$Y_1=2.72+0.01X_1+0.03X_2-0.01X_1X_2-0.05X_1^2-0.11X_2^2 \quad (3)$$

The model *F*-value of 41.84 from the ANOVA of production of CMCase implied that this model was also significant. The ANOVA indicated that this model was highly significant and those of X_1, X_2, X_1^2, and X_2^2 were significant for the production of CMCase by *C. lytica* LBH-14. The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R^2 was 0.9676.

Table 2. Parameter estimates and analysis of variance (ANOVA) of the design for cell growth and production of CMCase by *C. lytica* LBH-14 in a 7 L bioreactor

	Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*-value	Probe>F
Cell growth	Model	5	0.095	0.019	34.02	<0.0001
	X_1	1	0.002	0.002	3.72	0.0952
	X_2	1	0.004	0.004	8.69	0.0215
	X_1X_2	1	0.000	0.000	0.40	0.5462
	X_1^2	1	0.044	0.044	78.31	<0.0001
	X_2^2	1	0.056	0.056	99.29	<0.0001
	Error	4	0.004	0.001	-	-
	Total	12	0.099	-	-	-
CMCase	Model	5	1575.570	315.110	41.84	<0.0001
	X_1	1	354.940	354.940	47.13	0.0002
	X_2	1	795.820	795.820	105.67	<0.0001
	X_1X_2	1	17.220	17.220	2.29	0.1742
	X_1^2	1	158.780	158.780	21.08	0.0025
	X_2^2	1	298.680	298.680	39.66	0.0004
	Error	4	51.090	12.770	-	-
	Total	12	1628.290	-	-	-

The value of the adjusted determination coefficient (Adj. R^2=0.9445) was very high, indicating a high significance of this model. The predicted determination of coefficient of 0.9439 was also in reasonable agreement with the Adj. R^2 of 0.9445. From the statistical results obtained, the above models were adequate to predict the production of CMCase by *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 agitation speed and aeration rate for production of CMCase were 371 rpm and 0.70 vvm, respectively. The maximum production of CMCase of 120.8 U/mL was predicted by this model.

$$Y_2=120.94-8.42X_1-13.06X_2-0.27X_1X_2-7.94X_1^2-6.92X_2^2 \quad (4)$$

Cell growth and production of bacterial CMCases by *B. amyloliquefaciens* and *B. subtilis* subsp. *subtilis* as well as fungal CMCase by *T. reesei* were affected by the dissolved oxygen in the medium [13,16,17]. The optimal agitation speed and aeration rate for the production of CMCase by *B. amyloliquefaciens* as well as *B. subtilis* subsp. *subtilis* were 300 rpm and 1.0 vvm, which were lower than those for their cell growth [13,17]. The optimal agitation speed and aeration rate for cell growth were 432 rpm and 0.96 vvm, whereas those for production of CMCase by *C. lytica* LBH014 were 371 rpm and 0.70 vvm, which was the same as those for production of cellobiase. Higher agitation speed and serration rate, which resulted in increase of the concentration of dissolved oxygen in the medium, enhanced cell growth of *C. lytica* LBH-14. However, a higher than optimal concentration of dissolved oxygen for the production of CMCase by *C. lytica* LBH-14 seemed to lead the biosynthetic pathway to cell growth, but not to production of cellulases.

A three-dimensional (3D) response surface was generated to study the interaction between agitation speed and aeration rate and to visualize the combined effect of factors on the response of cell growth and the production of CMCase by *C. lytica* LBH-14, as shown Fig. 4. This kind of graphical visualization allows the relationships be-

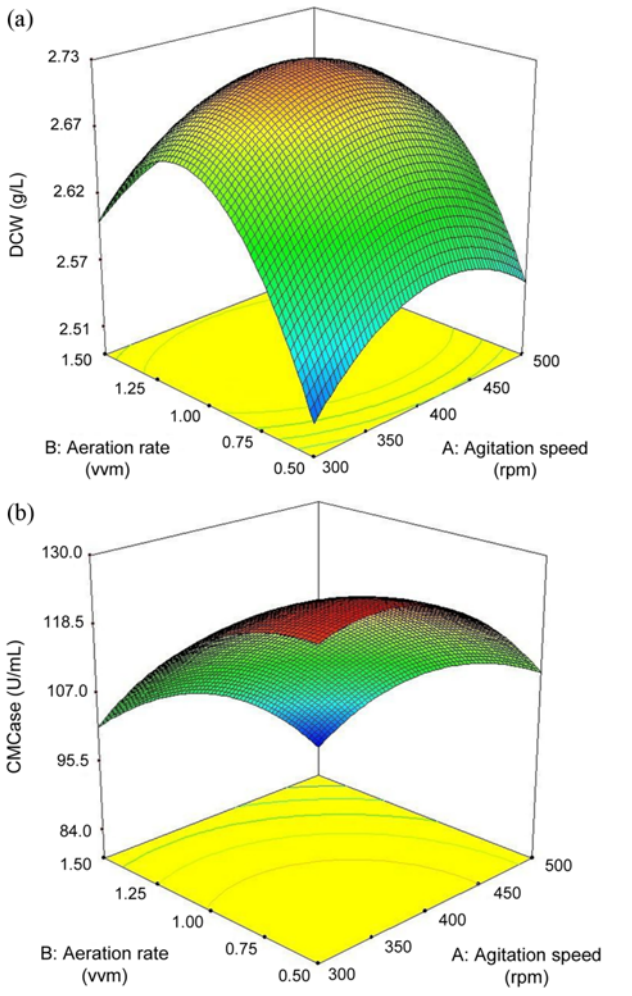

Fig. 4. Three-dimensional response surface plots displaying combined effect of agitation speed and aeration rate on cell growth (a) and production of CMCase (b) by *C. lytica* LBH-14 in 7 L bioreactors.

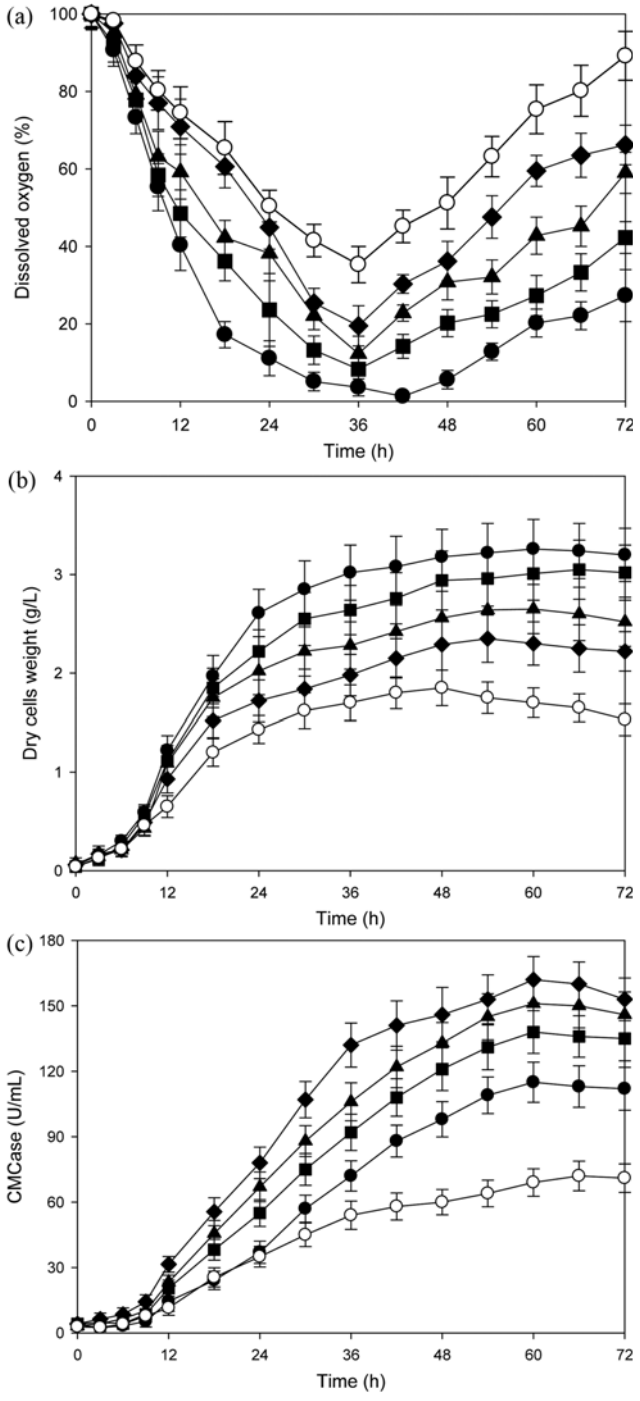

Fig. 5. Effect of inner pressure in a 100 L bioreactor on dissolved oxygen in medium (a), cell growth (b), and production of CMCase (c) by *C. lytica* LBH-14 in 100 L bioreactors (●, 0.00 Mpa; ■, 0.02 MPa; ▲, 0.04 MPa; ◆, 0.06 MPa; and ○, 0.08 MPa).

tween the experimental levels of each factor and the response to be investigated, and the type of interactions between test variables to be determined. In contrast to the circular shapes, the elliptical nature of curves indicates more significant mutual interactions between variables. The interactive effect of agitation speed and aeration rate on production of CMCase (*F*-value of 0.1742) was found to be more drastic than that on cell growth (*F*-value of 0.5462).

3. Effect of Inner Pressure on Production of CMCase

The effect of inner pressure on cell growth and the production of CMCase by *C. lytica* LBH-14 was investigated in a 100 L bioreactor. The inner pressure ranged from 0.00 to 0.08 MPa. The agitation speed and aeration rate of a 100 L bioreactor were 250 rpm and 0.7 vvm, respectively. The radius of the impeller in a 100 L bioreactor was bigger than that in a 7 L bioreactor. The angular velocity of a 100 L bioreactor at 250 rpm is almost same as that of a 7 L bioreactor at 370 rpm. Due to the rapid cell growth of early stage, the concentration of dissolved oxygen in the medium decreased until 36 h after cultivation, as shown in Fig. 5. Maximal cell growth occurred without an inner pressure in a 100 L bioreactor, whereas the optimal inner pressures for the production of CMCase by *C. lytica* LBH-14 was found to be 0.06 MPa. Higher dissolved oxygen in the medium, which resulted from higher inner pressure, enhanced the production of CMCase by *C. lytica* LBH-14. Productions of CMCase by *C. lytica* LBH-14 with inner pressure of 0.00, 0.02, 0.04, 0.06, and 0.08 MPa after 72 h of cultivation were 111.5, 135.6, 146.3, 153.6, and 71.4 U/mL, respectively. The production of CMCase with an inner pressure of 0.06 MPa was 1.38 times higher than that without an inner pressure.

Increased inner pressures of a 100 L bioreactor resulted in higher concentrations of dissolved oxygen in the medium, which might enhance the production of CMCase by *C. lytica* LBH-14 [18]. A variation in agitation speed and aeration rate results in a change in the concentration of dissolved oxygen in the medium, which in turn affects cell growth and the production of microbial metabolites such as lipase and β-mannanase [19,20]. The optimal agitation speed and aeration rate for cell growth were higher than for the production of CMCase by *C. lytica* LBH-14. However, the optimal inner pressure for cell growth was lower than that for production of CMCase. Higher inner pressures seemed to damage to cells, which resulted in inhibition of cell growth, even though higher inner pressures of a bioreactor can afford for higher concentration of dissolved oxygen enough to enhance production of CMCase.

CONCLUSION

The optimization of culture conditions by the traditional one-factor-at-a-time (OFAT) method requires a considerable amount of work and time. An alternate strategy is a statistical approach: for example, response surface methodology (RSM) involving a minimum number of experiments for a large number of factors [15]. The agitation speed and aeration rate for the production of CMCase by *C. lytica* LBH-14 at lab-scale using rice bran and ammonium chloride were optimized using RSM, and the inner pressure at pilot scale was established for industrial-scaled production of CMCase by *C. lytica* LBH-14 in this study. The time for the production of CMCase by a marine bacterium, *C. lytica* LBH-14, was 3 days, which is shorter than that by fungal strains [5,21]. Production of CMCase from cheap substrates with reduced time seemed to result in enhanced productivity of CMCase and decreased production cost. As shown in Table 3, the maximal production of CMCase by *C. lytica* under optimized conditions at pilot scale using rice bran and ammonium chloride was 153.6 U/mL, which was 1.39 times higher than that at flask scale. Moreover, this study is the first report using a pilot-scale bioreactor for industrial production of CMCase.

Table 3. Comparison of optimal conditions for cell growth and production of CMCase by *C. lytica* LBH-14 using two experimental methods

Scale	Optimal conditions	One factor at a time experiment		Response surface method		Ref.
		DCW	CMCase	DCW	CMCase	
Flask scale 1	Rice bran (g/L)	125	75	100.0	79.9	[12]
	Ammonium chloride (g/L)	5.0	7.5	5.00	8.52	
	Initial pH	7.0	6.0	7.0	6.1	
	Maximal production	3.19 g/L	70.0 U/mL	3.15 g/L	70.1 U/mL	
Flask scale 2	Temperature (°C)	35	25	-	-	
	Maximal production	3.18 g/L	110.8 U/mL	-	-	
Lab-scaled bioreactor	Agitation speed (rpm)	400	400	398	371	This study
	Aeration rate (vvm)	1.0	0.5	1.10	0.70	
	Maximal production	3.45 g/L	128.0 U/mL	3.21 g/L	120.8 U/mL	
Pilot-scaled bioreactor	Inner pressure (Mpa)	0.00	0.06	-	-	
	Maximal production	3.51 g/L	153.6 U/mL	-	-	

ACKNOWLEDGEMENTS

This paper was financially supported by Dong-A University Research Fund.

REFERENCES

1. J. C. Yi, J. C. Sandra, A. B. John and T. C. Shu, *Appl. Environ. Microbiol*., **65**, 553 (1999).
2. E. Tomás-Pejó, M. Carcía-Aparicio, M. J. Negr, J. M. Oliva and M. Ballesteros, *Bioresour. Technol*., **100**, 890 (2009).
3. G. Y. Wei, W. Gao, I. H. Jin, S. Y. Yoo, J. H. Lee, C. H. Chung and J. W. Lee, *Biotechnol. Bioprocess Eng.*, **14**, 828 (2009).
4. M. Z. Alam, S. A. Muyibi and R. Wahid, *Bioresour. Technol*., **99**, 4709 (2008).
5. H. Chen, Q. He and L. Liu, *Biotechnol. Bioprocess Eng*., **16**, 867 (2011).
6. H. J. Kim, Y. J. Lee, W. Gao, C. H. Chung and J. W. Lee, *Biotechnol. Bioprocess Eng*., **16**, 542 (2011).
7. H. J. Kim, W. Gao, C. H. Chung and J. W. Lee, *J. Life Sci*., **21**, 1083 (2011).
8. R. Rajulan, K. S. Dhar, M. Nampoothiri and A. Pandey, *Bioresour. Technol*., **102**, 8171 (2011).
9. M. Thiry and D. Cinogolani, *Trends Biotechnol*., **20**, 103 (2002).
10. B. H. Junker, *J. Biosci. Bioeng*., **97**, 347 (2004).
11. W. Gao, Y. J. Kim, C. H. Chung, J. Li and J. W. Lee, *J. Life Sci*., **20**, 1433 (2010).
12. W. Gao, E. J. Lee, S. U. Lee, J. Li, C. H. Chung, and J. W. Lee, *J. Microbiol. Biotechnol*., **22**, 1415 (2012).
13. K. I. Jo, Y. J. Lee, B. K. Kim, B. H. Lee, C. H. Chung, S. W. Nam, S. K. Kim and J. W. Lee, *Biotechnol. Bioprocess Eng*., **13**, 182 (2008).
14. B. K. Kim, B. H. Lee, Y. J. Lee, I. H. Jin, C. H. Chung and J. W. Lee, *Enzyme Microb. Technol*., **44**, 411 (2009).
15. H. J. Kim, Y. J. Lee, W. Gao, C. H. Chung and J. W. Lee, *Korean J. Chem. Eng*., **29**, 384 (2012).
16. R. Lejeune and G. V. Baron, *Appl. Microbiol. Biotechnol*., **43**, 249 (1995).
17. B. H. Lee, B. K. Kim, Y. J. Lee, C. H. Chung and J. W. Lee, *Enzyme Microb. Technol*., **46**, 38 (2010).
18. Y. J. Lee, H. J. Kim, W. Gao, C. H. Chung and J. W. Lee, *Biotechnol. Bioprocess Eng*., **17**, 227 (2012).
19. I. B. Bajaj and R. S. Singhal, *Biotechnol. Bioprocess Eng*., **15**, 635 (2010).
20. M. Elibol and D. Ozer, *Process Biochem*., **36**, 325 (2000).
21. D. Li, X. Fu and S. M. Kim, *Biotechnol. Bioprocess Eng*., **15**, 314 (2010).