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RESEARCH PAPER

Comparison of Optimal Conditions for Mass Production of Carboxymethylcellulase by *Escherichia coli* JM109/A-68 with Other Recombinants in Pilot-scale Bioreactor

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Abstract The optimal conditions for mass production of carboxymethylcellulase (CMCase) by *E. coli* JM109/A-68 were investigated and compared with other *E. coli* JM109 recombinants producing CMCase. The optimal agitation speed and aeration rate for cell growth of *E. coli* JM109/A-68 were 500 rpm and 0.50 vvm in a 7 L bioreactor, whereas those for production of CMCase were 416 rpm and 0.95 vvm. The optimal vessel pressures for cell growth as well as production of CMCase in a 100 L bioreactor were 0.04 MPa. The maximal production of CMCase by *E. coli* JM109/A-68 under the optimized conditions in a 100 L bioreactor was 11.0 times higher than its wild type, *B. velezensis* A-68. Optimal conditions for mass production of CMCase by recombinants were different from those for wild strains. The higher production of CMCase by *E. coli* JM109/A-68 and other recombinant of *E. coli* seemed to result from its higher cell growth under the optimal conditions for dissolved oxygen and its mixed-growth associated production pattern compared to the growth-associated production of *B. velezensis* A-68.

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1. Introduction

More than 80% of the total global energy is obtained by burning fossil fuels, of which 58% is consumed for transportation [1]. Production of bioethanol from food materials raised concern of global food security [2]. Agricultural wastes such as rice hulls and rice bran provides renewable resources for sustainable production of bioethanol [3]. The enzymatic saccharification of agricultural wastes for production of ethanol was performed by commercial cellulases, in which the major cellulase was carboymethylcellulase (CMCase) [4].

Most commercial cellulases have been produced with solid-state cultures of *Aspergillus* and *Trichoderma* species [5]. Unlike traditional production of cellulases, the CMCases were produced by batch fermentation of *B. amyloliquefaciens* DL-3 and *C. lytica* LBH-14 in stirred tank bioreactor [6,7]. The genes encoding CMCases were cloned and expressed in several expression systems to enhance its production [8,9]. The productions of CMCases by *E. coil* JM109/DL-3 and *E. coli* JM109/A-68 were 2.9 and 4.0 times higher than their wild types, respectively. In addition to the construction of recombinants with enhanced production, one of the most important steps for mass production is the optimization of parameters involved in dissolved oxygen [10,11]. The concentration of dissolved oxygen in the medium can be influenced by amount of supplied air, distribution by agitation, and the vessel pressure of bioreactors [12,13].

Bacillus velezensis A-68, which had been isolated from seawater, utilized rice hulls and produced CMCase [14,15].

The gene encoding the CMCase of *B. velezensis* A-68 was cloned in *E. coli* JM109 and the optimal condition for production of CMCase by *E. coli* JM109/A-68 at the flask scale were established [9]. In this study, the optimal agitation speed and aeration rate for the production of CMCase by *E. coli* JM109/A-68 were established using response surface methodology (RSM) [16,17]. The effects of vessel pressure on production of CMCase were also investigated. The cell growth and production of CMCase by *E. coli* JM109/A-68 was compared with that by *B. velezensis* A-68 in the pilot-scale bioreactor. Unlike the benchtop reactor, the pilot-scale bioreactor has the equipment to control the vessel pressure and is used for a pre-commercial production. The optimal conditions for production of CMCase in the pilot-scale bioreactor can be directly applied for mass production in the industrial-scale bioreactor.

2. Materials and Methods

2.1. Microorganism and medium

E. coli JM109/A-68 having a gene encoding CMCase of *B. velezensis* A-68 was used in this study for producing CMCase [9]. The medium used for production of CMCase consisted of 132.3 g/L rice bran, 4.68 g/L ammonium chloride, 5.0 g/L K_2HPO_4, 1.5 g/L NaCl, 0.6 g/L $MgSO_4 \cdot 7H_2O$, and 0.6 g/L $(NH_4)_2SO_4$.

2.2. Production of CMCase

Starter cultures for the production of CMCase by *E. coli* JM109/A-68 were prepared as described in the previous report [9]. The main culture was carried out in the above mentioned medium for 3 days under aerobic conditions. Samples during the main culture were periodically withdrawn to examine cell growth and production of CMCase.

Batch fermentations of *E. coli* JM109/A-68 were carried out in 7 and 100 L bioreactors (Ko-Biotech Co., Korea). Working volumes of 7 and 100 L bioreactors were 5 and 70 L, respectively, and inoculum sizes of batch fermentations were 5% (v/v). Temperatures for batch fermentations were maintained at 37°C.

2.3. Experimental design using response surface methodology

The independent variables in this experimental design using RSM were the agitation speed (X_1) and aeration rate (X_2) and the dependent output variables were cell growth (Y_1) and CMCase (Y_2). The model of a response function of the variables was constructed as a second-order polynomial as follows (Eq. 1):

$$Y = ß_0 + \sum ß_i X_i + \sum ß_{ii} X_i^2 + \sum ß_{ij} X_i X_j \quad (1)$$

where, y was the measured response, $ß_0$, $ß_i$, and $ß_{ij}$ are the regression coefficients, and X_i and X_j were the factors. For three variable systems, the model equation was given below (Eq. 2).

$$Y = ß_0 + ß_1 X_1 + ß_2 X_2 + ß_{11} X_1^2 + ß_{22} X_2^2 + ß_{12} X_1 X_2 \quad (2)$$

Statistical analysis of the model was performed for evaluating the analysis of variance (ANOVA) [18,19]. The statistical software, Design-Expert (Version 7.1.6, Stat-Ease Inc., Minneapolis, USA) was used for the regression analysis and estimation of coefficients.

2.4. Analytical methods

Dry cells weight as cell growth was determined as described in the previous report [9]. The 3,5-dinitrosalicylic acid (DNS) method was used to determine the production of CMCase [20]. Glucose and CMC (Sigma-Aldrich, UK) were used to prepare a calibration curve and determine the activity of CMCase. One unit of each CMCase was defined as described in the previous report [9].

3. Results and Discussion

3.1. Effect of agitation speed and aeration rate on production of CMCase

Effect of agitation speed on cell growth and the production of CMCase by *E. coli* JM109/A-68 was investigated in a 7 L bioreactor. The agitation speed ranged from 200 to 500 rpm and the aeration rate was fixed to be 1.0 vvm. As shown in Fig. 1, the optimal agitation speeds for cell growth and the production of CMCase by of *E. coli* JM109/A-68 were 400 rpm. The maximal cell growth and production of CMCase were 5.63 g/L and 897.5 U/mL. The effect of aeration rate on cell growth and production of CMCase was also investigated. The aeration rate in 7 L bioreactors ranged from 0.5 to 2.0 vvm and the agitation speed was fixed to be 400 rpm. Higher agitation speed as well as aeration rate enhanced cell growth of *E. coli* JM109/A-68, as shown in Fig. 2. The optimal aeration rate for the cell growth of *E. coli* JM109/A-68 was 1.5 vvm, whereas that for production of CMCase was 1.0 vvm. The cell growth and production of CMCase under optimized agitation rate and aeration rate were 5.63 g/L and 921.3 U/mL, respectively.

3.2. Statistical optimization of agitation speed and aeration rate for production of CMCase

Based on the results from one-factor-at-a-time experiment, the simultaneous effects of agitation speed and aeration rate on cell growth and production of CMCase were investigated using the response surface methodology (RSM). The coded values of minimum and maximum ranges of agitation speed

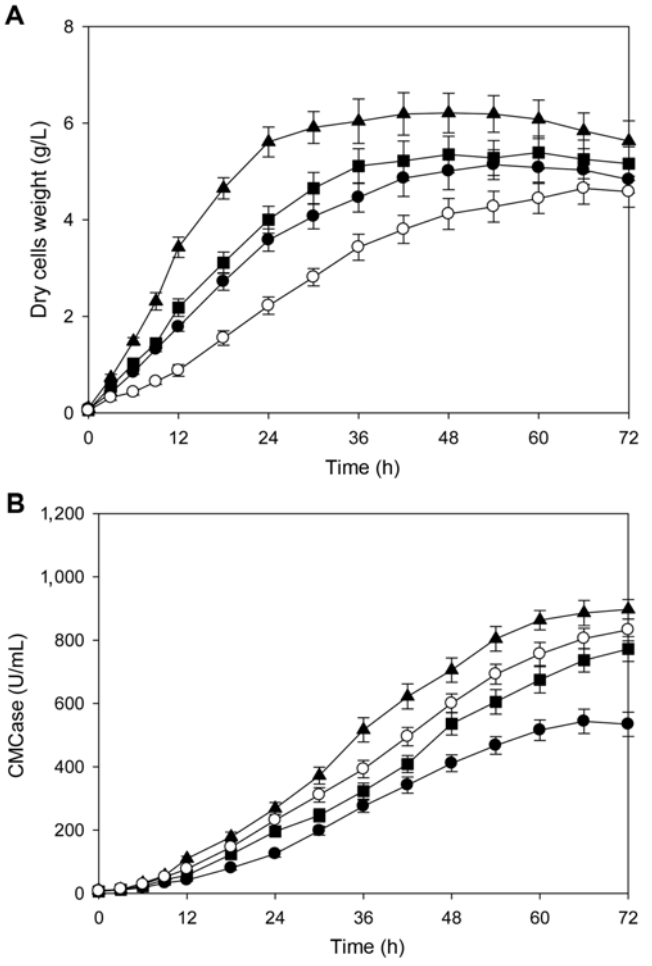

Fig. 1. Effect of agitation speed on cell growth (A) and production of CMCase (B) by *E. coli* JM109/A-68 in a 7 L bioreactor (●, 200 rpm; ■, 300 rpm; ▲, 400 rpm; ○, 500 rpm).

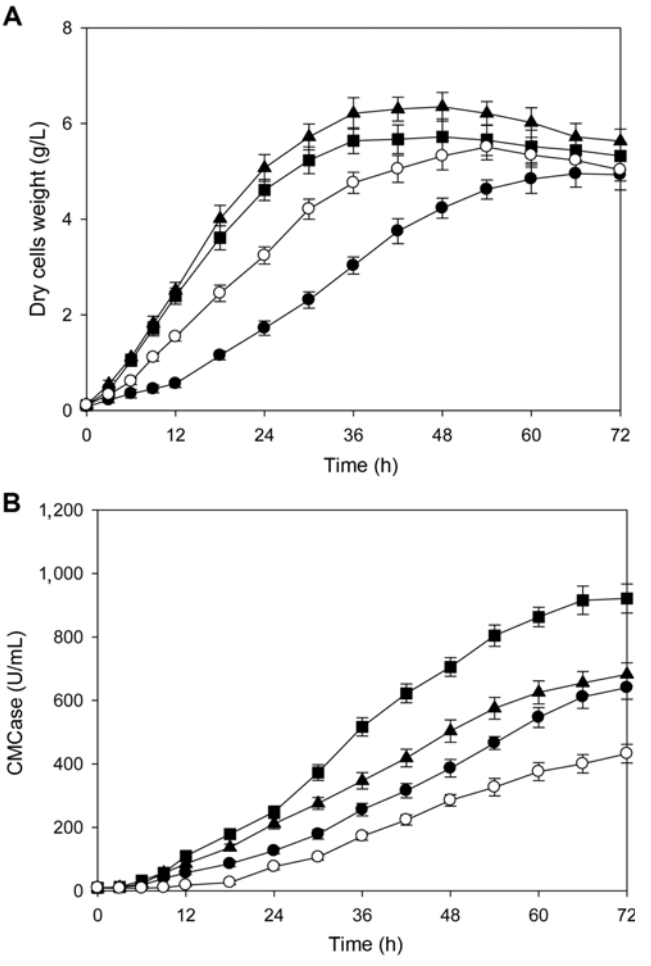

Fig. 2. Effect of aeration rate on cell growth (A) and production of CMCase (B) by *E. coli* JM109/A-68 in a 7 L bioreactor (●, 0.5 vvm; ■, 1.0 vvm; ▲, 1.5 vvm; ○, 2.0 vvm).

(X_1) and aeration rate (X_2) were 300 and 500 rpm and 0.5 and 1.5 vvm, respectively. The results of central composite design (CCD) experiments were shown in Table 1. Cell growth and production of CMCase from 13 different conditions ranged from 4.95 to 6.32 g/L and from 441.3 to 956.3 U/mL. As shown in Table 2, the model *F*-value of 13.50 from the ANOVA of cell growth implied that this model was significant. The smaller magnitudes of the *P* value mean the more significant corresponding coefficients [21]. The ANOVA indicated that this model and the model terms of X_1 and $X_1 \cdot X_2$ were significant ("probe > *F*" less 0.050) for cell growth of *E. coli* JM109/A-68. The multiple correlation coefficient of R^2 for cell growth was 0.9060. Multiple regression analysis for cell growth gave the following second-order polynomial equation in terms of coded factors (Eq. 3). The optimal agitation speed and aeration rate for cell growth were 500 rpm and 0.50 vvm. The maximum cell growth of 6.17 g/L was predicted by this model.

Table 1. Central composite design (CCD) for optimization of agitation speed (X_1) and aeration rate (X_2) and determined response values of DCW (Y_1) and CMCase (Y_2)

Run	X_1	X_2	Y_1 (g/L)		Y_2 (U/mL)	
			Predicted	Actual	Predicted	Actual
1	400	1.00	5.33	5.20	913.7	896.6
2	400	0.29	5.01	4.95	509.4	441.3
3	400	1.00	5.33	5.41	913.7	920.1
4	500	0.50	6.18	6.08	712.7	787.0
5	400	1.00	5.33	5.21	913.7	916.6
6	400	1.00	5.33	5.30	913.7	898.9
7	259	1.00	5.15	5.03	581.1	629.6
8	500	1.50	5.33	5.08	542.9	509.2
9	300	1.00	4.37	4.52	445.5	462.8
10	400	0.50	5.33	5.52	913.7	956.3
11	400	1.71	5.42	5.58	511.0	595.6
12	541	1.00	6.10	6.32	717.2	685.1
13	300	1.50	5.80	5.80	617.5	526.8

Table 2. Parameter estimates and analysis of variance (ANOVA) of the design for cell growth and production of CMCase by *E. coli* JM109/A-68 in a 7 L bioreactor

	Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*-value	Probe > *F*
Cell growth	Model	5	2.55	0.51	13.50	0.0018
	X_1	1	0.89	0.89	23.45	0.0019
	X_2	1	0.17	0.17	4.53	0.0709
	X_1X_2	1	1.30	1.30	34.34	0.0006
	X_1^2	1	0.16	0.16	4.11	0.0823
	X_2^2	1	0.02	0.02	0.57	0.4763
	Error	4	0.08	0.02	-	-
	Total	12	2.82	-	-	-
CMCase	Model	5	410,300	82,068	17.39	0.0008
	X_1	1	18,536	18,536	3.93	0.0879
	X_2	1	2	2	51,459.00	0.9825
	X_1X_2	1	29,207	29,207	6.19	0.0417
	X_1^2	1	121,700	121,700	25.80	0.0014
	X_2^2	1	283,100	283,100	59.99	0.0001
	Error	4	2,660	665	-	-
	Total	12	443,400	-	-	-

$$Y_1 = 5.33 + 0.33X_1 + 0.15X_2 - 0.57\ X_1X_2 + 0.15X_1^2 - 0.06X_2^2 \quad (3)$$

The model *F*-value of 17.39 from ANOVA of production of CMCase implied that this model was also significant. The multiple correlation coefficient of R^2 for production of CMCase was 0.9255. Multiple regression analysis for production of CMCase gave the following second-order polynomial equation (Eq. 4). The optimal agitation speed and aeration rate for production of CMCase were 416 rpm and 0.95 vvm. The maximum production of CMCase of 917.4 U/mL was predicted by this model.

$$Y_2 = 913.7 + 48.1X_1 + 0.6X_2 - 85.5X_1X_2 - 132.3X_1^2 - 201.7X_2^2 \quad (4)$$

As shown in Fig. 3, the three-dimensional response surface was generated in order to study the interaction among factors tested and to visualize the combined effects of agitation speed and aeration rate on cell growth and production of CMCase. The elliptical nature of curves in 3D plot related to circular shapes indicates more significant mutual interactions between variables. The interactive effect of agitation speed and aeration rate on cell growth (*P*-value of 0.0006) was more drastic than that on production of CMCase (*P*-value of 0.0417) [25].

As shown in Table 3, the optimal agitation speed and aeration rate for cell growth of some microorganisms including recombinants were are different from those for their production of cellulases [6,14]. Productions of CMCase by recombinants, *E. coli* JM109/A-53, *E. coli* JM109/A-68, and *E. coli* DL-3, were higher than those by their wild strains [8,11]. The optimal agitation speed for cell growth

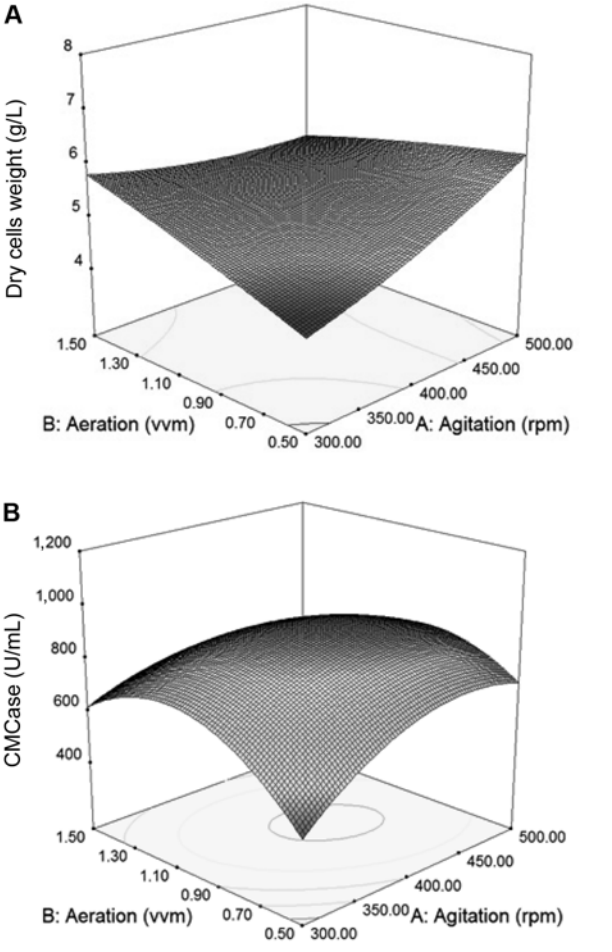

Fig. 3. 3D response surface displaying relative effect of two variables on cell growth and the production of CMCase by *E. coli* JM109/A-68 in a 7 L bioreactor; interaction between agitation speed and aeration rate for cell growth (A) and the production of CMCase (B).

Table 3. Comparison of optimal agitation speed and aeration rate for various cell growths and their productions of CMCases in bioreactors

Microorganism	Growth			CMCase			Ref.
	Agitation speed (rpm)	Aeration rate (vvm)	DCW (g/L)	Agitation speed (rpm)	Aeration rate (vvm)	CMCase (U/mL)	
B. amyloliquifaciens DL-3	500	1.50	1.82	300	1.00	220.2	[6]
B. atrophaeus LBH-18	324	0.90	3.01	343	0.60	105.2	[22]
B. subtilis subsp. *subtilis* A-53	400	1.50	3.62	300	1.00	147.2	[26]
B. velezensis A-68	323	1.46	1.49	380	0.54	88.3	[14]
C. lytica LBH-14	398	0.98	3.21	357	0.55	120.8	[7]
P. aquimaris LBH-10	400	1.50	3.36	300	1.00	320.3	[19]
E. coli JM109/A-53	395	1.38	3.73	396	0.55	650.9	[11]
E. coli JM109/A-68	500	0.50	6.17	416	0.95	917.4	This study
E. coli JM109/DL-3	498	1.40	2.88	395	0.60	629.5	[8]

Table 4. Effect of vessel pressure on growth and production of CMCase by *E. coli* JM109/A-68 in a 100 L bioreactor

Vessel pressure (MPa)	DCW (g/L)	CMCase (U/mL)	$Y_{x/s}$ (g/g)	$Y_{p/s}$ (U/g)	$Y_{p/x}$ (U/g)	μ (/h)	μ_{max} (/h)
0.00	5.16	918.3	0.04	6.94	178.0	0.12	0.59
0.02	7.02	1,013.2	0.05	7.66	144.3	0.13	0.65
0.04	7.50	1,192.3	0.06	9.01	159.0	0.12	0.67
0.06	7.34	1,053.1	0.06	7.96	143.5	0.12	0.63
0.08	6.83	828.1	0.05	6.26	121.4	0.12	0.60

Table 5. Comparison of optimal vessel pressure for various cell growths and their productions of cellulases in pilot-scale bioreactors

Microorganism	Cellulase	Cell growth		Production of CMCase		Ref.
		Inner pressure (MPa)	DCW (g/L)	Inner pressure (MPa)	CMCase (U/mL)	
B. atrophaeus LBH-18	CMCase	0.06	2.96	0.06	127.5	[22]
B. velezensis A-68	CMCase	0.00	1.46	0.04	108.1	[14]
Cellulophga lytica LBH-14	CMCase	0.00	3.51	0.06	153.1	[20]
Cellulophga lytica LBH-14	Cellobiase	0.00	3.20	0.06	140.1	[24]
E. coli JM109/A-53	CMCase	0.06	5.42	0.06	880.2	[25]
E. coli JM109/A-68	CMCase	0.04	7.50	0.04	1,192.3	This study
E. coli JM109/DL-3	CMCase	0.08	3.05	0.06	871.0	[8]

of *E. coli* JM109/A-68 was higher than that for production of CMCase whereas the optimal aeration rate for cell growth was lower than that for production of CMCase. Moreover, the optimal conditions for productions of CMCase by recombinants were different from each other.

3.3. Effect of vessel pressure on production of CMCase

The effect of vessel pressure on cell growth and the production of CMCase by *E. coli* JM109/A-68 was investigated in a 100 L bioreactor. The vessel pressure ranged from 0.00 to 0.08 MPa. The agitation speed and aeration rate of a 100 L bioreactor were 150 rpm and 0.95 vvm. The angular velocity of a 100 L bioreactor at 150 rpm is almost same as that of a 7 L bioreactor at 416 rpm. The concentration of the dissolved oxygen in the medium decreased until 48 h after cultivation, as shown in Fig. 4. The optimal inner pressures for cell growth and the production of CMCase by *E. coli* JM109/A-68 were found to be 0.04 MPa. The production of CMCase with a vessel pressure of 0.04 MPa was 1,192.3 U/mL, which was 1.3 times higher than that without vessel pressures, as shown in Table 4. The value of μ_{max} with a vessel pressure of 0.04 MPa was higher than those with other vessel pressure. The cell growth seemed to be related with production of CMCase.

Higher vessel pressures in the bioreactor resulted in the higher concentration of dissolved oxygen in the medium compared with the same amount of supplied air and also protect the culture from contamination [12,23]. As shown in Table 5, the optimal vessel pressures for cell growth of some microorganisms are different from those for production

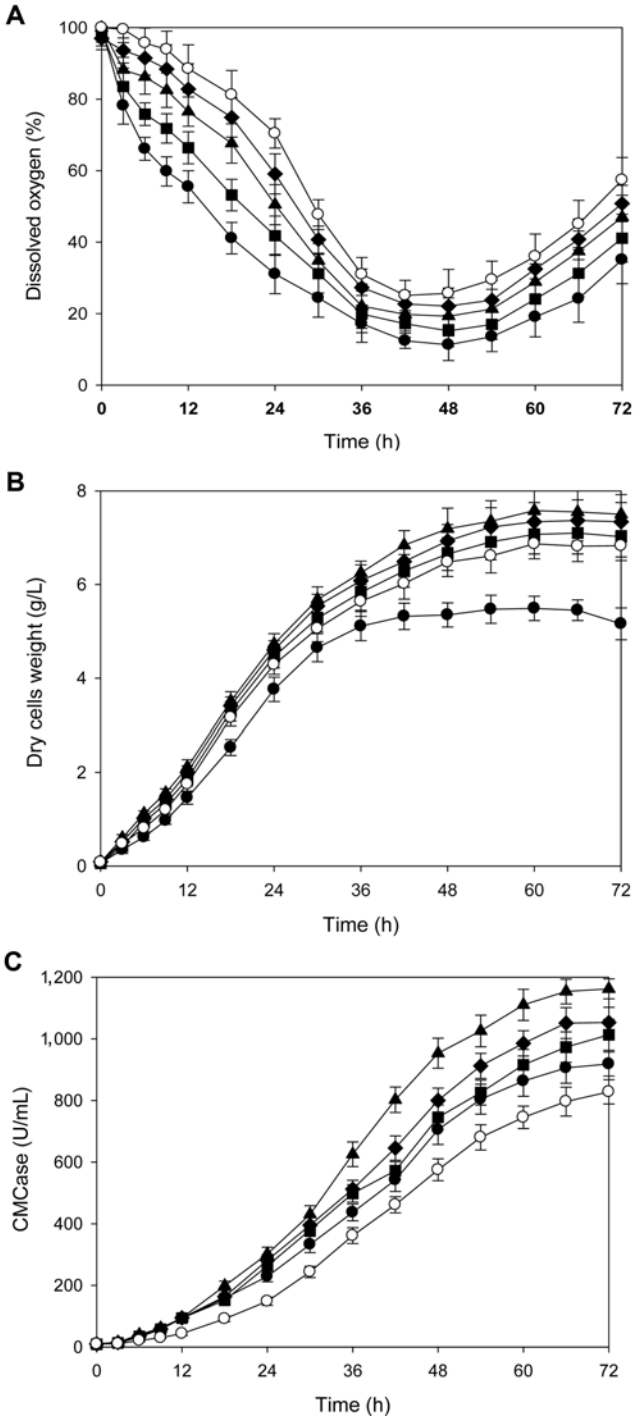

Fig. 4. Effect of the vessel pressure on dissolved oxygen (A), cell growth (B), and production of CMCase (C) by *E. coli* JM109/A-68 in a 100 L bioreactor (●, 0.00 MPa; ■, 0.02 MPa; ▲, 0.04 MPa; ◆, 0.06 MPa; ○, 0.08 MPa).

of CMCase. Productions of CMCases with higher vessel pressures are higher than those without vessel pressure. The optimal vessel pressures for production as well as cell growth of recombinants were different from each other. The production of CMCase by *B. velezensis* A-68 with a

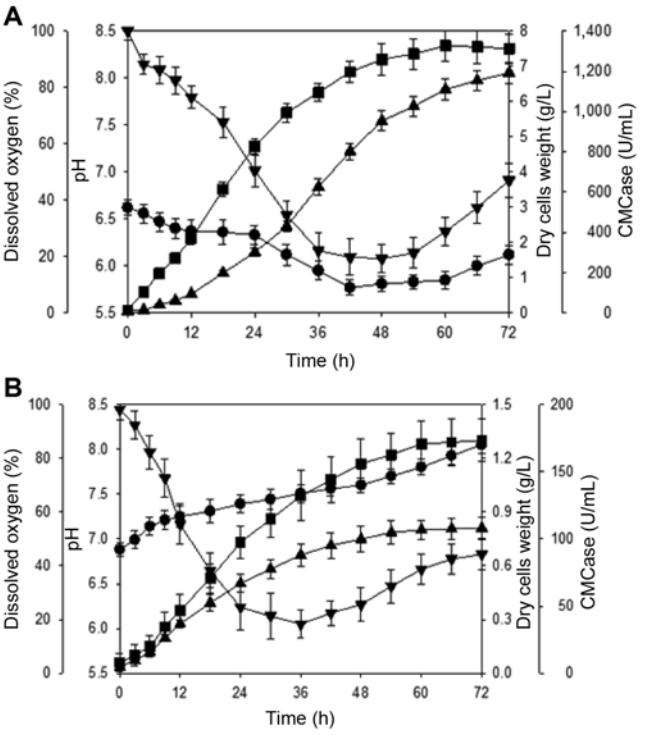

Fig. 5. Cell growth and production of CMCase by *E. coli* JM109/A-68 (A) and *B. velezensis* A-68 (B) in a 100 L bioreactor under optimized conditions (●, pH; ▼, DO; ■, DCW; and ▲, CMCase).

vessel pressure of 0.04 MPa was 1.2 times higher than that without vessel pressures [15]. The production of CMCase by *E. coli* JM109/A-53 with the optimized vessel pressure was 1.4 times higher than that without vessel pressures [11,22]. The higher concentration of dissolved oxygen in the medium due to the higher vessel pressure seemed to result in the higher cell growth of *E. coli* JM109/A-68 and enhanced production of CMCase.

3.4. Production of CMCase under optimized conditions in pilot-scale bioreactor

The production of CMCase by *E. coli* JM109/A-68 was conducted in a 100 L pilot-scale bioreactor under optimized conditions. The initial pH and cultural temperature were 7.1 and 37ºC. Agitation speed, aeration rate, and vessel pressure of a 100 L bioreactor for production of CMCase by *E. coli* JM109/A-67 were 150 rpm, 0.95 vvm, and 0.04 MPa, which were optimized in this study. During the batch fermentation, a decrease in the concentration of the dissolved oxygen in the culture media was observed as shown in Fig. 5. Cell growth of *E. coli* JM109/A-68 rapidly increased until 48 h. The production of CMCase by *E. coli* JM109/A-68 took place during the mid-log and stationary phases whereas that by *B. velezensis* A-68 took place during the log phase. It seemed that the production of CMCase by *B. velezensis* A-68 was proportional to its cell

Table 6. Comparison of optimal conditions for cell growth and production of CMCase by *E. coli* JM109/A-68 with those for *B. velezensis* A-68 [9]

Scale	Conditions	*E. coli* JM109/A-68		*B. velezensis* A-68	
		DCW	CMCase	DCW	CMCase
Lab-scale bioreactor	Agitation speed (rpm)	500	416	323	380
	Aeration rate (vvm)	0.50	0.95	1.46	0.54
	Maximal production	6.17 g/L	917.4 U/mL	1.49 g/L	88.3 U/mL
Pilot-scale bioreactor	Inner pressure (MPa)	0.04	0.04	0.00	0.04
	Maximal production	7.50 g/L	1,192.3 U/mL	1.46 g/L	108.1 U/mL

growth. Microbial products can be classified in three major categories; (1) growth-associated products, (2) non-growth-associated products, and (3) mixed-growth-associated products [25]. The production pattern of CMCase by *E. coli* JM109/A-68 was the mixed-growth associated whereas that of its wild type, *B. velensis* A-68, was the growth-associated.

The maximal production of CMCase by *E. coli* JM109/A-68 under the optimized conditions in pilot scale bioreactor is 1.4 times higher than that at the previously optimized flask scale [9]. Moreover its production was 11.0 times higher than that of *B. velensis* A-68 as shown in Table 6. The maximal production of CMCase by *E. coli* JM109/A-53 was 5.9 times higher than that of its wild strain, *B. subtilis* subsp. *subtilis* A-53 [22,26].

4. Conclusion

In this study, the pilot-scale optimization for production of CMCase by *E. coli* JM109/A-68 from rice bran and ammonium chloride was established using the statistical methodology. The production of CMCase by *E. coli* JM109/A-68 was 11.0 times higher than that of its wild type, *B. velensis* A-68. Productions of CMCases by recombinants were generally higher than those of wild strains. However, optimal conditions for productions of CMCases by recombinants were different from each other. The higher production of CMCase by *E. coli* JM109/A-68 resulted from its higher cell growth under the optimized conditions and its mixed-growth associated production pattern compared to the lower cell growth and the growth-associated production of CMCase by *B. velezensis* A-68. The economic process with the optimized conditions was developed in this study to apply for the industrial-scale production of CMCase and can overcome the major constrain in enzymatic saccharification of agricultural wastes.

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