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Enhanced Production of Carboxymethylcellulase by a Marine Bacterium, *Bacillus velezensis* A-68, by Using Rice Hulls in Pilot-scale Bioreactor under Optimized Conditions for Dissolved Oxygen

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The optimal conditions for the production of carboxymethylcellulase (CMCase) by *Bacillus velezensis* A-68 at a flask scale have been previously reported. In this study, the parameters involved in dissolved oxygen in 7 and 100 L bioreactors were optimized for the pilot-scale production of CMCase. The optimal agitation speed and aeration rate for cell growth of *B. velezensis* A-68 were 323 rpm and 1.46 vvm in a 7 L bioreactor, whereas those for the production of CMCase were 380 rpm and 0.54 vvm, respectively. The analysis of variance (ANOVA) implied that the highly significant factor for cell growth was the aeration rate, whereas that for the production of CMCase was the agitation speed. The optimal inner pressures for cell growth and the production of CMCase by *B. velezensis* A-68 in a 100 L bioreactor were 0.00 and 0.04 MPa, respectively. The maximal production of CMCase in a 100 L bioreactor under optimized conditions using rice hulls was 108.1 U/ml, which was 1.8 times higher than that at a flask scale under previously optimized conditions.

Keywords: *Bacillus velezensis*, carboxymethylcellulase, marine bacterium, response surface methodology, rice hulls

Introduction

Rice hulls, the outer coat of rice, represent about 20% of the dry weight of harvested rice. The world rice production in 2010 reached at 464 million tons (696 million tons of paddy) (Kim *et al.*, 2013). Hydrolysates of rice hulls contain mainly glucose and xylose, which can be used as substrates for the production of ethanol (Singh *et al.*, 2011). Enzymatic saccharification of rice hulls can be accomplished via a complex reaction involving three different types of cellulases (Jo *et al.*, 2008). Rice hulls have been hydrolyzed by commercial cellulases including carboxymethylcellulase (CMCase) (Wei *et al.*, 2010). However, a major constraint in the enzymatic saccharification of cellulosic materials is the cost of cellulases and low productivity of the process (Sukumaran *et al.*, 2009).

Enzymes produced by marine microorganisms can provide several advantages over traditional enzymes due to their ability to adapt to extreme and varied environmental conditions (Kim *et al.*, 2010). A microorganism producing CMCase with rice hulls as a substrate was isolated from seawater and identified as *Bacillus velezensis* by 16S rDNA analyses (Kim *et al.*, 2013). The time for the production of CMCase by this strain in suspension culture was reduced from 7 to 10 days to 3 days of fungal strains in a solid-state culture (Kim *et al.*, 2013).

The production of microbial metabolites on a large-scale is mostly performed via batch fermentation in stirred tank bioreactors (Jung *et al.*, 2013). Oxygen transfer can often be important when scaling-up reactions due to its low solubility in medium (Gao *et al.*, 2013a). The rate at which oxygen is transferred into the medium can be influenced by agitation speed, aeration rate, and the inner pressure of the bioreactor (Giavasis *et al.*, 2006). In this study, the optimal agitation speed and aeration rate for cell growth and the production of CMCase by *B. velezensis* A-68 were established using response surface methodology (RSM) (Kim *et al.*, 2011a; Gao *et al.*, 2012). The effects of inner pressure on cell growth and the production of CMCase were also investigated.

Materials and Methods

Production of CMCase by *B. velezensis* A-68

Starter cultures for the production of CMCase by *B. velezensis* A-68 were prepared as previously described (Kim *et al.*, 2013). The resulting cultures were incubated at 30°C for 2 days under aerobic conditions. Each starter culture was used to inoculate 150 ml of medium in 500 ml Erlenmeyer flasks. The medium of the main culture consisted of 50.0 g/L rice hulls, 5.0 g/L yeast extract, 7.5 g/L K_2HPO_4, 1.0 g/L NaCl, 0.1 g/L $MgSO_4 \cdot 7H_2O$, and 0.8 g/L $(NH_4)_2SO_4$ for 72 h under aerobic conditions. The initial pH of medium and temperature were 7.3 and 35°C, respectively. Batch fermentations for the production of CMCase were performed in 7 and 100 L bioreactors (Ko-Biotech Co., Korea). Working volumes of the 7 and 100 L bioreactors were 5 and 70 L, respectively, and 5% (v/v) inoculum was used for the production of CMCase in batch fermentation. Cultures were agitated by 3 six-flat-blade impellers in the 7 and 100 L bioreactors.

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Experimental design using response surface methodology

The agitation speed (X_1) and aeration rate (X_2) were chosen as independent variables, and cell growth (Y_1) and CMCase (Y_2) were used as dependent output variables. The following second-order polynomial model was constructed to serve as a response function of variables affecting cell growth and the production of CMCase (Eq. 1):

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

Where y is the measured response (cell growth as measured dry cells weight or the production of CMCase), β_0, β_i, and β_{ij} are 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).

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

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA) (Kim *et al.*, 2012a). Regression analysis and the estimation of the coefficient were performed using the statistical software, Design-Expert (Version 7.1.6, Stat-Ease Inc., USA). The contribution of individual parameters and their quadratic and interactive effects on cell growth and the production of CMCase were determined (Kim *et al.*, 2012b).

Analytic methods

Dry cells weight was measured as previously described (Jo *et al.*, 2008). The activity of CMCase was measured using the 3,5-dinitrosalicylic acid (DNS) method, in which the amount of reducing sugars liberated from CMC solubilized in 50 mM Tris-HCl buffer, pH 8.0 were determined (Kim *et al.*, 2010, 2011b). One unit of each CMCase was defined as the amount of enzyme that released 1 μmol of a reducing sugar equivalent to glucose per min under the assay conditions.

Results and Discussion

Effect of agitation speed and aeration rate on production of CMCase

The effect of agitation speed on cell growth of CMCase by

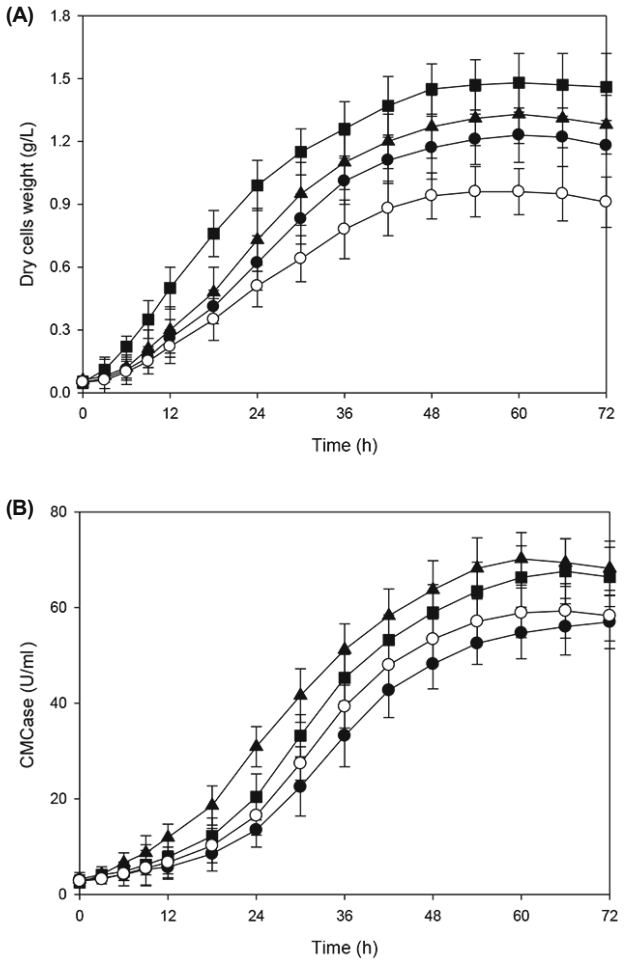

Fig. 1. Effect of agitation speed on cell growth (A) and production of CMCase by *B. velezensis* A-68 (B) (● , 200 rpm; ■ , 300 rpm; ▲, 400 rpm; and ○ , 500 rpm).

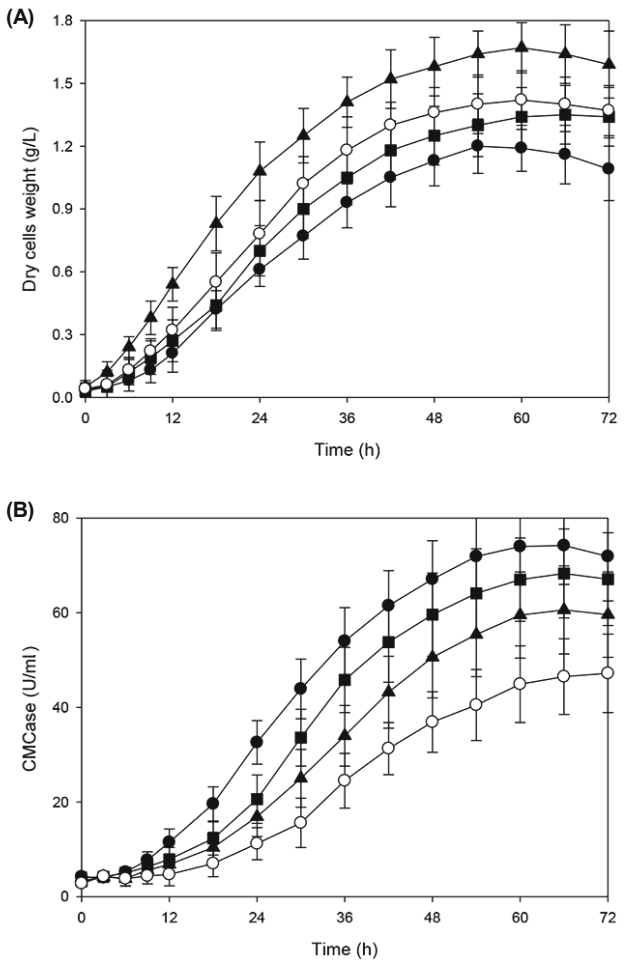

Fig. 2. Effect of aeration rate on cell growth (A) and production of CMCase by *B. velezensis* A-68 (B) (● , 0.5 vvm; ■ , 1.0 vvm; ▲, 1.5 vvm; and ○ , 2.0 vvm).

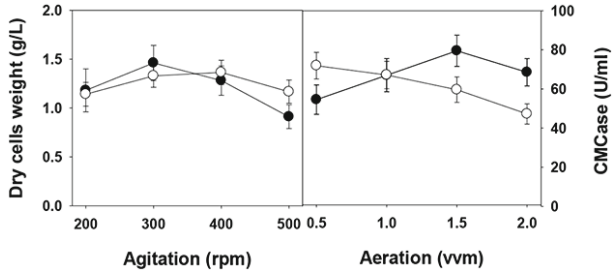

Fig. 3. Effect of agitation speed and aeration rate on cell growth (A) and production of CMCase by *B. velezensis* A-68 (B) (●, DCW and ○, CMCase).

B. velezensis A-68 in a 7 L bioreactor was investigated using the one-factor-at-a-time method. The carbon and nitrogen sources for production of CMCase were 50.0 g/L rice hulls and 5.0 g/L yeast extract (Kim *et al.*, 2013). The initial pH of the medium and temperature were 7.0 and 35°C, respectively. The agitation speed ranged from 200 to 500 rpm and aeration rate was 1.0 vvm. The optimal agitation speed for cell growth was 300 rpm, whereas that for production of CMCase was 400 rpm, as shown in Fig. 1. The maximal cell growth, measured by dry cells weight (DCW) and the production of CMCase were 1.46 g/L and 68.2 U/ml, respectively. The effect of aeration rate on cell growth and production of CMCase was also investigated. The aeration rate ranged from 0.5 to 2.0 vvm and the agitation speed was fixed at 400 rpm. The optimal aeration rate for cell growth was 1.5 vvm, whereas that for the production of CMCase was 0.5 vvm, as shown in Fig. 2. The maximal cell growth and production of CMCase were 1.59 g/L and 71.9 U/ml.

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

Based on the results from the one-factor-at-a-time experiment, as shown in Fig. 3, the simultaneous effects of agitation speed and aeration rate on cell growth and the production of CMCase by *B. velezensis* A-68 were investigated in a 7 L bioreactor. The coded values of minimum and maximum ranges of agitation speed (X_1) and aeration rate (X_2) were 200 and 400 rpm and 0.5 and 1.5 vvm. The results of central composite design (CCD) experiments consisted of experimental data, as shown in Table 1. Cell growth measured as dry cells weight and production of CMCase from 13 different conditions ranged from 1.14 to 1.50 g/L and from 78.3 to 89.4 U/ml. The model *F*-value of 65.34 from the analysis of variance (ANOVA) of cell growth implied that this model was significant, as shown in Table 2. The smaller the magnitude of the *P* value, the more significant the corresponding coefficient (Lee *et al.*, 2011). ANOVA indicated that this model and the model terms of X_2 and X_1^2 were both highly significant ("probe > F" less 0.001) for the cell growth of *B. velezensis* A-68. The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R^2 is 0.979. The value of the adjusted determination coefficient (Adj. R^2 = 0.964) is very high to advocate for a high significance of this model. A multiple regression analysis of the experimental data gave the following second-order polynomial equation in terms of coded

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	300	1.7	1.50	80.4
2	160	1.0	1.18	79.6
3	300	1.0	1.40	84.9
4	300	1.0	1.35	85.1
5	300	1.0	1.44	87.2
6	300	1.0	1.44	87.6
7	300	0.3	1.19	85.8
8	300	1.0	1.36	86.9
9	400	0.5	1.14	89.4
10	200	1.5	1.44	78.3
11	200	0.5	1.14	84.5
12	400	1.5	1.44	83.9
13	440	1.0	1.22	86.2

Table 2. Parameter estimates and analysis of variance (ANOVA) of the design for cell growth and production of CMCase by *B. velezensis* 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	0.20	0.04	65.34	<0.0001
	X_1	1	0.00	0.00	0.65	0.4454
	X_2	1	0.13	0.13	220.62	<0.0001
	X_1X_2	1	0.00	0.00	0.000	1.0000
	X_1^2	1	0.06	0.60	104.12	<0.0001
	X_2^2	1	0.00	0.00	6.09	0.0430
	Error	4	0.00	0.00	-	-
	Total	12	0.20	-	-	-
CMCase	Model	5	120.670	24.130	15.96	0.0010
	X_1	1	49.170	49.170	32.51	0.0007
	X_2	1	46.740	46.740	30.90	0.0009
	X_1X_2	1	0.120	0.120	0.08	0.7842
	X_1^2	1	14.900	14.900	9.85	0.0164
	X_2^2	1	12.940	12.940	8.55	0.0222
	Error	4	6.250	1.560	-	-
	Total	12	131.260	-	-	-

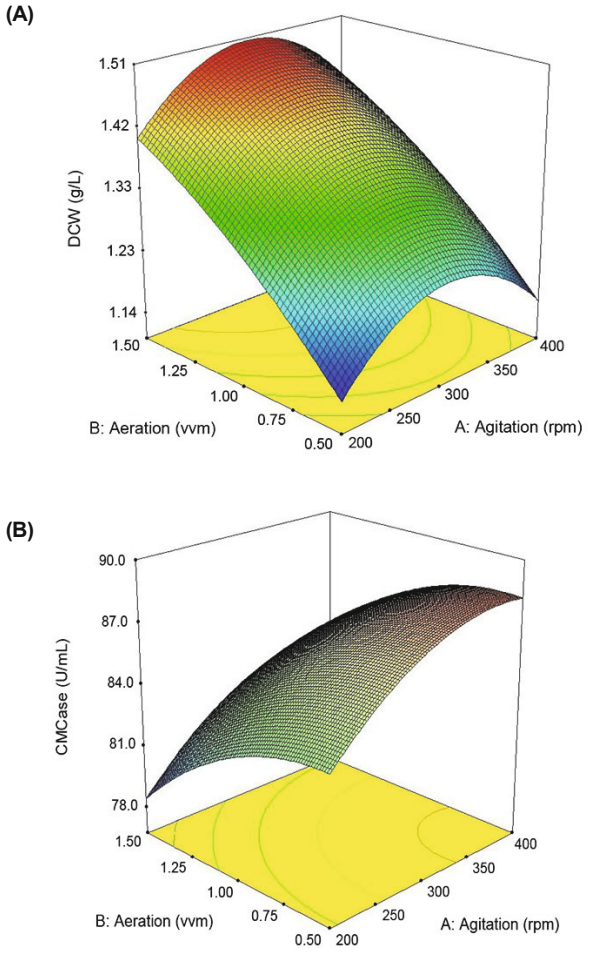

Fig. 4. 3D response surface plots displaying combined effect of agitation speed and aeration rate on cell growth (A) and production of CMCase (B) by *B. velezensis* A-68.

factors (Eq. 3). The optimal agitation speed and aeration rate for cell growth were determined to be 323 rpm and 1.46 vvm. The maximum cell growth of 1.49 g/L was predicted using the following model.

$$Y_1 = 1.40 + 0.01X_1 + 0.13X_2 - 0.10X_1^2 - 0.02X_2^2 \quad (3)$$

The model *F*-value of 15.96 from ANOVA for the production of CMCase implied that this model was also significant. Thus, ANOVA indicated that this model and the model terms of X_1, X_2, X_1^2, and X_2^2 were both significant. The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R^2 was 0.919. The value of the adjusted determination coefficient (Adj. R^2 = 0.862) was high to advocate for a high significance of this model. The predicted value of 0.691 for the coefficient of 0.691 was also in reasonable agreement with the Adj. R^2 of 0.862. Multiple regression analysis of the experimental data gave the following second-order polynomial equation in terms of coded factors (4). The optimal agitation speed and aeration rate for the production of CMCase were 380 rpm and 0.54 vvm. The maximum production of CMCase of

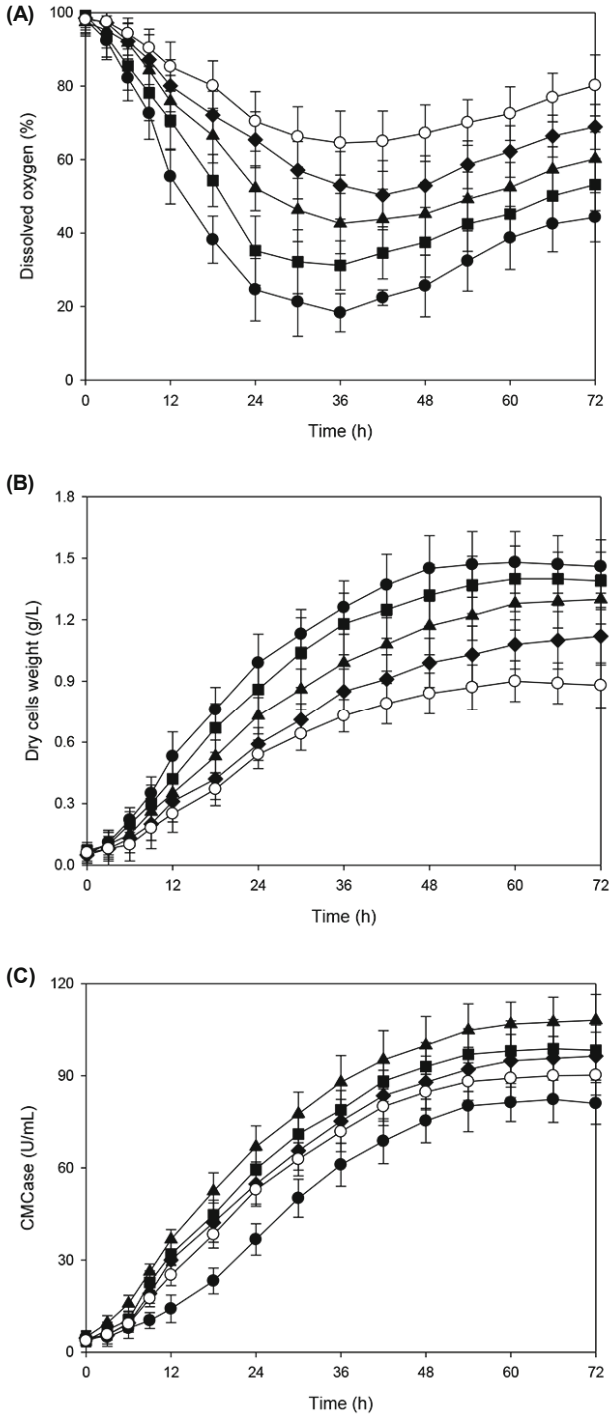

Fig. 5. Effect of inner pressure in a 100 L bioreactor on dissolved oxygen in medium (A), cell growth (B), and production of CMCase by *B. velezensis* A-68 (C) (● , 0.00 MPa; ■ , 0.02 MPa; ▲, 0.04 MPa; ◆ , 0.06 MPa; and ○ , 0.08 MPa).

88.3 U/ml was predicted by this model.

$$Y_2 = 86.34 + 2.48X_1 - 2.42X_2 + 0.18X_1X_2 - 1.46X_1^2 - 1.36X_2 \quad (4)$$

Table 3. Comparison of optimal agitation speed, aeration rate, and inner pressure for cell growth and production of CMCase by various microorganisms

Strain	Cell growth			Production of CMCase			Reference
	Agitation speed (rpm)	Aeration rate (vvm)	Inner pressure (MPa)	Agitation speed (rpm)	Aeration rate (vvm)	Inner pressure (MPa)	
Bacillus amyloliquefaciens DL-3	500	1.5	-	300	1.0	-	Jo *et al.* (2008)
Bacillus atrophaeus LBH-18	324	0.9	0.06	343	0.6	0.06	Kim *et al.* (2012b)
Bacillus subtilus subsp. *subtilis* A-53	400	1.5	-	300	1.0	-	Lee *et al.* (2010)
Bacillus velezensis A-68	323	1.5	0.00	380	0.5	0.04	This study
Cellulophaga lytica LBH-14	398	1.0	0.00	357	0.6	0.06	Gao *et al.* (2013b)
Psychrobacter aquimaris LBH-10	400	1.5	-	300	1.0	-	Kim *et al.* (2010)
Escherichia coli JM109/A-53	395	1.4	0.06	396	0.6	0.06	Lee *et al.* (2013)
Escherichia coli JM109/DL-3	498	1.4	0.08	395	0.6	0.06	Lee *et al.* (2012)

The three-dimensional response surface was generated to study the interactions among four factors tested and visualize the combined effects of agitation speed and aeration rate on the response of cell growth and the production of CMCase by *B. velezensis* A-68, as shown Fig. 4. In contrast to the circular shapes, the elliptical nature of the curves indicates more significant mutual interactions between variables. There were relatively more significant effects of agitation speed and aeration rate on the production of CMCase (*F*-value of 0.784) than cell growth (*F*-value of 1.000) (Lee *et al.*, 2010, 2011).

Agitation and aeration rates are the most critical parameters used for process scale-up and play significant roles in determining the productivity of the process (Gao *et al.*, 2013a). The concentration of dissolved oxygen in the medium is influenced by the agitation speed and aeration rate of bioreactors (Giavasis *et al.*, 2006). Variation in the agitation speed and aeration rate results in a change in the concentration of dissolved oxygen in the medium, which affects cell growth and the production of microbial metabolites (Gao *et al.*, 2013a). The optimal agitation speed and aeration rate for the cell growth of *Bacillus subtilis* subsp. *subtilis* A-53 were 400 rpm and 1.5 vvm, whereas those for the production of CMCase were 300 rpm and 1.0 vvm, as shown in Table 3. The optimal agitation speed and aeration rate for the cell growth of *B. velezensis* A-68 were also different from those for the production of CMCase. The higher than optimal concentration of dissolved oxygen for the production of CMCase induced the biosynthetic pathway to increase cell growth, but not to produce CMCase.

Effect of inner pressure on production of CMCase

The effect of inner pressure on cell growth and the production of CMCase by *B. velezensis* A-68 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 220 rpm and 0.5 vvm. 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 240 rpm is almost same as that of a 7 L bioreactor at 380 rpm. The concentration of dissolved oxygen in the medium with and without an inner pressure decreased until 36 h after cultivation, as shown in Fig. 5A. The production of CMCase by *B. velezensis* A-68 was paralleled with cell growth. The optimal inner pressure for cell growth of *B. velezensis* A-68 was 0.00 MPa, whereas that for production of CMCase was 0.04 MPa. The productions of CMCase by *B. velezensis* A-68 with inner pressures of 0.00, 0.02, 0.04, 0.06, and 0.08 MPa after 72 h cultivation were 81.0, 98.3, 108.1, 96.4, and 90.2 U/ml, respectively, as shown in Figs. 5B and 5C. The production of CMCase with an inner pressure of 0.04 MPa

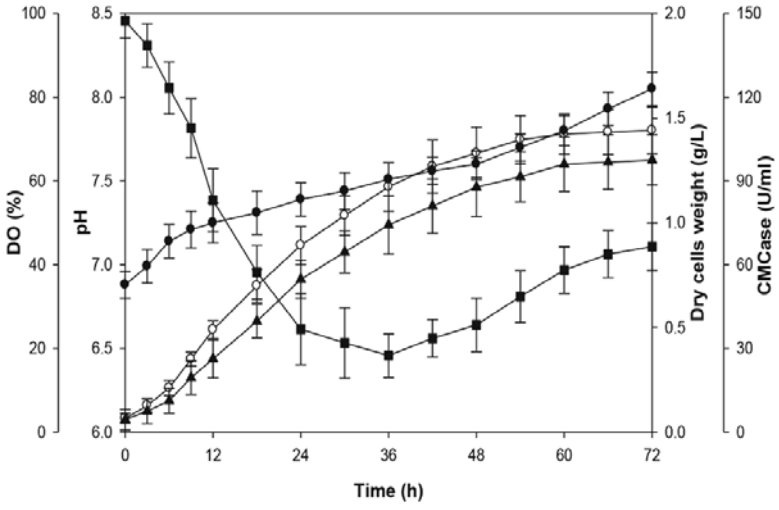

Fig. 6. Time-course of cell growth and production of CMCase by *B. velezensis* A-68 in a 100 L bioreactor under optimized conditions for production of CMCase (●, pH; ■, dissolved oxygen; ▲, cell growth; and ○, CMCase).

Table 4. Comparison of optimal conditions for cell growth and production of CMCase by *B. velezensis* A-68 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 hulls (g/L)	50.0	50.0	60.2	50.0	Kim *et al.* (2013)
	Yeast extract (g/L)	7.5	5.0	7.38	5.00	
	Initial pH	7.3	7.3	7.18	7.30	
	Maximal production	1.24 g/L	62.0 U/ml	1.23 g/L	61.3 U/ml	
Flask Scale-2	Temperature (°C)	30	35	-	-	
	Maximal production	1.46 g/L	83.8 U/ml	-	-	
Lab-scaled bioreactor	Agitation speed (rpm)	300	400	323	380	This study
	Aeration rate (vvm)	1.5	0.5	1.46	0.54	
	Maximal production	1.59 g/L	71.9 U/ml	1.49 g/L	88.3 U/ml	
Pilot-scaled bioreactor	Inner pressure (Mpa)	0.00	0.04	-	-	
	Maximal production	1.46 g/L	108.1 U/ml	-	-	

was 1.2 times higher than that without an inner pressure.

Higher inner pressures in pilot and industrial scale-bioreactors can increase concentration of the dissolved oxygen in the medium with the same amount of supplied air and protect the culture from contamination (Seo *et al.*, 2006). Increased inner pressures of a 100 L bioreactor resulted in higher concentrations of dissolved oxygen in the medium, which could potentially enhance the production of CMCase by *B. velezensis* A-68. The production of CMCase by another marine bacterium, *Cellulophaga lytica* LBH-14 with an inner pressure of 0.06 MPa was 1.38 times higher than that without an inner pressure, as shown in Table 3. Moreover, the amount of heteropolysaccharide-7 by *Beijerinckia indica* and pullulan by *Aureobasidium pullulans* with inner pressures of 0.04 MPa was 1.3 times higher than that without inner pressure (Seo *et al.*, 2004; Jung *et al.*, 2013). Increasing the driving force for diffusion from air bubbles to the medium by elevating the inner pressure of a bioreactor increased the oxygen transfer rate (OUR), which enhanced the production of β-glucosidase by *Pichia pastoris*, without cell growth (Charoenrat *et al.*, 2006). Higher agitation speeds and inner pressures, which can maintain higher concentrations of dissolved oxygen in the medium, resulted in the enhanced production of CMCase. However, increased inner pressures can damage cells, resulting in decreased cell growth of *B. velezensis* A-68.

The batch culture for the production of CMCase by *B. velezensis* A-68 was performed in a 100 L bioreactor under the optimized conditions, as shown in Fig. 6. Carbon and nitrogen sources were 50.0 g/L rice hulls and 5.0 g/L yeast extract. The initial pH and cultural temperature were 7.3 and 35°C. In this study, the optimized agitation speed, aeration rate, and inner pressure of a 100 L bioreactor were 240 rpm, 0.5 vvm, and 0.04 MPa, respectively. During the first 12 h, the pH of the medium increased from 6.88 to 7.25 and then increased gradually to 7.70 by 48 h of cultivation. The dissolved oxygen in the medium significantly decreased over 24 h and then gradually increased after 36 h and the cell growth of *B. velezensis* A-68 rapidly increased until 48 h. Furthermore, the production of CMCase by *B. velezensis* A-68 appeared to correlate with cell growth.

Conclusion

In this study, rice hulls were used as substrates for the production of CMCase by *B. velezensis* A-68 in a pilot-scale bioreactor, as shown in Table 4. The process developed in this study can be directly used for the mass production of CMCase. The maximal production of CMCase under optimized conditions in a 100 L bioreactor was 108.1 U/ml, which was 1.8 times higher than that at a flask scale under previously optimized conditions. The production of CMCase by wild type microorganisms was less than those by their recombinant strains, as shown in Table 5. Thus, the construction of recombinant strains of *B. velezensis* A-68 will

Table 5. Comparison of production of CMCases by various microorganisms under optimized conditions

Strain	Carbon source	Nitrogen source	Initial pH	Temperature (°C)	Productivity	Reference
Bacillus amyloliquefaciens DL-3	Rice hulls	Peptone	6.8	37	367 U/ml	Jo *et al.* (2008)
Bacillus atrophaeus LBH-18	Rice bran	peptone	7.0	30	128 U/ml	Kim *et al.* (2012b)
Bacillus licheniformis LBH-52	Rice hulls	Ammonium nitrate	7.0	36	75 U/ml	Kim *et al.* (2011a)
Bacillus subtilus subsp. *subtilis* A-53	Rice bran	Yeast extract	6.8	30	137 U/ml	Lee *et al.* (2010)
Bacillus velezensis A-68	Rice hulls	Yeast extract	7.3	35	108 U/ml	This study
Cellulophaga lytica LBH-14	Rice bran	Ammonium chloride	6.1	25	154 U/ml	Gao *et al.* (2013b)
Psychrobacter aquimaris LBH-10	Rice bran	peptone	8.0	30	339 U/ml	Kim *et al.* (2010)
Escherichia coli JM109/DL-3	Rice bran	Peptone	7.2	37	871 U/ml	Lee *et al.* (2012)
Escherichia coli JM109/A-53	Rice bran	Ammonium chloride	8.0	35	880 U/ml	Lee *et al.* (2013)
Aspergillus niger KK2	Rice straw	Yeast extract	7.0	28	129 U/g CS[a]	Kang *et al.* (2004)

[a] carbon source

overcome major limitations in the enzymatic saccharification of cellulosic materials, such as high costs and low productivity of cellulases (Sukumaran *et al.*, 2009). Time required for the production of cellulases by fungal species in solid-state fermentation normally takes 7 to 10 days (Kang *et al.*, 2004). In the present study, the time by a marine bacterium to produced CMCase was reduced to only 3 days, which increased the productivity of CMCase and decreased production costs.

Acknowledgements

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