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Enhanced Production of Carboxymethylcellulase by a Newly Isolated Marine Microorganism *Bacillus atrophaeus* LBH-18 Using Rice Bran, a Byproduct from the Rice Processing Industry

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A microorganism producing carboxymethylcellulase (CMCase) was isolated from seawater and identified as *Bacillus atrophaeus*. This species was designated as *B. atrophaeus* LBH-18 based on its evolutionary distance and the phylogenetic tree resulting from 16S rDNA sequencing and the neighbor-joining method. The optimal conditions for rice bran (68.1 g/l), peptone (9.1 g/l), and initial pH (7.0) of the medium for cell growth was determined by Design Expert Software based on the response surface method; conditions for production of CMCase were 55.2 g/l, 6.6 g/l, and 7.1, respectively. The optimal temperature for cell growth and the production of CMCase by *B. atrophaeus* LBH-18 was 30℃. The optimal conditions of agitation speed and aeration rate for cell growth in a 7-l bioreactor were 324 rpm and 0.9 vvm, respectively, whereas those for production of CMCase were 343 rpm and 0.6 vvm, respectively. The optimal inner pressure for cell growth and production of CMCase in a 100-l bioreactor was 0.06 MPa. Maximal production of CMCase under optimal conditions in a 100-l bioreactor was 127.5 U/ml, which was 1.32 times higher than that without an inner pressure. In this study, rice bran was developed as a carbon source for industrial scale production of CMCase by *B. atrophaeus* LBH-18. Reduced time for the production of CMCase from 7 to 10 days to 3 days by using a bacterial strain with submerged fermentation also resulted in increased productivity of CMCase and a decrease in its production cost.

Key words : *Bacillus atrophaeus*, carboxymethylcellulase, rice bran, response surface method

Introduction

Cellulosic biomass has an enormous potential and its conversion to fermentable sugars represents a major challenge in global efforts to utilize renewable resources [2]. A major constrain in enzymatic saccharification of cellulosic biomass for the production of fermentable sugars is low productivity and the cost of cellulases [31]. The enzymatic hydrolysis of cellulosic biomass required 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) [3,38]. The enzymatic saccharification of lignocellulosic materials for the production of ethanol was performed by commercial cellulases, in which the major cellulase was carboxymethylcellulase [35,36].

A number of cellulase-producing fungi and bacteria have been identified [10,21]. Most commercial cellulases have been produced by *Aspergillus* and *Trichderma* species with solid-state cultures [9]. Bacterial cellulase systems of *Clostridium, Cellomonas, Bacillus, Thermonospora, Ruminococcus, Bacteriodes, Erwinia,* and *Acetivibrio* species have been reported [14,22,28]. Enzymes and microbial metabolites produced by marine microorganisms can provide numerous advantages over traditional enzymes due to the severe and wide range of environments [11,16]. Activities of enzymes produced by psychrophilic microorganisms are reported to be much higher at low temperature than those of their mesophilic and thermophilic counterparts [17]. Cold-adapted peptidases were isolated from marine bacteria and a halo-tolerant marine bacterium, which produced κ-carrageenase, was studied [13].

In this study, a microorganism which utilized rice bran and produced carboxymethylcellulase (CMCase) was isolated from seawater and identified as *Bacillus atrophaeus*. *B. atrophaeus* is a commonly used model for the study of microbial development and cell differentiation in cell development [34]. However, the production of celluases by this strain had

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not been reported yet. Industrial scaled optimization for the production of CMCase by this strain was established using response surface method [15].

Materials and Methods

Isolation of a marine microorganism producing carboxymethylcellulase

To isolate microorganism producing carboxymethylcellulase (CMCase), seawater from the Kyungsang Province of Korea was suspended with 0.85% (w/v) NaCl. The suspension was then cultivated on marine agar plates at 30℃ for 3 day under aerobic conditions. Isolated cultures were prepared by transferring cells from the agar plate to 200 ml of medium in 500 ml Erlenmeyer flasks. The medium used for the production of CMCase consisted of 20.0 g/l carboxymethylcellulose (CMC), 2.5 g/l yeast extract, 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 $(NH_4)_2SO_4$. The resulting cultures were incubated at 30℃ for 3 day under aerobic conditions. Basis on the productivity of CMCase, one microorganism was selected for the production of CMCase and identified by sequencing of 16S rDNA.

Analysis of 16S rDNA sequences of the isolated microorganism

For the nucleotide sequence analysis of 16S rDNA, bacterial genomic DNA was extracted and purified using a Wizard Genomic DNA Prep. Kit (Promega Co., Madison, USA). Two primers annealing at the 5′ and 3′ end of the 16S rDNA were 5′-AGAGTTTGATCCTGGCTCAG-3′ (positions 8 to 27 [*E. coli* 16S rDNA numbering]) and 5′-AAGGAGGTGATCCAGCCGCA-3′ (positions 1541 to 1522 [*E. coli* 16S rDNA numbering]), respectively [37]. PCR amplification was performed as described in the previous report [14]. The PCR reaction was run for 35 cycles in a DNA thermal cycler (Model No. 9700, Perkin-Elmer Co. Wellesley, USA). The following thermal profile was used for the PCR: denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and extension at 72°C for 2 min. The final cycle included extension at 72°C for 10 min to ensure full extension of the products. The amplified PCR products were then analyzed in a 1.0% (w/v) agarose gel, excised from the gel, and purified. The purified products were cloned into a pGEM-T Easy vector (Promega Co., Madison, USA) and subsequently sequenced using an ALF Red automated DNA sequencer (Pharmacia, Sweden). The 16S rDNA sequence of the isolate was aligned with those in the GenBank database. Multiple alignments of sequences and calculations of levels of sequence similarity were performed by using CLUSTAL W [33]. Neighbor-joining phylogenetic analysis was carried out with MEGA program [20].

Production of CMCase by the isolated microorganism

Starter cultures were prepared by transferring cells from agar slants to 50 ml of the same medium except for agar in 250 ml Erlenmeyer flasks. The resulting cultures were incubated at 30℃ for 2 day under aerobic conditions. Each starter culture was used as an inoculum for 200 ml of medium in 500 ml Erlenmeyer flasks. The main culture was carried out in a medium containing 20 g/l glucose, 2.5 g/l yeast extract, 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 day under aerobic conditions. Samples were periodically withdrawn from the cultures to examine cell growth and the production of CMCase by the isolated microorganism [22].

Batch fermentations for the production of CMCase by an isolated microorganism 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 inoculum size of batch fermentations for the production of CMCase was 5% (v/v). Agitation was provided by three six-flat-blade impellers in a 7 l fermentor.

Experimental design and optimization for production of CMCase

The rice bran (X_1), ammonium chloride (X_2), and initial pH of the medium (X_3) were chosen as the independent variables and cell growth (Y_1) and CMCase (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 maximum values of cell growth and production of CMCase were taken as the responses of the design experiment. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). A multiple regression analysis of the data was carried out with the statistical software, Design-Expert (Version 7.1.6, Stat-Ease Inc., Minneapolis, USA).

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 interrelationships of the variables were also determined by fitting the second degree polynomial equation to data obtained from 13 experiments using mean values of the triplicates. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA).

Analytical methods

Dry cells weight (DCW) was measured as described in the previous report [10]. Activities of the CMCase produced by the isolated microorganism were 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, UK) 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

Identification of the isolated microorganism

A microorganism hydrolyzing carboxymethylcellulose (CMC) was isolated from seawater of the Kyungsang Province in Korea and designated strain LBH-18. The phylogenetic analysis of strain LBH-18 using the nucleotide sequence data of its 16S rDNA showed that this strain had the highest homology (99.8%) with *Bacillus atrophaeus* AB021181. Based on the evolution distance and the phylogenetic tree resulting from 16S rDNA sequencing and the neighbour-joining method [30], a microorganism isolated from seawater was identified as *Bacillus atrophaeus* and designated *B. atrophaeus* LBH-18, as shown in Fig. 1.

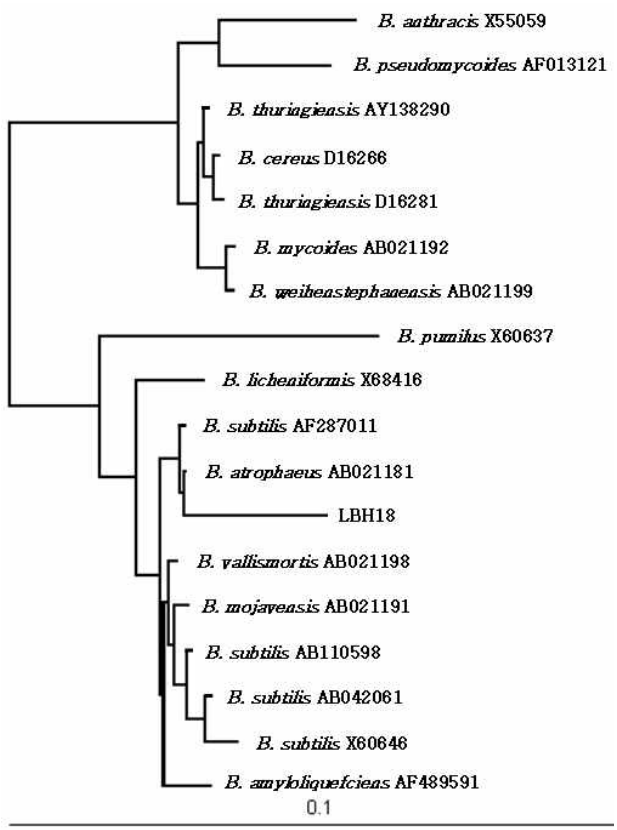

Fig. 1. Neighbour-joining tree based on 16S rDNA sequences of *Baillus atrophaeus*. Numbers at the nodes indicate the levels of bootstrap support based on a neighbour-joining analysis of 1,000 resampled dataset. Scale bar indicates 0.1 nucleotide substitution per nucleotide position.

Effects of carbon and nitrogen sources on production of CMCase

Effects of carbon and nitrogen sources on cell growth and the production of CMCase by *B. atrophaeous* LBH-18 were investigated. Carbon sources tested for production of CMCase were 20.0 g/l glucose, fructose, maltose, sucrose, rice bran, and rice hulls. Nitrogen sources tested were 2.5 g/l malt extract, peptone, tryptone, yeast extract, ammonium sulfate, and ammonium nitrate. Initial pH of the medium before sterilization was adjusted to 6.8. Rice bran and peptone were found to be the best combination of carbon and nitrogen sources for cell growth as well as the production of the CMCase by *B. atrophaeous* LBH-18, as shown in Fig. 2.

The best combination of carbon and nitrogen sources for the production of CMCase produced by *B. amyloliquefaciens* DL-3 was rice hulls and peptone, whereas that by *B. sutilis* subsp. *sutilis* A-53 was rice bran and yeast extract [10,22]. Rice bran was reported to be the best carbon source for the production of CMCase by *Bacillus* sp. CH43 and HR68 [27]. The composition of the rice bran used in this study was 48.0% carbohydrate, 6.9% fiber, 14.9% crude lipid, 13.1% crude protein, 7.6% ash, and 9.5% water [22]. The best combination of carbon and nitrogen sources for the production of CMCase produced by *B. sutilis* subsp. *sutilis* A-53, which was isolated form seawater, were rice bran and yeast extract [22]. All strains investigated to date for the production of cellulases are inducible by cellulose, lactose or sophorose, and repressible by glucose, which are reasons why the best carbon sour-

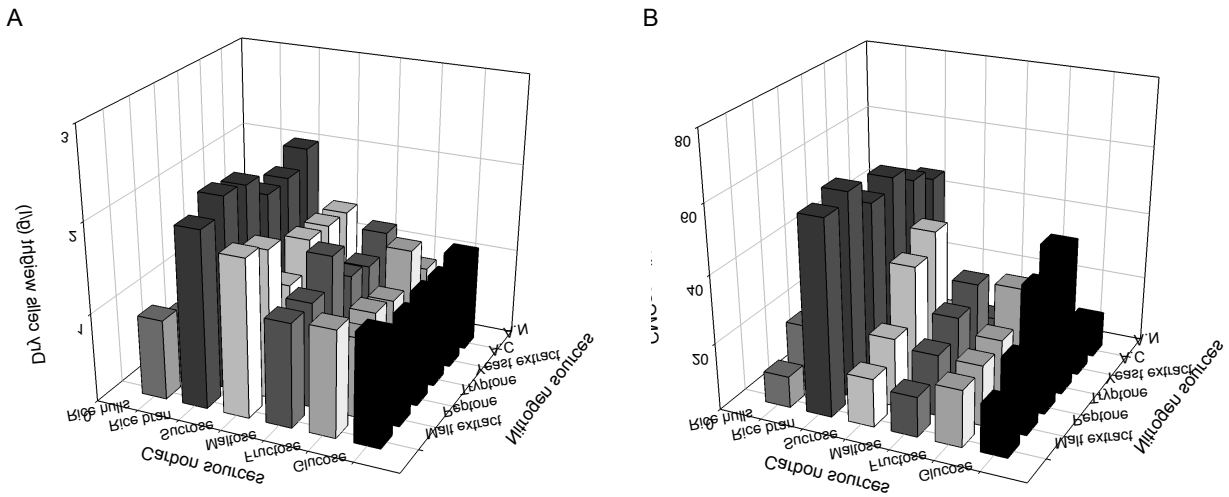

Fig. 2. Effect of carbon and nitrogen sources on cell growth (A) and production of CMCase (B) by *B. atrophaeus* LBH-18.

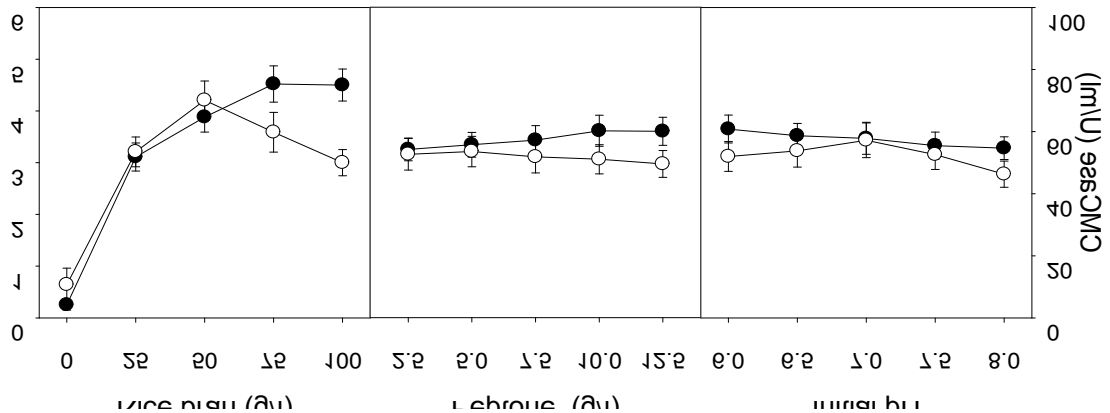

Fig. 3. Effect of rice bran, peptone, and initial pH on cell growth and production of CMCase by *B. atrophaeus* LBH-18 (●, DCW and ○, CMCase).

ces for the production of cellulases by bacterial and fungal microorganisms are rice hulls, rice bran or wheat bran [6,12]. Induction, synthesis, and secretion of the β-glucanase appear to be closely associated [29].

Effects of rice bran, peptone, and initial pH on production of CMCase

Effects of rice bran and peptone as carbon and nitrogen sources, and initial pH of the medium on cell growth and the production of CMCase by *B. atropphaeus* LBH-18 were investigated using one-factor-at-a-time method. Composition of basic medium and initial pH were 50.0 g/l rice bran, 7.5 g/l peptone, and pH 7.0. The optimal concentration of rice bran and peptone, and initial pH of the medium for cell growth were found to be 75.0 g/l, 10.0 g/l, and 6.0, whereas those for production of CMCase were 50.0 g/l, 5.0 g/l, and 7.0, as shown in Fig. 3. The optimal concentration of rice ran and peptone, and initial pH of the medium for cell growth were different from those for production of CMCase. Initial pH of the medium, especially during a batch culture without pH control, is one of the most important factors, which affects cell growth as well as its production of metabolites [1]. The optimal initial pHs of the medium for the production of CMCase by *B. amyloliquefaciens* DL-3 and *B. subtilis* subsp. *subtilis* were 6.8, whereas those for their cell growth were 7.3 [10,22].

Optimization of rice bran, peptone, and initial pH using response surface method

The simultaneous effects of rice bran, peptone, and initial pH of the medium on cell growth and the production of CMCase by *B. atropphaeus* LBH-18 were investigated using response surface method. The minimum and maximum range of variables, rice bran, peptone, and initial pH of medium, with respect to their coded values were 25.0 and 75.0 g/l, 5.0 and 10.0 g/l, and 6.5 and 7.5, respectively. Cell growth, measured as dry cells weight (DCW) and production of CMCase from 20 different conditions ranged from 2.82 to 3.88 g/l and from 44.5 to 60.7 U/ml, as shown in Table 1. The model *F*-value of 24.58 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 ANOVA indicated that this model and the model term of X_1 ("probe > F" less 0.0001) were highly significant [23]. The ANOVA also indicated that the model term of X_1^2 ("probe > F" less 0.0500) were significant for cell growth of *B. atropphaeus* LBH-18. The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R^2 was 0.9568. The model can explain 95.68% variation in the response. The value of the adjusted determination coefficient (Adj. R^2=0.9178) was very high to advocate for a high significance of this model [24]. The predicted determination of coefficient of 0.7649 was in reasonable agreement with the Adj. R^2 of 0.9178. From the statistical results obtained, it was shown that this model was adequate to predict the cell growth of *B. atropphaeus* LBH-18 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 (1). The optimal conditions of rice bran, peptone, and initial pH of the medium for cell growth extracted by Design Expert Software were 68.1 g/l, 9.1 g/l, and 7.0, respectively. The maximum cell growth of 3.77 g/l was predicted by this model.

$$Y_1=3.60+0.26X_1+0.04X_2-0.03X_3-0.09X_1^2+0.01X_2^2+0.01X_3^2 \quad (1)$$

The model *F*-value of 18.70 from the ANOVA of production of CMCase implied that this model was also significant. The ANOVA indicated that this model and model term of X_1^2 were highly significant and those of X_1 and X_3^2 were significant. The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R^2 was 0.9439. The value of the adjusted determination coefficient (Adj. R^2=0.8934) was high to advocate for a high significance of this model. The predicted determination of coefficient of 0.6141 was also in reasonable agreement with the Adj. R^2 of 0.8934. From the statistical results obtained, it was shown that this model was adequate to predict the production of CMCase by *B. atropphaeus* LBH-18 within the

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

Run	X_1	X_2	X_3	Y_1	Y_2
1	75.0	10.0	6.5	3.88	54.9
2	75.0	5.0	7.5	3.73	55.3
3	50.0	11.7	7.0	3.65	59.1
4	25.0	10.0	6.5	3.41	52.8
5	92.0	7.5	7.0	3.80	54.0
6	50.0	7.5	6.2	3.63	58.2
7	50.0	7.5	7.0	3.59	59.7
8	50.0	3.3	7.0	3.54	60.1
9	50.0	7.5	7.0	3.62	60.5
10	8.0	7.5	7.0	2.82	44.5
11	25.0	5.0	6.5	3.32	53.6
12	50.0	7.5	7.0	3.69	60.7
13	50.0	7.5	7.0	3.52	59.2
14	75.0	10.0	7.5	3.82	54.5
15	25.0	10.0	7.5	3.35	52.4
16	50.0	7.5	7.0	3.57	59.4
17	50.0	7.5	7.8	3.54	56.9
18	75.0	5.0	6.5	3.79	55.7
19	50.0	7.5	7.0	3.63	59.5
20	25.0	5.0	7.5	3.26	53.2

Table 2. Parameter estimates and analysis of variance (ANOVA) of the design for cell growth and production of CMCase by *B. atrophaeus* LBH-18

	Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*-value	Probe>F
Cell growth	Model	9	1.060	0.120	24.58	<0.0001
	X_1	1	0.910	0.910	189.37	<0.0001
	X_2	1	0.022	0.022	4.52	0.0595
	X_3	1	0.011	0.011	2.33	0.1579
	X_1^2	1	0.110	0.110	22.59	0.0008
	X_2^2	1	0.003	0.003	0.58	0.4646
	X_3^2	1	0.002	0.002	0.32	0.5833
	Error	5	0.017	0.003	-	-
	Total	19	1.110	-	-	-
CMCase	Model	9	281.620	32.290	18.70	<0.0001
	X_1	1	43.510	43.510	26.00	0.0005
	X_2	1	1.750	1.750	1.04	0.3312
	X_3	1	1.050	1.050	0.63	0.4467
	X_1^2	1	228.830	228.830	136.76	<0.0001
	X_2^2	1	1.530	1.530	0.91	0.3619
	X_3^2	1	15.900	15.900	9.50	0.0116
	Error	5	1.910	0.380	-	-
	Total	19	298.360	-	-	-

range of variables studied. Multiple regression analysis of the experimental data gave the following second-order polynomial equation in terms of coded factors (2). The optimal conditions of rice bran, peptone, and initial pH of the medium for production of CMCase were 55.2 g/l, 6.6 g/l, and 7.1, respectively. The maximum production of CMCase of 60.0 U/ml was predicted by this model.

$$Y_2=59.87+1.78X_1-0.36X_2-0.28X_3-3.98X_1^2-0.33X_2^2-1.05X_3^2 \quad (2)$$

Effect of temperature on production of CMCase

The effect of temperature on cell growth and the production of CMCase by *B. atropphaeus* LBH-18 was examined. Temperatures for cell growth and production of CMC ranged from 25 to 45℃. The optimal temperature for cell growth as well as the production of CMCase of *B. atrophaeus* LBH-18 was found to be 30℃, as shown in Fig. 4. Maximal cell growth and production of CMCase at 30℃ were 3.71 g/l and 104.6 U/ml. Significance of each value for cell growth and production of CMCase was analyzed by DPS software version 3.01 (DPS Co., Middlesex, UK). The optimal temperatures for cell growth of *B. amyloliquefaciens* DL-3 and *B. subtilis* subsp. *subtilis* were 32 and 35℃, respectively, whereas those for production of CMCases were 37 and 30℃, respectively [10,22]. The optimal temperatures for productions of cellulases by *T. reesei* QM9414 and *T. reesei* MCG77 in solid-state fermentation using rice bran as a substrate were 30 and 25℃, respectively [21]. Unlike other strains producing cellulases, the optimal temperature for cell growth of *B. atrophaeus* LBH-18 was the same as that for production of CMCase.

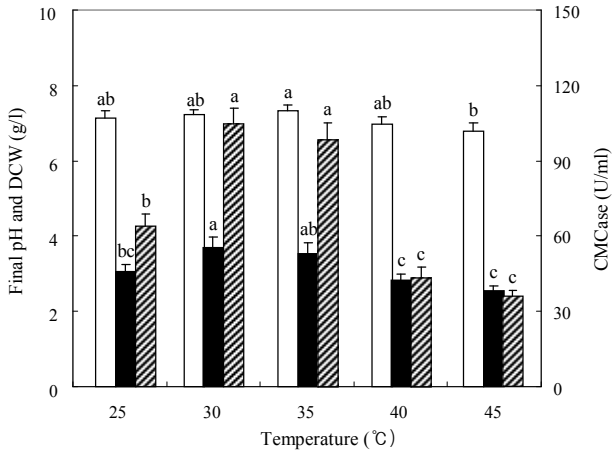

Fig. 4. Effect of temperature on cell growth and production of CMCase by *B. atrophaeus* LBH-18 (□, final pH; ■, DCW; and ▨, CMCase).

Effects of agitation speed and aeration rate on production of CMCase

Effects of agitation speed and aeration rate on cell growth

and the production of CMCase by *B. atrophaeus* LBH-18 were investigated in a 7 l bioreactor. The minimum and maximum ranges of agitation speed and aeration rate with respect to coded values were 200 and 400 rpm and 0.5 and 1.5 vvm. Cell growth and production of CMCase from 13 different conditions ranged from 2.37 to 3.10 g/l and from 87.0 to 106.6 U/ml, as shown in Table 3. The model *F*-value of 22.16 from the ANOVA of cell growth implied that this model was significant, as shown in Table 4. The ANOVA indicated that the model and model terms of X_1, X_2, X_1^2 and X_2^2 were significant for cell growth of *B. atrophaeus* LBH-18. The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R^2 was 0.9406. The value of the adjusted determination coefficient (Adj. R^2=0.8981) was high to advocate for a high significance of this model. The predicted determination of coefficient of 0.7612 was in reasonable agreement with the Adj. R^2 of 0.8981. From the statistical results obtained, it was shown that the above models were adequate to predict the cell growth of *B. atrophaeus* LBH-18 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 (3). The optimal conditions of agitation speed and aeration rate for cell growth were 324 rpm and 0.9 vvm. The maximum cell growth of 3.01 g/l was predicted by this model.

$$Y_1' = 2.99 + 0.07X_1 - 0.11X_2 - 0.19X_1^2 - 0.23X_2^2 \quad (3)$$

Table 3. Central composite design and determined response values

Run	X_1	X_2	Y_1	Y_2
1	400	0.5	2.84	103.7
2	300	1.7	2.39	92.4
3	300	1.0	2.99	102.4
4	200	0.5	2.67	97.1
5	300	1.0	3.04	104.2
6	200	1.5	2.37	87.0
7	300	0.3	2.61	103.1
8	441	1.0	2.67	97.8
9	300	1.0	3.10	106.6
10	159	1.0	2.50	88.1
11	300	1.0	2.91	100.5
12	300	1.0	2.93	99.8
13	400	1.5	2.54	93.5

Table 4. Parameter estimates and analysis of variance (ANOVA) of the design for cell growth and production of CMCase by *B. atrophaeus* LBH-18 in a 7 l bioreactor

	Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*-value	Probe>F
Cell growth	Model	5	0.700	0.140	22.16	0.0004
	X_1	1	0.042	0.042	6.70	0.0360
	X_2	1	0.100	0.100	16.51	0.0048
	X_1X_2	1	0.000	0.000	0.00	1.0000
	X_1^2	1	0.250	0.250	39.48	0.0004
	X_2^2	1	0.370	0.370	59.24	0.0001
	Error	4	0.025	0.006	-	-
	Total	12	0.740	-	-	-
CMCase	Model	5	436.660	87.330	17.88	0.0007
	X_1	1	89.900	89.900	18.41	0.0036
	X_2	1	156.930	156.930	32.13	0.0008
	X_1X_2	1	0.003	0.003	0.00	0.9826
	X_1^2	1	165.750	165.750	33.94	0.0006
	X_2^2	1	42.830	42.830	8.77	0.0211
	Error	4	30.800	7.700	-	-
	Total	12	470.850	-	-	-

The model *F*-value of 17.88 from the ANOVA of production of CMCase implied that this model was also significant. The ANOVA indicated that this model and the model term of X1, X_2, X_1^2, and X_2^2 were significant for the production *B. atrophaeus* LBH-18. The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R^2 was 0.9274. The value of the adjusted determination coefficient (Adj. R^2=0.8755) was very high to advocate for a high significance of this model. The predicted determination of coefficient of 0.8466 was also in reasonable agreement with the Adj. R^2 of 0.8755. From the statistical results obtained, it was shown that the above models were adequate to predict the production of CMCase by *B. atrophaeus* LBH-18 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 (4). The optimal conditions of agitation speed and aeration rate for production of CMCase were 343 rpm and 0.60 vvm. The maximum production of CMCase of 105.2 U/ml was predicted by this model.

$$Y_2' = 102.70 + 3.35X_1 - 4.43X_2 - 0.03X_1X_2 - 4.88X_1^2 - 2.482X_2^2 \quad (4)$$

The three-dimensional response surface plots were generated to investigate the interaction between agitation speed and aeration rate to visualize their combined effect on the response of cell growth and the production of CMCase by *B. atrophaeus* LBH-18, as shown in Fig. 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 of agitation speed and aeration rate [26]. In contrast to the circular shape in the 3D plot, the elliptical nature of the curve in the 3D plot indicated significant mutual interactions between variables. Interactive effect of agitation speed and aeration rate on the production of CMCase by *B. atrophaeus* LBH-18 was relatively more significant than that on production of cell growth.

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 [10,22]. The optimal agitation speeds and aeration rates for the production of CMCase by *B. amyloliquefaciens* and *B. subtilis* subsp. *subtilis* were 300 rpm and 1.0 vvm, which were lower than those for their cell growth [10,22]. Higher agitation speed and aeration rate, which resulted in increase of the concentration of dissolved oxygen in the medium, enhanced cell growth of *B. atrophaeus* LBH-18. However, higher than optimal concentration of dissolved oxygen for the production of CMCase by *B. atrophaeus* LBH-18 seemed to lead the biosynthetic pathway to cell growth, but not to production of CMCase. It seems that a higher than optimal concentration of dissolved oxygen for the production of CMCase by *B. atrophaeus* LBH-18 leads the

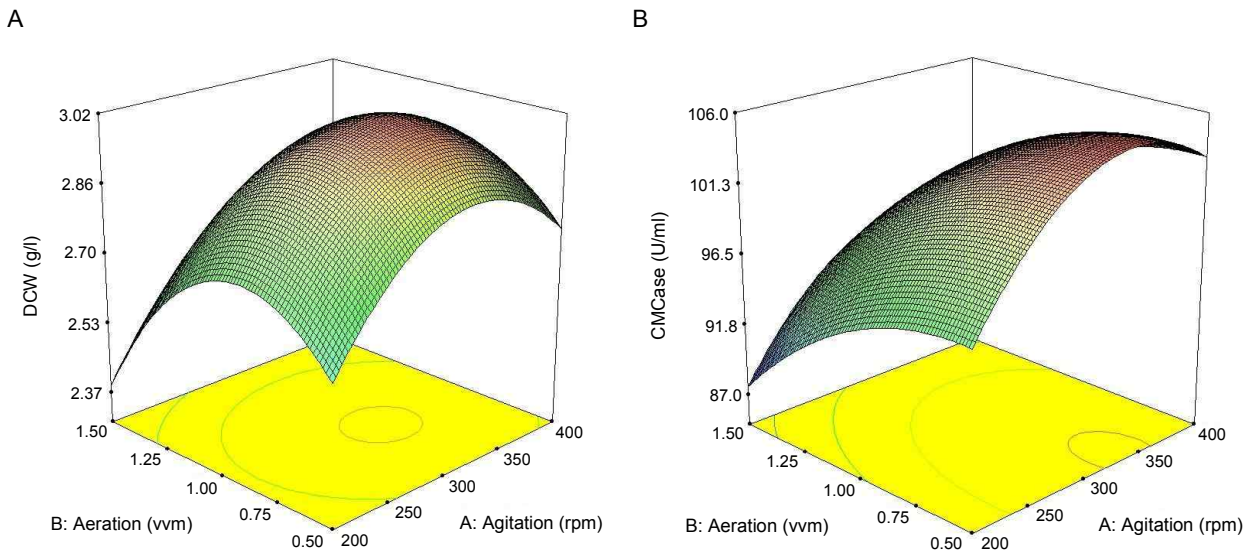

Fig. 5. 3D response surface displaying relative effect of two variables on cell growth and the production of CMCase by *B. atrophaeus* LBH-18 in a 7 l bioreactor; interaction between agitation speed and aeration rate for cell growth (A) and the production of CMCase (B).

biosynthetic pathway to cell growth, but not to production of CMCase.

Effect of inner pressure on production of CMCase in a 100 l bioreactor

The effect of inner pressure on cell growth and the production of CMCase by *B. atrophaeous* LBH-18 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 0.6 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 250 rpm is almost the same as that of a 7 l bioreactor at 343 rpm. The pH of the medium rapidly decreased and reached about 6.5 after 12 hr of cultivation, then gradually increased, as shown in Fig. 6. The concentration of the dissolved oxygen in the medium dramatically decreased and then reached 0% at the middle of the log phase, and the production of CMCase by *B. atrophaeus* LBH-18 started. The optimal inner pressure of a 100 l bioreactor for cell growth of *B. atrophaeus* LBH-18 was 0.06MPa, which was the same as that for the production of CMCase.

Productions of CMCase by *B. atrophaeus* LBH-18 with an inner pressure of 0.00, 0.02, 0.04, 0.06, and 0.08 MPa after 72 hr of cultivation were 96.5, 106.8, 119.0, 127.5, and 76.2 U/ml, respectively. The production of CMCase by *B. atrophaeus* LBH-18 with an inner pressure of 0.06 MPa was 1.32 times higher than that without inner pressure. Increased inner pressure in a 100 l bioreactor resulted in a higher concentration of dissolved oxygen in the medium, which might be enhance cell growth as well as the production of CMCase by *B. atrophaeus* LBH-18 [8]. Variation in agitation speed and

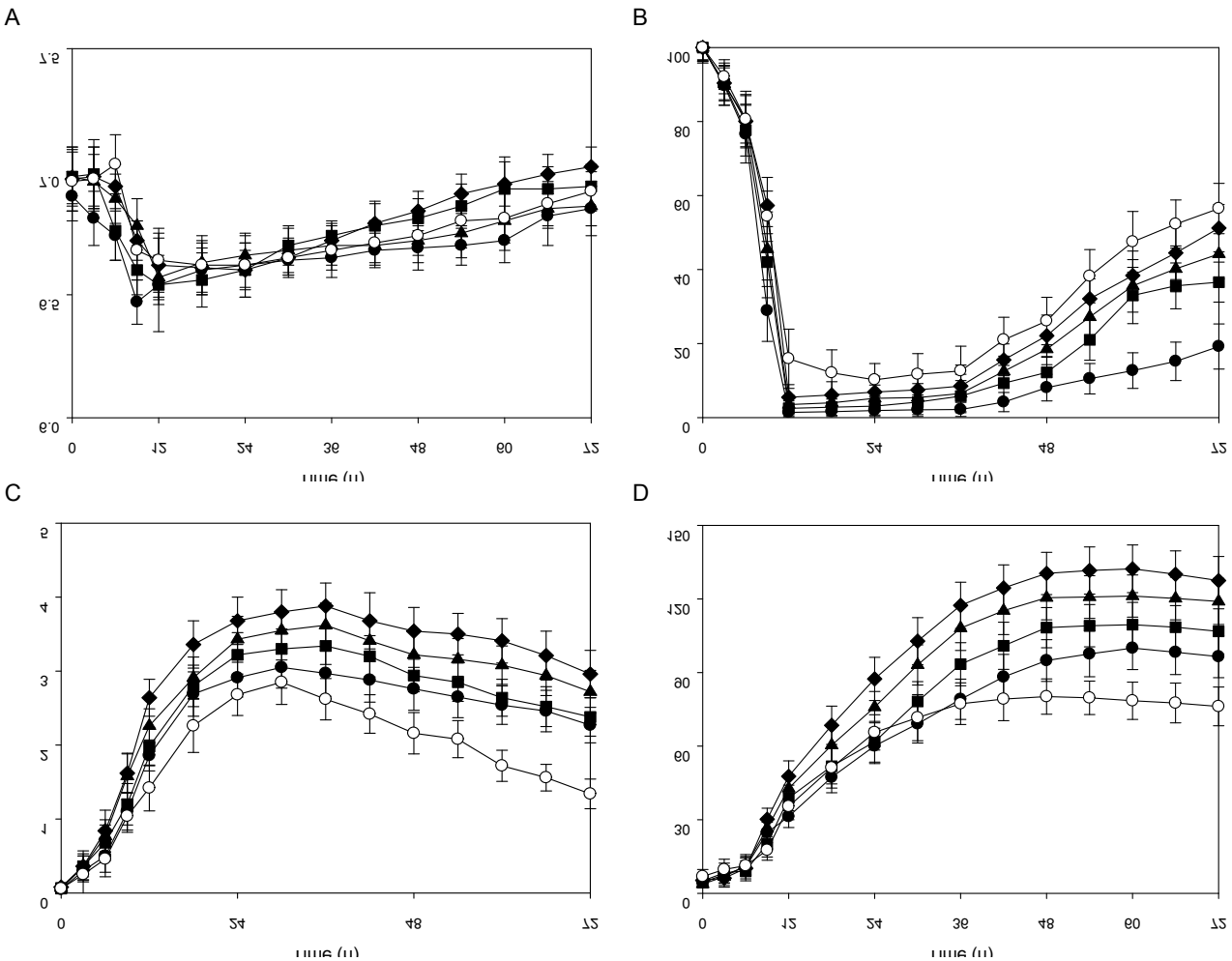

Fig. 6. Effect of the inner pressure in a 100 l bioreactor on pH of medium (A), dissolved oxygen (B), cell growth (C), and production of CMCase (D) by *B. atrophaeus* LBH-18 in a 100 l bioreactor (●, 0.00 MPa; ■, 0.02 MPa; ▲, 0.04 MPa; ◆, 0.06 MPa; and ○, 0.08 MPa).

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 [5,7]. The optimal aeration rate for cell growth was higher than that for the production of CMCase by *B. atrophaeus* LBH-18. The optimal inner pressure for cell growth was the same as that for the production of CMCase. Higher inner pressure can afford for higher concentration of dissolved oxygen to enhance cell growth unless it damage to cells. *B. atrophaeus* LBH-18 seemed to be strong enough to endure higher inner pressure, which resulted in enhanced production of CMCase.

Various carbon sources have been used for the production of cellulases, but their prices are too expensive to apply commercially [4,25]. Rice bran from the rice processing industry is produced in large amounts in Korea, as well as other rice producing countries. In this study, rice bran was developed to be a carbon source and the optimal conditions for the production of CMCase by *B. atrophaeus* LBH-18 was established using an orthogonal array method, as shown in Table 5. The optimal conditions for production of CMCase by bacterial and fungal species were compared, as shown in Table 6. Production of CMCase by fungal species with solid cultures normally takes 7 to 10 days. In this study, it took 3

Table 5. Optimal conditions for cell growth and production of CMCase by *B. asrophaeus* LBH-18

Scale	Conditions	Optimal conditions			
		One-factor-at-a-time method		Response surface method	
		DCW	CMCase	DCW	CMCase
Flask scale	Rice bran (g/l)	75.0	50.0	68.1	55.2
	Peptone (g/l)	10.0	5.0	9.1	6.6
	Initial pH	6.0	7.0	7.0	7.1
	Maximal production	4.52 g/l	70.1 U/ml	3.77 g/l	60.0 U/ml
Bioreactor scale	Agitation speed (rpm)	-	-	324	343
	Aeration rate (vvm)	-	-	0.9	0.6
	Maximal production	-	-	3.01 g/l	105.2 U/ml
Pilot scale	Inner pressure (MPa)	0.06	0.06	-	-
	Maximal production	2.96 g/l	127.5 U m/l	-	-

Table 6. Comparison of optimal conditions for the production of various CMCases by bacterial and fungal microorganisms

Strain	Carbon source	Nitrogen source	Initial pH	Temperature (℃)	Productivity	Reference
Bacillus amyloliquefaciens DL-3	Rice hulls	Peptone	6.8	37	367 U/ml	[10]
Bacillus atrophaeus LBH-18	Rice bran	peptone	7.0	30	128 U/ml	This study
Bacillus licheniformis LBH-52	Rice hulls	Ammonium nitrate	7.0	36	75 U/ml	[18]
Bacillus subtilus subsp. *subtilis* A-53	Rice bran	Yeast extract	6.8	30	137 U/ml	[22]
Bacillus subtilis CBTK 106	Banana fruit stalk waste	Yeast extract	7.0	35	10 U/g CS[a]	[19]
Cellomonas sp.	Wheat straw	Peptone	-	28	3 U/ml	[6]
Psychrobacter aquimaris LBH-10	Rice bran	peptone	8.0	30	339 U/ml	[16]
Aspergillus niger KK2	Rice straw	Yeast extract	7.0	28	129 U/g CS	[12]
Trichoderma harizanum	Wastewater sludge and wheat flour	-	5.0	33	10 U/ml	[1]
Trichoderma reesei	Wheat bran and avicel	Peptone	4.0[b]	28	133 U/ml	[39]
Trichoderma reesei Rut-C30	Solks Floc	Protease peptone	3.9-5.0[b]	25	184 U/ml	[25]
Trichoderma viride SL-1	Wheat bran	Yeast extract	-	32	1,400 U/g CS	[32]

[a]carbon source
[b]maintenance pH

days to produce CMCase by *B. atrophaeus* LBH-18. Reduced time for production of CMCase using a bacterial strain with submerged fermentations also results in increase in productivity of CMCase and decrease in its production cost.

Acknowledgement

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초록 : 미강을 이용한 해양미생물 *Bacillus atrophaeus* LBH-18 유래의 carboxymethylcellulase 생산의 최적화

김이준[1,3] · 고와[1,2] · 이유정[1,2] · 이상운[1,2] · 정정한[3] · 이진우[2,3]*
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Carboxymethylcellulase를 생산하는 미생물을 해수에서 분리하여 16S rDNA의 염기서열을 분석하고 계통 발생학 방법으로 비교한 결과, *Bacillus atrophaeus*로 확인되었다. 이 해양 미생물을 *B. atrophaeus* LBH-18로 명명하였으며 response surface method (RSM)를 사용하여 carboxymethylcellulase의 생산 조건을 최적화하였다. 이 균주의 생육에 최적인 미강, 펩톤 및 배지의 초기 pH는 68.1 g/l, 9.1 g/l 및 7.0이었으나, carboxymethylcellulase의 생산에 최적인 조건은 각각 55.2 g/l, 6.6 g/l 및 7.1이었다. 이 균주의 생육과 carboxymethylcellulase의 생산에 최적인 온도는 30℃이었다. 이 균주의 생육에 최적인 생물배양기의 교반속도 및 통기량은 324 rpm 및 0.9 vvm이었으나, carboxymethylcellulase의 생산에 최적인 조건은 각각 343 rpm 및 0.6 vvm이었다. 파이롯트 규모의 생물배양기를 사용하여 실험한 결과, 이 균주의 생육과 carboxymethylcellulase의 생산에 최적인 내압은 0.06 MPa이었다. 최적 조건의 내압으로 배양한 결과, 이 균주의 carboxymethylcellulase의 생산성은 127.5 U/ml이었으며, 이 결과는 내압을 가하지 않고 배양한 경우에 비하여 1.32배 향상된 것이다. 본 연구를 통하여 쌀 도정 공정의 부산물인 미강을 기질로 개발하였으며 해양 미생물을 사용하여 carboxymethylcellulase의 생산기간을 7~10일에서 3일로 단축시켰다.