Statistical Optimization for Production of Carboxymethylcellulase from Rice Hulls by a Newly Isolated Marine Microorganism *Bacillus licheniformis* LBH-52 Using Response Surface Method

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A microorganism utilizing rice hulls as a substrate for the production of carboxymethylcellulase (CMCase) was isolated from seawater and identified as *Bacillus lincheniformis* by analyses of its 16S rDNA sequences. The optimal carbon and nitrogen sources for production of CMCase were found to be rice hulls and ammonium nitrate. The optimal conditions for cell growth and the production of CMCase by *B. lincheniformis* LBH-52 were investigated using the response surface method (RSM). The analysis of variance (ANOVA) of results from central composite design (CCD) indicated that a highly significant factor ("probe>F" less than 0.0001) for cell growth was rice hulls, whereas those for production of CMCase were rice hulls and initial pH of the medium. The optimal conditions of rice hulls, ammonium nitrate, initial pH, and temperature for cell growth extracted by Design Expert Software were 48.7 g/l, 1.8 g/l, 6.6, and 35.7℃, respectively, whereas those for the production of CMCase were 43.2 g/l, 1.1 g/l, 6.8, and 35.7℃. The maximal production of CMCase by *B. lincheniformis* LBH-52 from rice hulls under optimized conditions was 79.6 U/ml in a 7 l bioreactor. In this study, rice hulls and ammonium nitrate were developed to be substrates for the production of CMCase by a newly isolated marine microorganism, and the time for production of CMCase was reduced to 3 days using a bacterial strain with submerged fermentation.

Key words : *Bacillus licheniformis*, marine microorganism, carboxymethylcellulase, rice hulls, optimization, response surface method

Introduction

Cellulose can be widely used to produce sustainable biomass and bioenergy to replace depleting fossil fuels [38]. Rice hulls are the main byproducts of the paddy process and accounts for 20-25% of the whole weight. More than 113 million metric tons of rice hulls are generated each year throughout the world. Due to its widespread availability and relatively low cost, rice hulls have the potential to serve as the feed stock for the production of fuel ethanol [33]. Three different types of cellulases, endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21), are considered to degrade crystalline cellulose to fermentable sugars [10]. Many efforts have been made to transform cellulosic biomass into fermentable sugars using cellulases [6,19].

Recently, endoglucanases (carboxymethylcellulases) have

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been applied in the textile and detergent industries. In the textile industry, endoglucanases are used for removing fuzz from the surface of cellulosic fibers, and enhancing the softness and the brightness of cotton fabric [9]. They are also used to facilitate the removal of soil by swelling the cotton fabric in the detergent industry. Cellulases are also used in improving nutritional quality and digestibility of animal feeds, in processing of fruit juices, and in baking, while de-inking of paper is yet another emerging application [25].

Enzymes produced by marine microorganisms can provide numerous advantages over traditional enzymes due to severe conditions and wide range of environments [20,31]. Hyperthermophilic bacteria have been isolated from marine sediments for biomass conversion of hemicellulose such as xylans and mannans [4]. A κ-carrageenase produced by a halo-tolerant marine bacterium was purified and characterized [16]. In this study, a microorganism utilizing rice hulls as a substrate for production of carboxymethylcellulase (CMCase) was isolated from seawater and the optimal conditions for the production of CMCase by this marine micro-

organism were investigated using response surface methodology (RSM).

Materials and Methods

Isolation of marine microorganisms producing carboxymethylcellulase

To isolate microorganism producing cellulases, 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 days under aerobic conditions. Isolated cultures were prepared by transferring cells from the agar plates to 200 ml of medium in 500 ml Erlenmeyer flasks. The medium used for the production of CMCase by a carboxymethylcellulase (CMC) hydrolyzing isolated microorganism contained the following components: 20.0 g/l CMC, 2.5 g/l yeast extract, 5.0 g/l K_2HPO_4, 1.0 g/l NaCl, 0.2 g/l $MgSO_4·7H_2O$, and 0.6 g/l $(NH_4)_2SO_4$. The resulting cultures were incubated at 30℃ for 3 days under aerobic conditions. Based on the productivity of CMCase, one microorganism was selected for production of CMCase and identified by sequencing of 16S rDNA nucleotides.

Identification of the isolated microorganism

For the nucleotide sequence analysis, 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′ ends of the 16S rDNA were 5′-AGAGTTTGATCCTGGCTCAG-3′ (positions 8 to 27, *E. coli* 16S rDNA numbering) and 5′-AAGGAGGTGATCC AGCCGCA-3′ (positions 1541 to 1522), respectively [39]. PCR amplification was performed as described in the previous report [18]. 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. Amplified PCR products were then analyzed in a 1.0% (w/v) agarose gel, excised from the gel, and purified. 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 [37]. Neighbor-joining phylogenetic analysis was carried out with a MEGA program [22].

Experimental design and optimization for production of CMCase

The rice bran (X_1), ammonium nitrate (X_2), initial pH of the medium (X_3), and temperature (X_4) were chosen as independent variables and cell growth (Y_1, g/l) and CMCase (Y_2, U/ml) were used as dependent output variables for response surface optimization. The total number of experiments was 30 (=2^k+2k+6), where k is the number of independent variables [34]. The interrelationships of variables were determined by fitting the second degree polynomial equation to data obtained from 30 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 8.0, Stat-Ease Inc., Minneapolis, USA) and the second order polynomial equation (1) that defines predicted response (Y) in terms of the independent variables (X_1, X_2, X_3 and X_4) was obtained:

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_4 + A_{12}X_1X_2 + A_{13}X_1X_3 + A_{14}X_1X_4 + A_{23}X_2X_3 + A_{24}X_2X_4 + A_{34}X_3X_4 + A_{11}X_1^2 1 + A_{22}X_2^2 + A_{33}X_3^2 + A_{44}X_4^2$$

(1)

Where X_1, X_2, X_3 and X_4 are input variables; A_0 is constant; A_1, A_2, A_3, and A_4 are linear coefficients; A_{12}, A_{13}, A_{14}, A_{23}, A_{24}, and A_{34} are cross-product coefficients; A_{11}, A_{22}, A_{33}, and A_{44} are quadratic coefficients. Combinations of factors (such as X_1X_2) represent an interaction between the individual factors in that term. Then the response is a function of the levels of factors.

Batch fermentation for production of CMCase

Batch fermentations for the production of CMCase by an isolated microorganism were performed in 7 l bioreactors (Ko-Biotech Co., Korea). The working volume of 7 l bioreactors was 5 l, and inoculum size of batch fermentation was 5% (v/v). Agitation was provided by three six-flat-blade

impellers in the 7 1 bioreactor.

Scanning electron microscopic observations

Samples were fixed by 3.0% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) with 0.1% (w/v) MgSO$_4$ at 4℃ for 30 min. Fixed samples were post-fixed by 1.0% (v/v) osmium tetroxide in 0.2 M cacodylate buffer (pH 7.4) at 4℃ for 24 hr and serially dehydrated in ethyl alcohol. After critical-point drying, they were coated with gold using a sputter-coating system (sputter-coater 5150A, Edward High Vacuum International, Crawley, England) and then examined with a scanning electron microscope (JSM-35CF, JEOL, Japan) which operated with an accelerating voltage of 5 KV. Images were digitized and stored in tagged image file format in the microscope computer.

Analytical methods

Dry cells weight was measured by directly weighing the biomass after drying to a constant weight at 100-105℃ after collection of cells by centrifugation at 12,000× g for 10 min. CMCase activity was measured by mixing 0.1 ml of enzyme solution with 0.1 ml of 10.0 g/l CMC in 10 mM sodium phosphate buffer, pH 6.5 at 50℃ for 20 min. The reaction was stopped by adding 3,5-dinitrosalicylic acid (DNS) reagent. The mixture was boiled for 10 min, cooled in ice and its optical density at 550 nm was determined [28]. Glucose (Sigma-Aldrich, UK) was used to prepare a calibration curve. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of glucose per min at 50℃.

Results and Discussion

Identification of the isolated microorganism

A microorganism hydrolyzing carboxymethylcellulose (CMC) was isolated from seawater in Kyungsang Province in Korea and designated as strain LBH-52. The phylogenetic analysis of strain LBH-52 using its 16S rDNA nucleotide sequence data showed that this strain had more than 99% homology with *Bacillus lincheniformis* strians, as shown in Table 1. Based on the evolution distance and the phylogenetic tree resulting from 16S rDNA sequencing and the neighbour-joining method [7], this strain was identified as a *Bacillus lincheniformis* and designated as *B. lincheniformis* LBH-52, as shown in Fig. 1. The scanning electron microscopic observations show that *B. lincheniformis* LBH-52 is a

Table 1. Similarity of the isolated microorganism with *Bacillus* species based on the 16S rDNA sequences

| Strain	Similarity (%)	Nucleotide differences/ compared
Bacillus licheniformis AY871102.1	99.38	9/1456
Bacillus licheniformis AY786999.1	99.44	8/1452
Bacillus licheniformis AY750906.1	99.44	8/1452
Bacillus sp. MO12 AY553105.1	99.44	8/1452
Bacillus licheniformis DQ372686.1	99.51	7/1445
Bacillus licheniformis AB219153.1	99.51	7/1445
Bacillus licheniformis DQ082996.1	99.44	8/1452
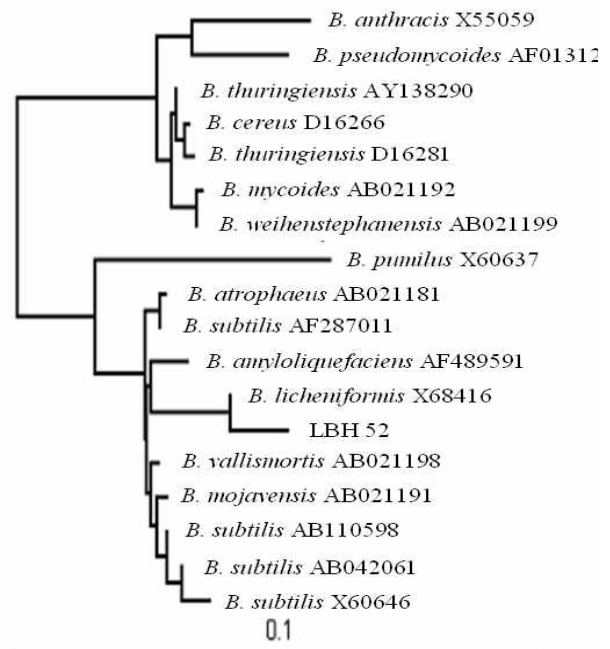
Fig. 1. Neighbour-joining tree based on 16S rDNA sequences of *B. licheniformis* complex. Numbers at the nodes indicate the levels of bootstrap support based on a neighbour-joining analysis of 1,000 re-sampled dataset. Scale bar indicates 0.1 nucleotide substitution per nucleotide position.

rod-shaped bacterium, as shown in Fig. 2. *B. licheniformis* forms spores and produces various extracellular enzymes that are associated with the cycling of nutrients in nature [5,11].

Effects of carbon and nitrogen sources on production of CMCase

The effects of carbon and nitrogen sources on cell growth and the production of the CMCase by *B. licheniformis* LBH-52 were investigated. Carbon sources tested in this
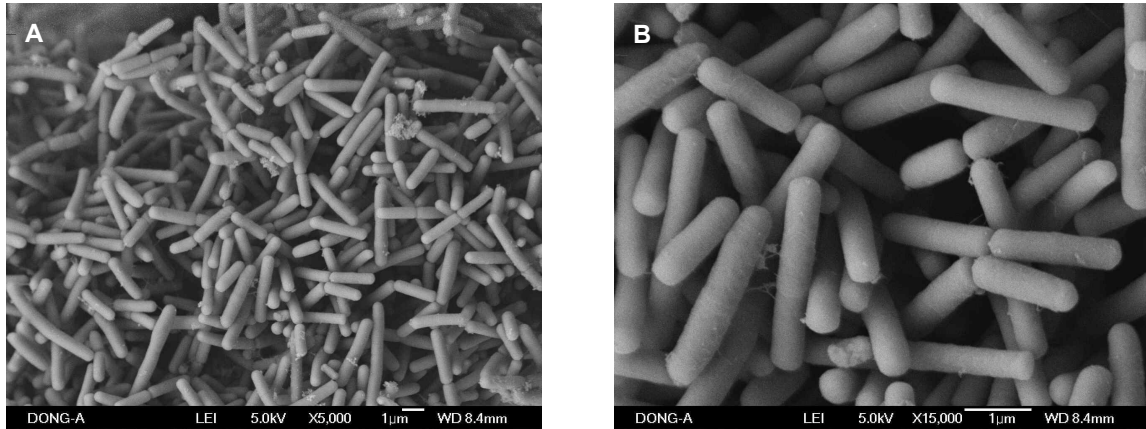
Fig. 2. Scanning electron microscopic observations of *B. licheniformis* LBH-52 (A, ×5,000 and B, ×15,000).

study were 20.0 g/l glucose, fructose, maltose, sucrose, rice bran, and rice hull. Nitrogen sources were 2.5 g/l malt extract, peptone, tryptone, yeast extract, ammonium sulfate, and ammonium nitrate. The best combination of carbon and nitrogen source for cell growth was sucrose and yeast extract, whereas that for production of CMCase was maltose and ammonium chloride, as shown in Fig. 3. Production of CMCase from 20.0 g/l maltose and 2.5 g/l ammonium chloride was 60.6 U/ml, whereas that from 20.0 g/l rice hulls and 2.5 g/l ammonium nitrate was 50.4 U/ml. Based on their cost and availability as well as productivity of CMCase, rice hulls and ammonium nitrate were chosen as carbon and nitrogen sources for next examination to find out the optimal conditions for the production of CMCase by *B. licheniformis* LBH-52.

The best combination of carbon and nitrogen sources for

the production of CMCase produced by *B. amyloliquefaciens* DL-3 were rice hulls and peptone, whereas that by *B. subtilis* subsp. *subtilis* A-53, which was isolated form seawater, were rice bran and yeast extract [23]. The composition of the rice hulls used in this study was as follows: 47.0% fiber, 0.2% crude lipid, 2.4% crude protein, 14.1% ash, and 7.1% water [13]. Rice bran was reported to be the best carbon source for the production of CMCase by *Bacillus* sp. CH43 and HR68 [27]. A major carbon source for the production of fungal CMCases by *Aspergillus* and *Trichoderma* species was reported to be wheat bran [12,24]. Wheat bran was also found to be the best substrate for the production of an extracellular and thermostable xylanase by *B. lichemiformis* in solid-state fermentation (SSF) [2]. All strains investigated to date for production of cellulases are inducible by cellulose, lactose or sophorose, and repressible by glucose, which are reasons
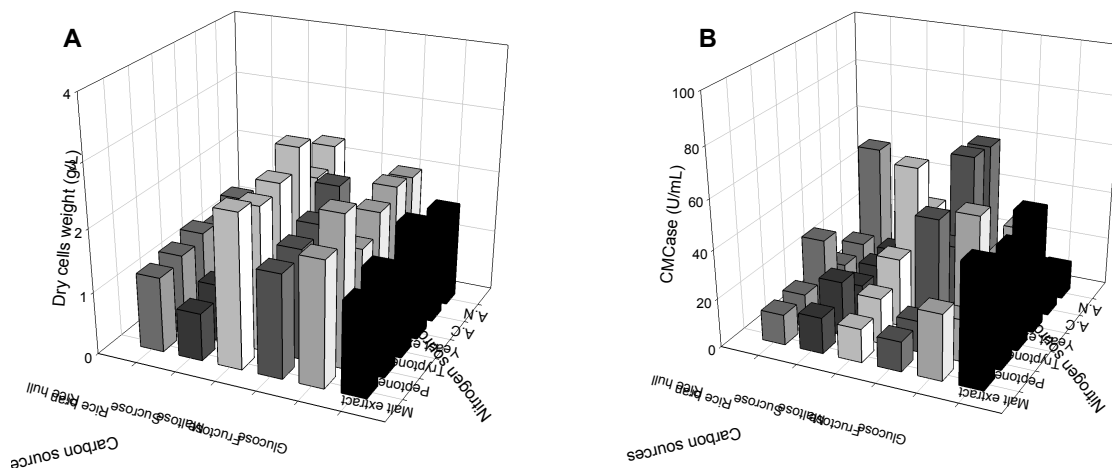

Fig. 3. Effect of carbon and nitrogen sources on cell growth (A) and production of CMCase by *B. licheniformis* LBH-52 (B) (AC, ammonium chloride and AN, ammonium nitrate).
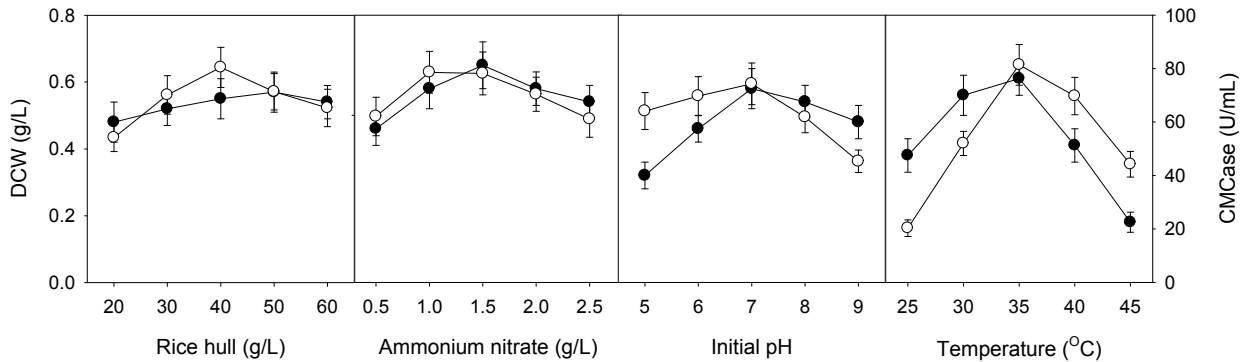
Fig. 4. Effects of rice hulls, ammonium nitrate, initial pH, and temperature on cell growth and production of CMCase by *B. licheniformis* LBH-52 within single-factor experiments (●, pH and ○, CMCase).

why the best carbon source for the production of cellulases by bacterial and fungal microorganisms is rice hulls, rice bran or wheat bran [8,15]. Induction, synthesis, and secretion of the β-glucanase appear to be closely associated [32].

Optimization of rice hulls, ammonium nitrate, initial pH, and temperature for production of CMCase using 'one-factor-at-a-time' experiments

Initial pH of the medium and temperature as well as carbon and nitrogen sources are also very essential factors to cell growth and production of CMCase. Optimal conditions of rice hulls, ammonium nitrate, initial pH, and temperature were investigated using 'one-factor-at-a time' experiments. Composition of basic medium and culture conditions for 'one-factor-at a time' experiment were 50.0 g/l rice hulls, 1.5 g/l ammonium nitrate, initial pH of 6.8, and temperature of 30℃. The optimal conditions of rice hulls, ammonium nitrate, initial pH, and temperature for cell growth of *B. licheniformis* LBH-52 were 50 g/l, 1.5 g/l, 7.0, and 35℃, respectively, whereas those for production of CMCase were 40 g/l, 1.0 g/l, 7.0, and 35℃, as shown in Fig. 4.

Optimization of rice hulls, ammonium nitrate, initial pH, and temperature for production of CMCase using response surface method

The optimal conditions of rice hulls, ammonium nitrate, initial pH, and temperature on cell growth and the production of CMCase by *B. licheniformis* LBH-52 were investigated using the response surface methodology (RSM). The minimum and maximum ranges of variables and the full experimental plan with respect to their actual and coded values were shown in Table 2. The results of central composite design (CCD) experiments consisted of experimental and

Table 2. Process variables used central composite design (CCD) with actual factor levels corresponding to coded factor levels

Variables	Symbol	Coded levels		
		-1	0	1
Rice hulls (g/l)	X_1	20.0	35.0	75.0
Ammonium nitrate (g/l)	X_2	1.0	1.5	2.0
Initial pH	X_3	6.0	6.5	7.0
Temperature (℃)	X_4	30	35	40

predicted values of four independent variables, as shown in Table 3. Cell growth, measured as dry cells weight (DCW), and production of CMCase from 30 different conditions ranged from 0.55 to 0.66 g/l and from 60.2 to 76.6 U/ml.

The analysis of variance (ANOVA) of the design for cell growth of *B. licheniformis* LBH-52 indicated that the model *F*-value was 31.39, which implied that this model was significant, as shown in Table 4. There is only a 0.01% chance that a "Model *F*-value" this large could occur due to noise. The ANOVA also indicated that the model term of X_1 was highly significant ("probe>F" less than 0.0001) and those of X_2, X_4, X_1^2, X_3^2, and X_4^2 were significant ("probe>F" less than 0.0500) for cell growth. However, the interactive effects of X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4, and X_3X_4 were not significant. The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R^2 was 0.9668. The model can explain 96.68% variation in the response. The value of the adjusted determination coefficient (Adj. R^2=0.9358) was very high to advocate for a high significance of this model [17]. The predicted determination of coefficient of 0.8910 was in reasonable agreement with the Adj. R^2 of 0.9358. From the statistical results obtained, it was shown that the above model was adequate to predict the cell growth

Table 3. Central composite design and determined response values

Run	X_1 (g/l)	X_2 (g/l)	X_3	X_4 (℃)	Experimental		Predicted	
					Y_1 (g/l)	Y_2 (U/ml)	Y_1 (g/l)	Y_2 (U/ml)
1	50	2.0	7.0	40	0.64	68.2	0.64	68.9
2	35	1.5	6.5	35	0.61	75.4	0.61	65.3
3	35	2.5	6.5	35	0.61	68.5	0.62	67.1
4	50	1.0	7.0	40	0.62	69.1	0.62	69.8
5	35	1.5	6.5	35	0.60	76.6	0.61	65.3
6	20	2.0	6.0	40	0.56	60.2	0.55	60.9
7	20	1.0	7.0	40	0.55	64.1	0.55	64.7
8	35	1.5	5.5	35	0.58	66.5	0.59	65.1
9	35	1.5	6.5	35	0.62	74.2	0.61	75.3
10	35	1.5	6.5	35	0.63	74.4	0.61	75.3
11	20	2.0	6.0	30	0.57	61.7	0.57	62.3
12	50	1.0	6.0	30	0.63	67.5	0.63	68.2
13	20	1.0	7.0	30	0.56	65.5	0.56	66.1
14	65	1.5	6.5	35	0.66	70.6	0.66	69.2
15	20	1.0	6.0	40	0.55	61.1	0.55	61.7
16	35	1.5	6.5	35	0.61	75.8	0.61	75.3
17	35	1.5	6.5	45	0.57	63.7	0.58	62.4
18	50	1.0	7.0	30	0.63	70.5	0.63	71.1
19	50	2.0	7.0	30	0.65	69.6	0.65	70.3
20	20	1.0	6.0	30	0.56	62.5	0.56	63.1
21	35	0.5	6.5	35	0.59	70.0	0.59	68.8
22	20	2.0	7.0	40	0.57	63.2	0.56	63.8
23	35	1.5	6.5	25	0.60	66.5	0.60	65.2
24	35	1.5	6.5	35	0.60	75.2	0.61	65.3
25	5	1.5	6.5	35	0.51	60.3	0.52	59.1
26	20	2.0	7.0	30	0.58	64.7	0.58	65.3
27	35	1.5	7.5	35	0.59	72.3	0.60	71.1
28	50	1.0	6.0	40	0.62	66.1	0.62	66.8
29	50	2.0	6.0	40	0.63	65.2	0.63	65.9
30	50	2.0	6.0	30	0.64	66.6	0.64	67.3

Table 4. Parameter estimates and analysis of variance (ANOVA) of the design for cell growth of *B. licheniformis* LBH-10

Source of variation	Degree of freedom	Sum of squares	Mean squares	F-value	Probe>F
Model	14	0.035	0.002	31.19	<0.0001
X_1	1	0.031	0.031	387.90	<0.0001
X_2	1	0.001	0.001	13.43	0.0023
X_3	1	0.000	0.000	1.89	0.1896
X_4	1	0.001	0.001	10.28	0.0059
$X_1 X_2$	1	0.000	0.000	0.00	1.0000
$X_1 X_3$	1	0.000	0.000	0.00	1.0000
$X_1 X_4$	1	0.000	0.000	0.00	1.0000
$X_2 X_3$	1	0.000	0.000	1.23	0.2795
$X_2 X_4$	1	0.000	0.000	0.00	1.0000
$X_3 X_4$	1	0.000	0.000	0.00	1.0000
X_1^2	1	0001	0.001	9.37	0.0079
X_2^2	1	0.000	0.000	0.73	0.4050
X_3^2	1	0.001	0.001	9.37	0.0079
X_4^2	1	0.001	0.001	9.37	0.0079
Error	5	0.001	0.000	-	-
Total	29	0.036	-	-	-

of *B. licheniformis* LBH-52 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 (2) and the optimal conditions of rice hulls, ammonium nitrate, initial pH of the medium, and temperature for cell growth extracted by Design Expert Software were 48.7 g/l, 1.8 g/l, 6.6, and 35.7℃, respectively. The maximum cell growth of 0.64 g/l was predicted by this model.

$$Y_1 \text{ (cell growth)} = 0.610 + 0.036X_1 + 0.007X_2 + 0.003X_3 - 0.006X_4$$
$$+ 0.03X_2X_3 - 0.005X_1^2 - 0.001X_2^2 - 0.005X_3^2$$
$$-0.005X_4^2 \qquad (2)$$

The ANOVA of the design for the production of CMCase by *B. licheniformis* LBH-52 indicated that the model *F*-value was 29.62, which also implied that this model was significant, as shown in Table 5. The ANOVA also indicated that the model terms of X_1, X_3, X_1^2, X_2^2, X_3^2 and X_4^2 were highly significant and that of X_4 was significant for production of CMCase. The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R^2 was 0.9651. The model can explain 96.51% variation in the response. The value of the adjusted determination coefficient (Adj. R^2=0.9325) was very high to advocate for a high significance of this model. The predicted determination of coefficient of 0.8238 was in reasonable agreement with the Adj. R^2 of 0.9325. From the statistical results obtained, it was shown that the above models were adequate to predict the production of CMCase by *B. licheniformis* LBH-52

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) and The optimal conditions of rice hulls, ammonium nitrate, initial pH of the medium, and temperature for production of CMCase were 43.2 g/l, 1.1 g/l, 6.8, and 35.7℃, respectively. The maximum production of CMCase of 75.3 U/ml was predicted by this model.

$$Y_2 \text{ (CMCase)} = 75.27 + 2.52X_1 - 0.42X_2 + 1.48X_3 - 0.71X_4 - 0.01X_1X_2$$
$$-0.01X_1X_4 - 0.01X_2X_4 - 2.78X_1^2 - 1.83X_2^2 - 1.79X_3^2$$
$$-2.86X_4^2 \qquad (3)$$

The three-dimensional (3D) response surface plots were generated to investigate the interaction among variables and to visualize the combined effects of those on the response of cell growth and production of CMCase. When the effect of two factors was plotted, the other two factors were set at the coded value zero. This kind of graphical visualization allows the relationships between the experimental levels of each factor and the response to be investigated, and the type of interactions between test variables to be determined, which is necessary to establish the optimal conditions for cell growth and production of CMCase [26]. In contrast to the circular shapes, the elliptical nature of curves indicates significant mutual interactions between variables. There was found to be a more drastic interactive effect of ammonium nitrate and initial pH on cell growth than production of CMCase, as shown in Fig. 5.

Table 5. Parameter estimates and analysis of variance (ANOVA) of the design for the production of CMCase by *B. licheniformis* LBH-10

| Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*-value	Probe>F
Model	14	665.420	47.530	29.62	<0.0001
X_1	1	152.010	152.010	94.72	<0.0001
X_2	1	4.170	4.170	2.60	0.1279
X_3	1	52.810	52.810	32.90	<0.0001
X_4	1	12.040	12.040	7.50	0.0152
$X_1 X_2$	1	0.003	0.003	0.00	0.9690
$X_1 X_3$	1	0.000	0.000	0.00	1.0000
$X_1 X_4$	1	0.003	0.003	0.00	0.9690
$X_2 X_3$	1	0.000	0.000	0.00	1.0000
$X_2 X_4$	1	0.003	0.003	0.00	0.9690
$X_3 X_4$	1	0.000	0.000	0.00	1.0000
X_1^2	1	211.530	211.530	131.81	<0.0001
X_2^2	1	91.560	91.560	57.05	<0.0001
X_3^2	1	87.840	87.840	54.74	<0.0001
X_4^2	1	225.070	225.070	140.25	<0.0001
Error	5	3.970	0.790	-	-
Total	29	689.490	-	-	-
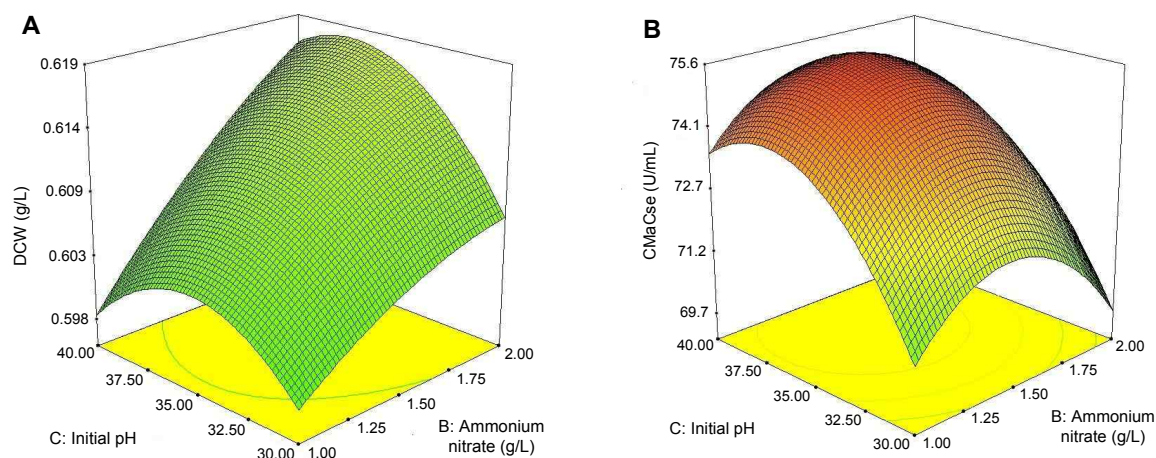
Fig. 5. 3D response surface displaying relative effect of two variables - ammonium nitrate and initial pH on cell growth (A) and production of CMCase by *B. licheniformis* LBH-52 (B).

The optimal concentrations of rice bran and yeast extract for cell growth of *B. subtilis* subsp. *subtilis* A-53 were 10.0 and 2.5 g/l, respectively, whereas those for the production of CMCase were 50.0 and 1.0 g/l [23]. Like other production of CMCases by *Bacillus* species, optimal concentrations of carbon and nitrogen sources for cell growth of *B. licheniformis* LBH-52 were different from those for production of CMCase. Optimal concentrations of carbon and nitrogen sources for cell growth seem to be generally higher than those for production of CMCase. The optimal initial pH of the medium and temperature for cell growth of *B. amyloliquefaciens* DL-3 were 7.2 and 32℃, whereas those for the production of CMCase were 6.8 and 37℃ [13], as shown in Table 6. The optimal initial pH and temperature for cell growth of *B. subtilis* subsp. *subtilis* A-53 was also different from those for the production of CMCase. Optimal initial pHs for the production of CMCases by bacterial and fungal microorganisms ranged from 4.0 to 7.3 [21,29]. Optimal temperatures for production of their CMCases ranged from 25 to 37℃, except for thermophilic microorganisms such as *Thermoascus aurantiacu*, which optimal temperature for the production of CMCase is 50℃ [14]. Generally speaking, optimal initial pHs for the production of CMCases by bacterial strains are higher than those by fungal strains. The highest production of CMCase by *B. lichemiformis* LBH-52 was about 75.3 U/ml under optimized conditions in this study. Even though the productivity of the CMCase by *B. lichemiformis* LBH-52 was not higher than those by other reported microorganisms, a newly isolated *B. lichemiformis* LBH-52 can be used for mass production of CMCase for commercial applications due to cost and availability of carbon and nitrogen sources.
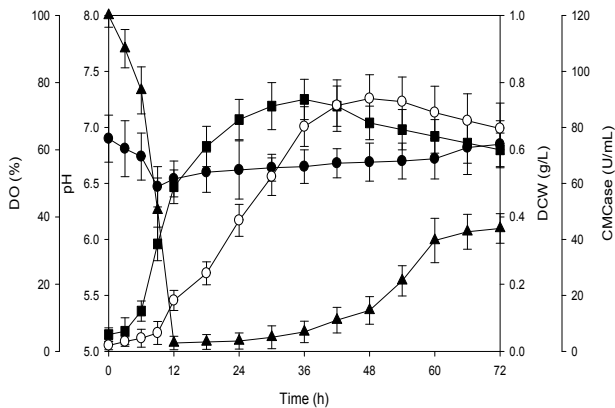
Batch fermentation for production of CMCase

Batch cultures for the productions of CMCase by *B. licheniformis* LBH-52 were performed in 7 l bioreactors. An agitation speed and an aeration rate were 400 rpm and 1.0 vvm. Concentrations of rice hulls and ammonium nitrate, initial pH, and temperature were 43.2 g/l, 1.1 g/l, 6.8, and 35.7℃, respectively. The pH of the culture rapidly decreased until 9 h after cultivation, and then steadily increased until around 6.9, as shown in Fig. 6. The concentration of the dissolved oxygen in the medium dramatically decreased until 12 hr, and the production of cellulases by *B. licheniformis* LBH-52 started. Cell growth of *B. licheniformis* LBH-52 rapidly increased in the first 12 hr of cultivation. The production of CMCase by *B. licheniformis* LBH-52 seemed to be parallel with cell growth. The maximal production of CMCase in a 7 l bioreactor for 72 hr under optimized conditions was 79.6 U/ml.

It normally takes 7 to 10 days to produce cellulases by fungal species in solid-state fermentation normally [15]. The times for batch cultures of submerged fermentation for the production of cellulases by bacterial species are shorter than those of solid-state fermentation by fungal ones [13,23]. In this study, it took 3 days to produce the CMCase by *B. licheniformis* LBH-52 in submerged fermentation. Reduced time for production of CMCase in a submerged fermentation can result in increase in productivity and decrease in their production cost.

Enzymatic saccharification of cellulosic materials such as

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

Strain	Carbon	Nitrogen	Initial pH	Temperature (℃)	Productivity	Reference
Bacillus amyloliquefaciens DL-3	Rice hulls	Peptone	6.8	37	367 U/ml	[13]
Bacillus licheniformis LBH-52	Rice hulls	Ammonium nitrate	7.0	36	75 U/ml	This study
Bacillus subtilus subsp. *subtilis* A-53	Rice bran	Yeast extract	6.8	30	137 U/ml	[23]
Bacillus subtilis CBTK 106	Banana fruit stalk waste	Yeast extract	7.0	35	10 U/g CS[a]	[21]
Cellomonas biazotea	Kallar grass straw	Yeast extract	7.3	30	38 U/ml	[29]
Cellomonas sp.	Wheat straw	Peptone	-	28	3 U/ml	[8]
Aspergillus niger KK2	Rice straw	Yeast extract	7.0	28	129 U/g CS	[15]
Thermoascus aurantiacus	Wheat straw	Ammonium sulphate	4.0	50	1,572 U/g CS	[14]
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	[40]
Trichoderma reesei Rut-C30	Solks Floc	Protease peptone	3.9-5.0[b]	25	184 U/ml	[24]
Trichoderma viride SL-1	Wheat bran	Yeast extract	-	32	1,400 U/g CS	[36]

[a] carbon source
[b] maintenance pH

Fig. 6. A batch fermentation for the production of CMCase by *B. licheniformis* LBH-52 in a 7 l bioreactor (●, pH; ▲, DO; ■, DCW; and ○, CMCase)

rice hulls has been performed by commercial cellulases, in which a major cellulase is CMCase [3,38]. A major constrain in enzymatic saccharification of cellulosic biomass for the production of fermentable sugars is low productivity and the cost of cellulases [35]. In this study, rice hulls and ammonium nitrate were developed to be substrates for the production of CMCase by a newly isolated *B. licheniformis*. Rice hulls are a byproduct from the rice processing industry and ammonium nitrate is one of the cheapest nitrogen sources. The process developed in this study can reduce the cost for production of CMCase and solve a significant problem to the ecology and environment, which is mainly due to their

low digestibility, peculiar size, low bulk density, high ash contents, and abrasive characteristics. Reduced time for production of CMCase using bacterial strain with submerged fermentations results in increase in productivity of cellulases and decrease in their production cost. The next study will be focused on characterization of the CMCase produced by this strain with an expectation of distinctive features such as cold-adapted, halo-tolerant or acidophilic CMCase due to its living in severe conditions and wide ranges of environments.

Acknowledgement

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초록 : 통계학적인 방법과 왕겨를 기질로 사용하여 해양에서 분리한 Bacillus licheniformis LBH-52를 사용한 carboxymethylcellualse의 생산조건 최적화

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왕겨를 기질로 사용하여 carboxymethylcellualse (CMCase)를 생산하는 미생물을 해수에서 분리하였으며 16S rDNA의 염기서열을 분석하여 동정한 결과, *Bacillus licheniformis*로 확인되었다. CMCase를 생산하기 위한 최적의 탄소원과 질소원은 왕겨와 암모니움 나이트레이트이었다. 통계학적인 방법인 response surface method (RSM)을 사용하여 CMCase를 생산하기 위한 조건을 최적화하였다. 통계학적인 분석 결과, 왕겨가 균체의 생육에 미치는 영향이 가장 높았으며, 왕겨와 배지의 초기 pH가 CMCase 생산에 미치는 영향이 높았다. Design Expert Software를 사용하여 결과를 분석한 결과, 균체의 생장에 최적인 조건은 48.7 g/l 왕겨, 1.8 g/l 암모니움 나이트레이트, 배지의 초기 pH 6.8 및 배양온도 35.7℃이었으나, CMCase의 생산에 최적인 조건은 43.2 g/l 왕겨, 1.1 g/l 암모니움 나이트레이트, 배지의 초기 pH 6.8 및 배양온도 35.7℃이었다. 최적화된 조건에서 왕겨를 기질로 사용하여 *B. lincheniformis* LBH-52가 생산하는 CMCase는 79.6 U/ml이었다. 본 연구를 통하여 왕겨와 암모니움 나이트레이트를 CMCase를 생산하는 기질로 개발하였으며, 해수에서 분리한 미생물을 사용하여 생산기간을 3일로 단축하였다.