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Rapid Statistical Optimization of Cultural Conditions for Mass Production of Carboxymethylcellulase by a Newly Isolated Marine Bacterium, *Bacillus velezensis* A-68 from Rice Hulls

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A microorganism producing carboxymethylcellulase (CMCase) was isolated from seawater, identified as *Bacillus velezensis* by analyses of 16S rDNA and partial sequences of the *gyr*A, and designated as *B. velezensis* A-68. The optimal conditions for production of CMCase by *B. velezensis* A-68 were established using response surface methodology (RSM). The optimal concentrations of rice hulls and yeast extract, and initial pH of the medium for cell growth were 60.2 g/l, 7.38 g/l, and 7.18, respectively, whereas those for production of CMCase were 50.0 g/l, 5.00 g/l, and 7.30. The analysis of variance (ANOVA) implied that the most significant factor for cell growth as well as production of CMCase was yeast extract. The optimal concentrations of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ in the medium for cell growth were 7.50, 1.00, 0.10, and 0.80 g/l, respectively, which were the same as those for production of CMCase. The optimal temperatures for cell growth and production of CMCase were 30 and 35℃, respectively. The maximal production of CMCase under optimized conditions was 83.8 U/ml, which was 3.3 times higher than that before optimization. In this study, rice hulls, agro-by-product, were developed as a substrate for production of CMCase and time for production of CMCase was reduced to 3 days using a newly isolated marine bacterium.

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

Introduction

Conversion of lignocellulosic biomass to fermentable sugars represents a major challenge in global efforts to utilize renewable resources [1]. The world rice production in 2010 reaches at 464 million tone (696 million tons paddy) [24]. The annual waste product from the rice milling industry in the Republic of Korea is about 900 thousand tons of rice hulls. Hydrolysates of rice hulls contain mainly glucose and xylose, which can be used as substrates for the production of ethanol [27, 33]. However, rice hulls are mostly disposed of in land-fill sites or burned in rice fields, and have become a significant problem to the ecology and environment due to constraints such as their low digestibility, peculiar size, low bulk density, high ash contents, and abrasive characteristics [29].

Production of ethanol from lignocellulosic biomass by simultaneous saccharification and fermentation (SSF) was first reported in 1977 [31]. Enzymatic saccharification of cellulosic materials could be accomplished through a complex reaction of three different types of cellulases; endoglucanase (carboxymethylcellulase), exocellobiohydrolase (avicelase), and β-glucosidase [4]. Rice hulls were hydrolyzed by commercial cellulases, in which the major cellulase was carboxymethylcellulase [33]. Higher exogenously added cellulases result in increased yield of SSF process [25]. A major constrain in enzymatic saccharification of cellulosic materials is the cost of cellulases and low productivity [29].

Most commercial cellulases are produced by solid state fermentations of fungal species [7]. Productions of cellulases by bacterial systems of *Acetivibrio, Bacillus, Bacteriodes, Cellomonas, Clostridium, Erwinia, Thermonospora,* and *Ruminococcus* species have been reported [26]. Enzymes produced by marine microorganisms can provide numerous advantages over traditional enzymes due to their severe living condition and wide ranges of environment [14, 15]. Hyperthermophilic bacteria isolated from marine sediments

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and seawater can utilize glucans as well as hemicellulose such as xylans and mannans [1]. Cold-adapted amylase and arginine kinase were produced by marine bacteria [23, 30].

In this study, a microorganism producing carboxymethylcellulase (CMCase) was isolated from seawater and identified as *Bacillus velezensis*. The optimal conditions for the production of CMCase by *B. velezensis* using rice hulls were established using response surface methodology (RSM) [17, 22].

Materials and Methods

Isolation of a marine microorganism producing carboxymethylcellulase

To isolate microorganism producing carboxymethylcellulase (CMCase), seawater from Kyungsang Province of Korea was diluted with 0.85% (w/v) NaCl. The suspension was then cultivated on the marine agar plate (Difco Marine Agar 2216, Difco Lab., Franklin, USA) at 30℃ for 3 days under aerobic conditions. Starter cultures of isolated microorganisms were prepared by transferring cells from the agar plate to 200 ml of the medium in 500 ml Erlenmeyer flasks. The medium used for production of CMCase contained the following components: 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 $(NH_4)_2SO_4$, which had been previously optimized [15, 16]. The resulting cultures were incubated at 30℃ for 3 days under aerobic conditions. After the cultivation, cells were removed from the culture broth by centrifugation at 12,000× *g* for 20 min and the supernatants were dialyzed overnight against distilled water. Based on productivity of CMCase, one microorganism was selected for production of CMCase and identified by sequencing of 16S rDNA and gyres A gene (*gyrA*).

Analyses of 16S rDNA and gyrase A gene sequences

For 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′ end of the 16S rDNA were 5′-AGGAGG AAAAGATCAGATATGAAACGGTCAATC-3′ and 5′-TCCA GTATTTCATCCACAACGACCTCC-3′, respectively. The *gyrA* region was amplified using two oligonucleotide primers, 5′-CAGTCAGGAAATGCGTACGTCCTT-3′ and 5′-CAAGGTAATGCTCCAGGCATT GCT-3′ [3]. PCR amplification was performed as described in the previous report [12]. The PCR was run for 35 cycles in a DNA thermal cycler (Model No. 9700, Perkin-Elmer Co. Wellesley, USA). 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 [32]. Neighbor-joining phylogenetic analysis was carried out with a MEGA program [18].

Production of carboxymethylcellulase by isolated microorganism

The main culture for production of CMCase was carried out in the medium containing 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 $MgSO_4 \cdot 7H_2O$, and 0.6 g/l $(NH_4)_2SO_4$ for 3 days under aerobic conditions. Batch fermentations for production of CMCase by an isolated microorganism were performed in a 100 l bioreactor (Ko-Biotech Co., Korea). Working volumes of 100 l bioreactors were 70 l and inoculum sizes of batch were 5% (v/v). Agitation was provided by three six-flat-blade impellers. Samples were periodically withdrawn from the cultures to examine cell growth and production of CMCase.

Experimental design using response surface methodology

The rice hulls (X_1), yeast extract (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 model constructed as a response function of the variables on cell growth and production of CMCase was a second-order polynomial as follows (Eq. 1):

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

Where, y is the measured response (cell growth as measured dry cells weight or production of CMCase), β_0, β_i, and β_{ij} are the regression coefficients, and X_i and X_j are the factors under study. For three variable systems, the model equation is given below (Eq. 2). Regression analysis and esti-

mation of the coefficient were performed using the statistical software, Design-Expert (Version 7.1.6, Stat-Ease Inc., Minneapolis, USA). The contribution of individual parameters and their quadratic and interactive effects on cell growth and production of CMCase were determined.

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3 \quad (2)$$

The K_2HPO_4 (X_1), NaCl (X_2), $MgSO_4 \cdot 7H_2O$ (X_3), and $(NH_4)_2SO_4$ (X_4) were also chosen as the independent variables, and each variable was designated as -1, 0, and 1, respectively. Cell growth (Y_1') and CMCase (Y_2') were used as dependent output variables. For four variable systems, the model equation is given below (Eq. 3).

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{44}X_4^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{14}X_1X_4+\beta_{23}X_2X_3+\beta_{24}X_2X_4+\beta_{34}X_3X_4 \quad (3)$$

Analytic Methods

Cell growth as the 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. The activity of CMCase was measured by the 3,5-dinitrosalicylic acid (DNS) method [9], through the determination of the amount of reducing sugars liberated from CMC solubilized in 50 mM Tris-HCl buffer, pH 8.0. The cellulase activity was determined by using a calibration curve for glucose (Sigma-Aldrich, UK). One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of glucose for 1 min.

Results and Discussion

Identification of isolated microorganism

A microorganism producing CMCase isolated from seawater from Kyungsang Province in Korea was designated as strain A-68. The phylogenetic analysis of the strain A-53 using its 16S rDNA nucleotide sequence data showed that this strain had the highest homology (99.77%) with *Bacillus velezensis* LMG 22478T, as shown in Table 1. The partial sequences of the gyr A gene of strain A-53 also showed the highest homology (99.77%) with *B. velezensis* LMG 22478T. Based on the evolution distance and the phylogenetic tree resulting from partial sequencing of the gyr A gene and the neighbour-joining method [2, 3], this strain was identified as a *Bacillus velezensis* and designated as *B. velezensis* A-68, as shown in Fig. 1.

Effect of carbon and nitrogen sources on production of CMCase

The effect of carbon and nitrogen sources on cell growth and the production of CMCase by *B. velezensis* A-68 was investigated. Carbon sources used in this study were 20.0 g/l glucose, fructose, maltose, sucrose, rice bran, and rice hulls. Nitrogen sources were 2.5 g/l malt extract, peptone, tryptone, yeast extract, ammonium sulfate, and ammonium nitrate. Rice hulls used in this study consisted of 47.0% fiber, 0.2% crude lipid, 2.4% crude protein, 14.1% ash, and 7.1% water [9]. Maltose and yeast extract were found to be the best combination of carbon and nitrogen sources for cell

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

Strain	Similarity (%)	Nucleotide differences/compared
Bacillus velezensis LMG 22478T	99.77	2/871
Bacillus amyloliquefaciens ATCC 23350T	99.65	3/865
Bacillus atrophaeus JCM 9070T	99.54	4/874
Bacillus vallismortis DSM 1103T	99.43	5/874
Bacillus subtilis subsp. *subtilis* NCDO 1769T	99.31	6/870
Bacillus majavensis IFO 15718T	99.08	8/874
Bacillus subtilis subsp. *subtilis* DSM 10T	99.08	8/873
Bacillus licheniformis DSM 13T	97.59	21/873
Bacillus pumilus NCDO 1766T	96.47	30/850
Bacillus carboniphilus JCM 973T	95.06	43/870
Bacillus oleronius DSM 9356T	94.68	46/865
Bacillus sporothermodurans DSM 10599T	94.59	47/868
Bacillus indicus Sd/3T	94.54	47/861
Bacillus firmus IAM 12464	94.23	50/866
Bacillus methanolicus NCIMB 13114T	94.15	51/872
Bacillus azotoformans ATCC 29788T	93.98	50/830

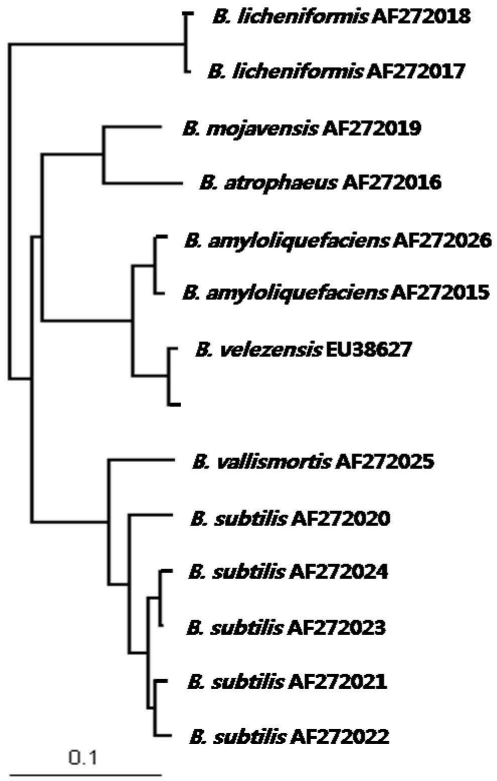

Fig. 1. Neighbour-joining tree based on *gyrase* A gene sequences of *Bacillus subtilis* complex. Scale bar indicates 0.1 nucleotide substitution per nucleotide position.

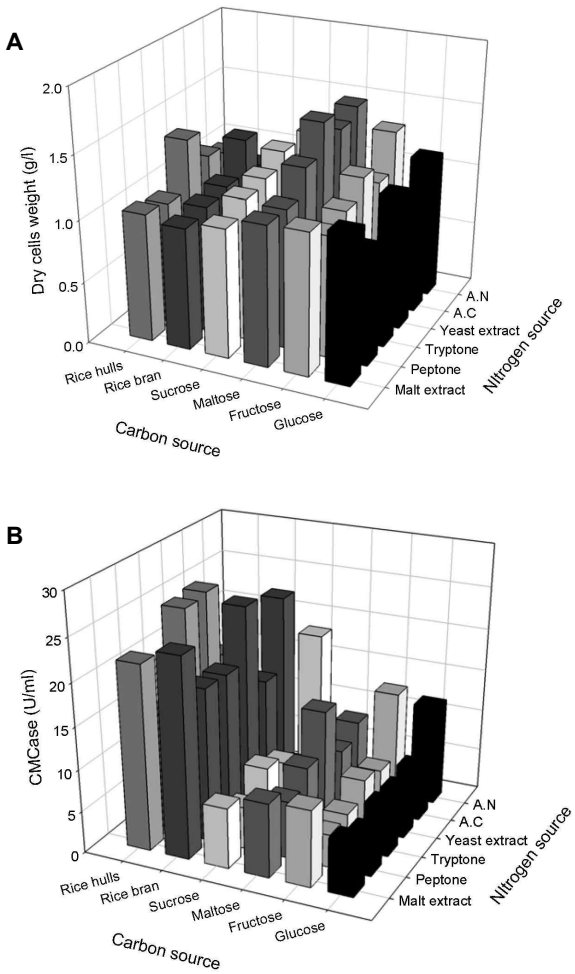

Fig. 2. Effect of carbon and nitrogen sources on cell growth (A) and production of CMCase (B) by *B. velezensis* A-68.

growth of *B. velezensis* A-68, whereas rice hulls and yeast extract were the best one for production of CMCase, as shown in Fig. 2. The best combination of carbon and nitrogen sources for cell growth of *B. velezensis* A-68 was different from that for the production of CMCase. The highest production of CMCase by *B. velezensis* A-68 was 25.3 U/ml from 20.0 g/l rice hulls and 2.5 g/l yeast extract.

The best combination of carbon and nitrogen sources for the production of CMCase by *B. amyloliquefaciens* DL-3 was rice hulls and peptone and that by *B. licheniformis* LBH-52 was rice hulls and ammonium nitrate [9, 16], whereas that for the production of CMCase by a marine bacterium, *B. sutilis* subsp. *sutilis* A-53 was rice bran and yeast extract [12, 19]. The best carbon and nitrogen source for the production of CMCase by a psychrophilic marine bacterium, *Psychrobacter aquimaris* LBH-10 were reported to be rice bran and peptone [15]. Wheat bran has been reported to be a major carbon source for the production of CMCases by *Aspergillus* and *Trichoderma* [11]. All strains investigated to date for the production of cellulases are inducible by cellulose, lactose or sophorose, and all are repressible by glucose, which are reasons why most carbon sources for the production of CMCase are rice hulls, rice bran or wheat bran [11]. Induction, synthesis, and secretion of the β-glucanase appear to be closely associated [4].

Optimization of rice hulls, yeast extract, and initial pH for production of CMCase

The optimal conditions of rice hulls, yeast extract, and initial pH of the medium for cell growth and the production of CMCase by *B. velezensis* A-68 were investigated using one-factor-at-a-time experiment. Composition of basic medium and culture conditions were 50.0 g/l rice hulls, 5.0 g/l yeast extract, and initial pH of 7.3, as shown in Fig. 3. The optimal concentrations of rice hulls and initial pH of the medium for cell growth as well as production of CMCase by *B. velezensis* A-68 were 50.0 g/l and 7.3, respectively. The optimal concentration of yeast extract for cell growth was

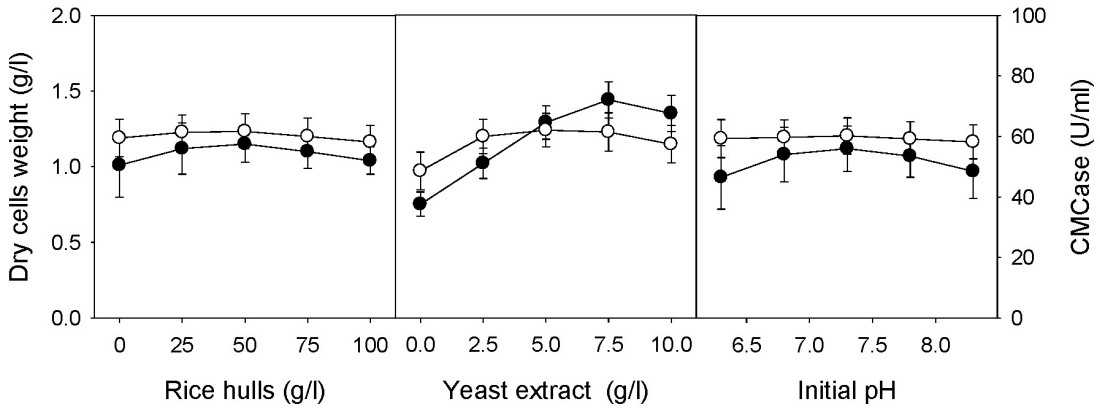

Fig. 3. Effect of rice hulls, yeast extract, and initial pH of medium cell growth and production of CMCase by *B. velezensis* A-68. ●, DCW and ○, CMCase

Table 2. Central composite design (CCD) and determined response values for optimization of rice hulls, yeast extract, and initial pH of medium

Run	X_1 (g/l)	X_2 (g/l)	X_3	Y_1 (g/l)	Y_2 (U/ml)
1	50	5.0	7.3	1.19	61.3
2	50	5.0	7.3	1.22	61.7
3	50	5.0	6.5	1.14	61.1
4	75	2.5	7.8	1.06	59.7
5	92	5.0	7.3	1.14	60.3
6	25	2.5	6.8	1.07	60.2
7	50	5.0	7.3	1.20	60.8
8	50	5.0	7.3	1.21	61.5
9	50	9.2	7.3	1.22	59.3
10	75	2.5	6.8	1.07	59.5
11	50	5.0	8.1	1.15	60.8
12	50	0.8	7.3	1.03	58.2
13	25	7.5	6.8	1.21	60.8
14	75	7.5	7.8	1.20	60.2
15	8	5.0	7.3	1.13	60.8
16	25	7.5	7.8	1.21	60.7
17	75	7.5	6.8	1.21	60.4
18	50	5.0	7.3	1.18	61.2
19	50	5.0	7.3	1.21	61.4
20	25	2.5	7.8	1.07	60.2

7.5 g/l, whereas that for production of CMCase was 5.0 g/l.

Based on results from one-factor-at-a-time experiment, the optimal conditions of rice bran, yeast extract, and initial pH of the medium on cell growth and production of CMCase was also investigated using response surface methodology. The coded values of minimum and maximum ranges of rice hulls (X_1), yeast extract (X_2), and initial pH of the medium (X_3) were 25.0 and 75.0 g/l, 2.5 and 7.5 g/l, and 6.8 and 7.8, respectively. Cell mass, measured as dry cells weight (DCW), and production of CMCase from 20 different conditions ranged from 1.03 to 1.22 g/l and from 58.2 to 61.7 U/ml, as shown in Table 2. The model *F*-value of 41.10 from the analysis of variance (ANOVA) of cell growth implied that this model was significant, as shown in Table 3. The *P* values were used as a tool to check the significance of each of the coefficients, which, in turn were necessary to understand the pattern of the mutual interactions between the test variables. The smaller the magnitude of the *P* value, the more significant is the corresponding coefficient. The ANOVA indicated that this model and the model terms of X_2 and X_2^2 ("probe > F" less 0.001) were highly significant and those of X_1^2 and X_3^2 ("probe > F" less 0.050) were sig-

Table 3. Parameter estimates and analysis of variance (ANOVA) of the design for optimization of rice hulls, yeast extract, and initial pH for cell growth and production of CMCase by *B. velezensis* A-68

	Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*-value	Probe>F
Cell growth	Model	9	0.075	0.008	41.10	<0.001
	X_1	1	0.000	0.000	0.00	0.953
	X_2	1	0.057	0.057	279.91	<0.001
	X_3	1	0.000	0.000	0.00	0.953
	X_1^2	1	0.007	0.007	35.06	0.001
	X_2^2	1	0.010	0.010	47.13	<0.001
	X_3^2	1	0.005	0.005	24.78	0.001
	Error	5	0.001	0.000	-	-
	Total	19	0.077	-	-	-
CMCase	Model	9	13.15	1.45	21.63	<0.001
	X_1	1	0.63	0.63	9.37	0.012
	X_2	1	1.39	1.39	20.51	0.001
	X_3	1	0.03	0.03	0.40	0.543
	X_1^2	1	0.75	0.75	11.09	0.008
	X_2^2	1	10.77	10.77	159.38	<0.001
	X_3^2	1	0.11	0.11	1.60	0.235
	Error	5	0.47	0.09	-	-
	Total	19	13.83	-	-	-

nificant for cell growth of *B. velezensis* A-68. The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R^2 was 0.974. The model can explain 97.4% variation in the response. The value of the adjusted determination coefficient (Adj. R^2=0.950) was very high to advocate for a high significance of this model [22]. The predicted determination of coefficient of 0.887 was in reasonable agreement with the Adj. R^2 of 0.950. From the statistical results obtained, it was shown that the above models were adequate to predict the cell growth of *B. velezensis* A-68 within the range of variables studied. Multiple regression analysis of the experimental data gave the following second-order polynomial equation in terms of coded factors (Eq. 4). The optimal conditions of rice hulls, yeast extract, and initial pH of the medium for cell growth extracted by Design Expert Software were 60.2 g/l, 7.38 g/l, and 7.18, respectively. The maximum cell growth of 1.23 g/l was predicted by this model. The maximal actual value of cell growth was 1.22 g/l when concentrations of rice hulls and yeast extract and initial pH of medium were 50.0 g/l, 5.0 g/l, and 7.3.

$$Y_1=1.20+0.06X_2-0.02X_1^2+0.03X_2^2-0.03X_3^2 \quad (4)$$

The model *F*-value of 21.63 from the ANOVA of production of CMCase implied that this model was also significant. The ANOVA indicated that this model and model terms of X_2^2 were highly significant and those of X_1, X_2, and X_2^2 were significant for the production of CMCase by *B. velezensis* A-68. The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R^2 was 0.951. The value of the adjusted determination coefficient (Adj. R^2=0.907) was high to advocate for a high significance of this model. From the statistical results obtained, it was shown that the above models were adequate to predict the production of CMCase by *B. velezensis* A-68 within the range of variables studied. Multiple regression analysis of the experimental data gave the following second-order polynomial equation in terms of coded factors (Eq. 5). The optimal conditions of rice hulls, yeast extract, and initial pH of the medium for production of CMCase were 50.0 g/l, 5.00 g/l, and 7.30, respectively. The maximum production of CMCase of 61.3 U/ml was predicted by this model. The maximal actual value of CMCase production was 61.7 U/ml when concentrations of rice hulls and yeast extract and initial pH of medium were 50.0 g/l, 5.0 g/l, and 7.3.

$$Y_2=61.31-0.22X_1+0.32X_2-0.04X_3+0.04X_1X_2+0.10X_1X_3 -0.06X_2X_3-0.236X_1^2-0.86X_22+0.09X_3^2 \quad (5)$$

The three-dimensional response surface plots were generated to investigate their combined effects of rice hulls and yeast extract, rice hulls and initial pH, and yeast extract and initial pH on the response of cell growth and the production of CMCase by *B. velezensis*A-68, as shown in Fig. 4. The P-values of rice hulls and yeast extract, rice hulls and initial

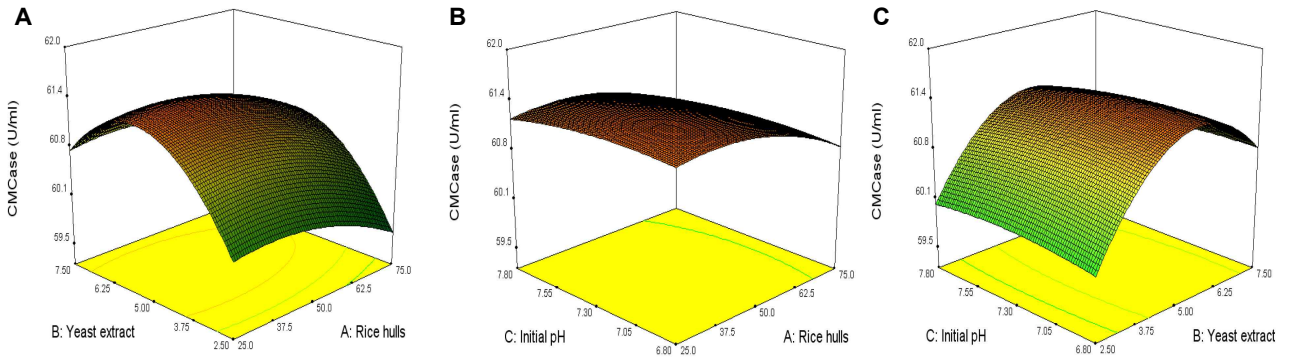

Fig. 4. 3D response surface displaying relative combined effects of rice hulls and yeast extract (A), rice hulls and initial pH (B), and yeast extract and initial pH (C) on production of CMCase by *B. velezensis* A-68.

pH, and yeast extract and initial pH were 0.6918, 0.8945, and 0.5116, respectively. Interactive effect of yeast extract and initial pH of medium on the production of CMCase by *B. velezensis*A-68 was relatively more significant than those of rice hulls and yeast extract as well as rice hulls and initial pH.

The optimal concentrations of rice bran and yeast extract for the production of CMCase by *B. sutilis* subsp. *sutilis* A-53 were 50.0 and 1.0 g/l, respectively. However, those for its cell growth were higher than those for production of CMCase [19]. The optimal concentrations of carbon and nitrogen source for cell growth of *B. velezensis* A-68 were also different from those for production of CMCase. The optimal concentrations of rice hulls and yeast extract for the production of CMCase by *B. amyloliquefaciens* DL-3 were 50.0 and 2.0 g/l, respectively, whereas those for cell growth was 50.0 and 3.0 g/l [9].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℃ [9]. The production of CMCase by another marine microorganism, *B. subtilis* subsp. *subtilis* A-53 was 136.8 U/ml when initial pH of the medium and temperature were 6.8 and 30℃ [19]. The optimal initial pHs for the production of CMCases by bacterial and fungal microorganisms ranged from 4.0 to 7.3 [14]. Generally speaking, optimal initial pHs for the production of CMCases by bacterial strains are higher than those by fungal strains.

Optimization of salts in medium for production of CMCase

The optimal concentrations of 4 salts in the medium, K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$, for cell growth and the production of CMCase by *B. velezensis* A-68 were investigated using one-factor-at-a-time experiment. Concentrations of rice hulls and yeast extract and initial pH of the medium were 50.0 g/l, 5.0 g/l, and 7.3. Concentrations of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ in the basic medium were 5.0, 2.0, 0.5, and 1.0 g/l. The optimal concentrations of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ for cell growth 7.5, 2.0, 0.25, and 1.0 g/l, respectively, which were the same as those for production of CMCase, as shown in Fig. 5.

Based on results from one-factor-at-a-time experiment, the optimal concentrations of 4 salts in the medium on cell growth and production of CMCase were also investigated using RSM. Levels of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ ranged from 2.5 to 7.5 g/l, from 1.0 to 3.0 g/l, from 0.25 to 0.75 g/l, and from 0.5 to 1.5 g/l, respectively. The results of central composite design (CCD) experiments consisted of experimental data to investigate effects of four independent variables, as shown in Table 4. Cell growth and production of CMCase from 30 different conditions ranged from 1.15 to 1.36 g/l and from 71.1 to 79.0 U/ml. The analysis of variance (ANOVA) of cell growth of *B. velezensis* A-68 indicated that this model and the model term of X_1 were highly significant ("probe > F" less than 0.0001) and that of X_2^2 was significant ("probe > F" less than 0.0500) for cell growth, as shown in Table 5. The model *F*-value of 12.89 implied that this model was significant. The regression equation obtained from the ANOVA indicated that the multiple correlation coefficient of R^2 was 0.9232. The predicted determination of coefficient of 0.7626 was in reasonable agreement with the Adj. R^2 of 0.8516. Multiple regression analysis of the experimental data gave the following second-order

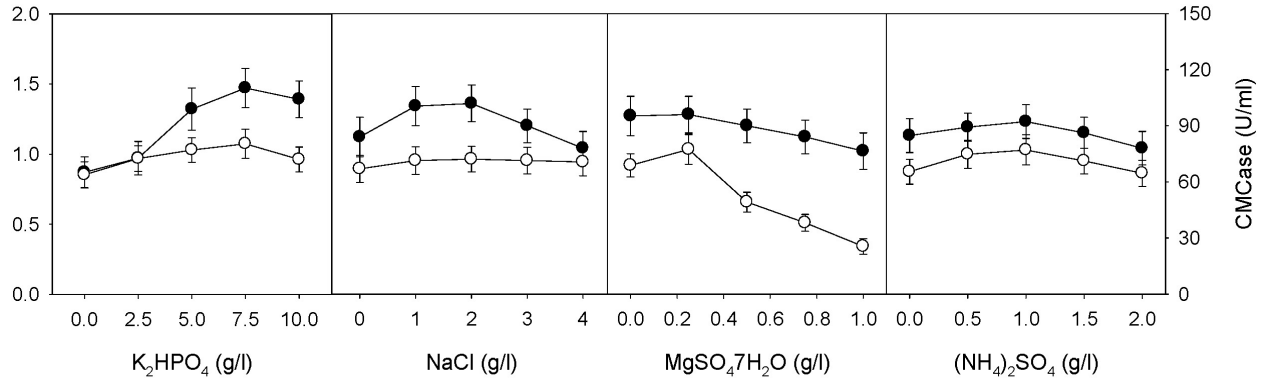

Fig. 5. Effect of salts in the medium on cell growth and production of CMCase by *B. velezenssis* A-68. ●, DCW and ○, CMCase

Table 4. Central composite design (CCD) for optimization of four salts in medium and determined response values

Run	X_1	X_2	X_3	X_4	Y_1 (g/l)	Y_2 (U/ml)
1	2.5	3.0	0.1	0.3	1.15	75.0
2	5.0	2.0	0.2	0.6	1.28	76.8
3	5.0	2.0	0.0	0.6	1.28	75.7
4	5.0	0.0	0.2	0.6	1.22	75.5
5	7.5	3.0	0.3	0.9	132	75.4
6	10.0	2.0	0.2	0.6	1.35	75.5
7	7.5	1.0	0.3	0.3	1.31	73.4
8	0.0	2.0	0.2	0.6	1.17	73.5
9	2.5	1.0	0.1	0.9	1.24	77.0
10	7.5	3.0	0.3	0.3	1.27	73.4
11	5.0	2.0	0.2	0.6	1.24	75.9
12	5.0	2.0	0.2	0.0	1.28	74.0
13	5.0	4.0	0.2	0.6	1.20	76.4
14	2.5	3.0	0.3	0.9	1.20	73.4
15	2.5	3.0	0.1	0.9	1.20	77.0
16	5.0	2.0	0.2	0.6	1.33	77.3
17	2.5	1.0	0.1	0.3	1.19	75.0
18	5.0	2.0	0.2	0.6	1.30	77.5
19	5.0	2.0	0.2	0.6	1.27	76.2
20	7.5	1.0	0.1	0.3	1.31	77.0
21	7.5	1.0	0.1	0.9	1.36	79.0
22	7.5	1.0	0.3	0.9	1.36	75.4
23	5.0	2.0	0.4	0.6	1.27	71.1
24	2.5	3.0	0.3	0.3	1.15	71.4
25	7.5	3.0	0.1	0.3	1.28	77.0
26	7.5	3.0	0.1	0.9	1.33	79.0
27	5.0	2.0	0.2	1.2	1.35	76.4
28	2.5	1.0	0.3	0.3	1.18	71.4
29	2.5	1.0	0.3	0.9	1.23	73.4
30	5.0	2.0	0.2	0.6	1.25	76.5

polynomial equation in terms of coded factors (Eq. 6) and the optimal concentrations of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ for cell growth were 7.50, 1.00, 0.10, and 0.80 g/l, respectively. The maximum cell growth of 1.35 g/l was predicted by the model. The maximal actual value of cell growth was 1.36 g/l when concentrations of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ were 7.5, 1.0, 0.1, and 0.9 g/l, respectively.

$$Y_1=5.33+0.03X_1+0.03X_3+0.04X_4+0.01X_1X_3+0.01X_2X_3-0.01X_2$$

$$X_4+0.01X_3X_4+0.02X_1^2+0.01X_2^2+0.01X_3^2+0.01X_4^2 \quad (6)$$

The ANOVA of the production of CMCase by *B. velezensis* A-68 indicated that this model and the model terms of X_1, X_3, X_4, and X_3^2 were highly significant and that of X_3^2 was significant for cell growth. The model *F*-value of 16.07 also implied that this model was significant. The regression equation obtained from the ANOVA indicated that the multiple correlation coefficient of R^2 was 0.9375. The value of the adjusted determination coefficient (Adj. R^2=0.8792) was very high to advocate for a high significance of this model. Multiple regression analysis of the experimental data gave the following second-order polynomial equation in terms of coded factors (Eq. 7) and the optimal concentrations of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ for cell growth were 7.50, 1.00, 0.10, and 0.80 g/l, respectively. The maximum production of CMCase of 78.3 U/ml was predicted by the model. The maximal actual value of CMCase production was 79.0 U/ml when concentrations of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ were 7.5, 3.0, 0.1, and 0.9 g/l, respectively.

$$Y_2=76.70+0.83X_1+0.08X_2-1.58X_3+0.87X_4-0.48X_1^2-0.11X_2^2 -0.75X_3^2-0.30X_4^2 \quad (7)$$

Many studies including types of strains, culture conditions, and substrates on production of cellulases have been reported [9, 13, 19]. However, there have been few reports on optimization of mineral salts in the medium on production of cellulases [15]. Results from RSM indicated that K_2HPO_4 was significant for cell growth of *B. velezenssis* A-68, whereas K_2HPO_4, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ were significant for production of CMCase. Potassium phosphate is one of the major salts in the medium for productions of microbial polysaccharides and enzymes as well as a well-known ingredient in buffer solutions [8, 12, 21]. Sodium chloride was reported to be used as a physiological modulator of biosynthetic pathway of biopolymers [15]. Magnesium sulfate added to medium assist with spore germination and initial growth of *A. fisheri*, which results in 1.9 fold increased production of xylanase [28].

Effect of temperature on production of CMCase

The effect of temperature on cell growth and the production of CMCase by *B. velezensis* A-68 was investigated. The temperature for cell growth and production of CMC ranged from 25 to 45℃. The carbon and nitrogen source and initial pH of the medium were 50.0 g/l rice hulls, 5.00 g/l

Table 5. Parameter estimates and analysis of variance (ANOVA) of the design for optimization of four salts in medium for cell growth and production of CMCase by *B. velezensis* A-68

	Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*-value	Probe>F
Cell growth	Model	14	0.110	0.008	12.89	<0.0001
	X_1	1	0.077	0.077	130.13	<0.0001
	X_2	1	0.004	0.004	7.20	0.0170
	X_3	1	0.000	0.000	0.25	0.6221
	X_4	1	0.012	0.012	20.52	0.0004
	X_1^2	1	0.001	0.001	1.81	0.1986
	X_2^2	1	0.010	0.010	16.28	0.0011
	X_3^2	1	0.000	0.000	0.29	0.5985
	X_4^2	1	0.002	0.002	2.61	0.1273
	Error	5	0.005	0.001	-	-
	Total	29	0.120	-	-	-
CMCase	Model	14	115.050	8.220	16.07	<0.0001
	X_1	1	16.670	16.670	32.59	<0.0001
	X_2	1	0.140	0.140	0.26	0.6149
	X_3	1	60.170	60.170	117.65	<0.0001
	X_4	1	18.030	18.030	35.25	<0.0001
	X_1^2	1	6.240	6.240	6.24	0.0033
	X_2^2	1	0.360	0.360	0.36	0.4146
	X_3^2	1	15.510	15.510	15.51	<0.0001
	X_4^2	1	2.500	2.500	2.50	0.0429
	Error	5	1.940	0.390	-	-
	Total	29	122.720	-	-	-

yeast extract, and pH 7.30, respectively. The optimal temperatures for cell growth and the production of CMCase by *B. velezensis* A-68 were found to be 30 and 35℃, respectively, as shown in Fig. 6. Cell growth and the production of CMCase by *B. velezensis* A-68 under optimized conditions were 1.38 g/l and 83.8 U/ml.

The optimal temperatures for cell growth and the production of CMCase by *B. subtilis* subsp. *subtilis* A-53 were 35 and 30℃, respectively [19]. The optimal temperatures for production of CMCases ranged from 25 to 37℃, except for thermophilic microorganisms such as *Thermoascus aurantiacu*, which optimal temperature for the production of CMCase is 50℃ [10]. The optimal temperature for cell growth of *B. amyloliquefaciens* DL-3 was 32℃, whereas that for production of CMCase was 37℃ [9]. The optimal temperature for cell growth of *B. velezensis* A-68 was also different from that for production of CMCase as production of other CMCases.

Mass production of CMCase under optimized conditions in a 100 l bioreactor

Batch culture for the production of CMCase by *B. velezensis* A-68 was performed in a 100 l bioreactor under optimized conditions in this study. Carbon and nitrogen source was 50.0 g/l rice hulls and 5.0 g/l yeast extract. The initial pH and cultural temperature were 7.3 and 35℃. Agitation speed and aeration rate of a 100 l bioreactor were 200 rpm and 1.0 vvm. The pH in the medium dramatically decreased until 12 h of cultivation, and then gradually increased to approximately 7.5 thereafter, as shown in Fig. 7. Dissolved oxygen in the medium also dramatically decreased until 24 h and gradually increased after 36 h. Cell growth of *B. velezensis* A-68 rapidly increased until 36 h. The production of CMCase by *B. velezensis* A-68 appeared to be paralleled with cell growth. Production of CMCase by *B. amyloliquefacience* DL-3 had been reported to be paralleled with cell growth [9]. Production of cellulases by a psychrophilic marine bacterium, *Psychrobacter aquimaris* LBH-10 was also paralleled with cell growth [13]. The cell growth and production of CMCase in a 100 l bioreactor was 1.46 g/l and 83.0 U/ml.

In this study, rice hulls were developed as a substrate for the production of CMCase by a newly isolated marine bacterium, *B. velezensis* A-68. The optimal conditions for production of CMCase were established using response surface method, as show in Table 6. The production of CMCase by *B. velezensis* A-68 in a 100 l bioreactor under optimized conditions established in the flask scale was almost the same as the maximal production of CMCase obtained in the flask scale, which meant that the optimized conditions in this study would be directly applied for mass production of CMCase in an industrial scale [5, 20]. Time for production of cellulases by fungal species in solid-state fermentation normally takes 7 to 10 days [11]. However, the time for production of CMCase by *B. velezensis* A-68 reduced to 3 days in suspension culture. The optimal conditions for production of CMCase by bacterial and fungal species were compared, as shown in Table 7. The productivities of CMCase by recombinant strains were normally higher than those by wild types. Next study will be focused on construction of recombinant strains and characterization of their CMCases with an expectation of distinctive features such as cold-adapted, halo-tolerant or acidophilic CMCase due to its living in severe conditions and

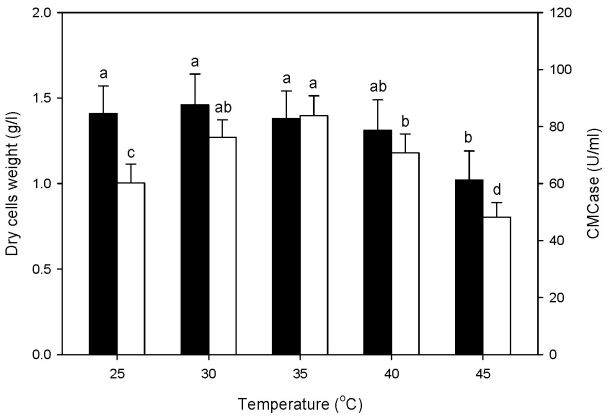

Fig. 6. Effect of temperature on cell growth and production of CMCase by *B. velezensis* A-68. ■, DCW and □, CMCase

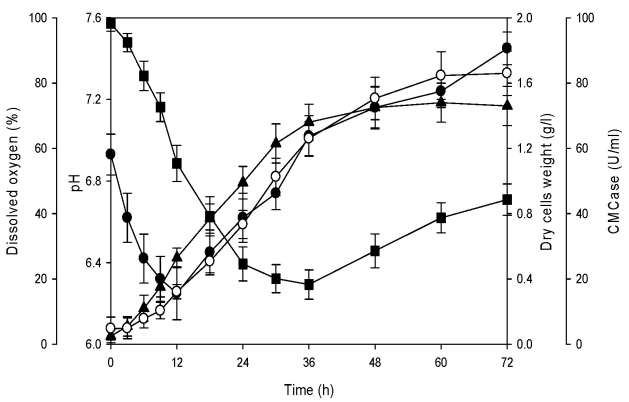

Fig. 7. Pilot-scaled production of CMCase by *B. velezensis* A-68 in a 100 L bioreactor. ●, pH; ■, DO; ▲, DCW; and ○, CMCase

Table 6. Optimal conditions for cell growth and production of CMCase by *B. velezensis* A-68

Scale	Condition	Optimal conditions					
		One-factor-at-a-time experiment		Response surface method			
				Predicted value		Actual value	
		DCW	CMCase	DCW	CMCase	DCW	CMCase
Flask scale-1	Rice hulls (g/l)	50.0	50.0	60.2	50.0	50.0	50.0
	Yeast extract (g/l)	7.5	5.0	7.38	5.00	5.0	5.0
	Initial pH	7.3	7.3	7.18	7.30	7.3	7.3
	Maximal production	1.24 g/l	62.0 U/ml	1.23 g/l	61.3 U/ml	1.22 g/l	61.7 U/ml
Flask scale-2	K_2HPO_4 (g/l)	7.5	7.5	7.50	7.50	7.5	7.5
	NaCl (g/l)	2.0	2.0	1.00	1.00	1.0	3.0
	$MgSO_4 \cdot 7H_2O$ (g/l)	0.25	0.25	0.10	0.10	0.1	0.1
	$(NH_4)_2SO_4$ (g/l)	1.0	1.0	0.80	0.80	0.9	0.9
	Maximal production	1.34 g/l	76.8 U/ml	1.35 g/l	78.3 U/ml	1.36 g/l	79.0 U/ml
Flask scale-3	Temperature (℃)	30	35	-	-	-	-
	Maximal production	1.46 g/l	83.8 U/ml	-	-	-	-

Table 7. 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	[9]
Bacillus atrophaeus LBH-18	Rice bran	peptone	7.0	30	128 U/ml	[17]
Bacillus licheniformis LBH-52	Rice hulls	Ammonium nitrate	7.0	36	75 U/ml	[13]
Bacillus subtilus subsp. *subtilis* A-53	Rice bran	Yeast extract	6.8	30	137 U/ml	[19]
Bacillus velezensis A-68	Rice hulls	Yeast extract	7.3	35	84 U/ml	This study
Cellulophaga lytica LBH-14	Rice bran	Ammonium chloride	6.1	25	154 U/ml	[5]
Psychrobacter aquimaris LBH-10	Rice bran	peptone	8.0	30	339 U/ml	[14]
Escherichia coli JM109/DL-3	Rice bran	Peptone	7.2	37	871 U/ml	[22]
Escherichia coli JM109/A-53	Rice bran	Ammonium chloride	8.0	35	880 U/ml	[20]
Aspergillus niger KK2	Rice straw	Yeast extract	7.0	28	129 U/g CS[a]	[11]

[a] carbon source

wide ranges of environments [6].

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초록 : 통계학적 방법을 사용한 해양미생물 *Bacillus velezensis* A-68균주의 섬유소 분해효소 생산 조건 최적화

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섬유소 분해효소(carbxymethylcellulase)를 생산하는 미생물을 해수에서 분리하여 16S rDNA 및 gyrase 유전자의 염기서열을 분석하여 동정한 결과, *Bcaillus velezensis*로 확인되었으며 *B. velezensis* A-68로 명명하였다. 이 균주가 생산하는 섬유소 분해효소의 생산 조건을 최적화하기 위하여 통계학적 방법인 response surface method (RSM)를 사용하였다. 이 균주의 생육에 최적인 왕겨, 효모 추출물 및 배지의 초기 pH는 60.2 g/l, 7.38 g/l 및 7.18이었으나, 섬유소 분해효소 생산에 최적인 왕겨, 효모 추출물 및 배지의 초기 pH는 50.0 g/l, 5.99 g/l 및 7.30이었다. 통계학적인 분석 결과, 균주의 생육 및 균주의 섬유소 분해효소 생산에 가장 큰 영향을 미치는 것은 효모 추출물이었다. 이 균주의 생육과 섬유소 분해효소의 생산에 최적인 K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$ 및 $(NH_4)_2SO_4$의 농도는 각각 7.50, 1.00, 0.10, and 0.80 g/l이었다. 이 균주의 생육 및 섬유소 분해효소 생산에 최적인 온도는 각각 30℃ 및 35℃로 생육에 최적인 조건과 섬유소 분해효소 생산에 최적인 조건이 다름을 알 수 있었다. 이 균주가 생산하는 섬유소 분해효소의 생산성은 83.8 m/l이며, 이는 최적화하기 전의 생산성에 비하여 3.3배 증가한 것이다. 이 연구를 통하여 농업 부산물인 왕겨를 섬유소 분해효소 생산을 위한 기질로 개발하였으며, 해수에서 분리한 미생물을 사용함으로써 섬유소 분해효소의 생산기간을 3일로 단축할 수 있었다.