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Construction of Recombinant *Escherichia coli* JM109/LBH-10 and Comparison of Its Optimal Condition for Production of Carboxymethyl cellulase with Its Wild Type, *Psychrobacter aquimaris* LBH--10

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Abstract

A gene encoding the Carboxymethyl cellulase (CMCase) of a psychrophilic marine bacterium, *Psychrobacter aquimaris* LBH-10 was cloned in *Escherichia coli* JMB109. The optimal conditions of rice bran, ammonium chloride, and initial pH of the medium for cell growth of *E. coli* JM109/LBH-10 were 73.4 g/L, 5.8 g/L, and 6.5, respectively, whereas those for production of CMCase were 57.1 g/L, 6.4 g/L, and 6.7. The optimal temperatures for cell growth and the production of CMCase by *E. coli* JM109/LBH-10 were found to be and 40 and 35°C, respectively. The optimal agitation speeds and aeration rates of a 7 L bioreactor for cell growth and production of CMCase were 480 rpm and 1.0 vvm. In this study, the optimal conditions for cell growth and the production of CMCase by *E. coli* JM109/LBH-10 were found to be different from those for its wild type, *P. aquimaris* LBH-10. The maximal production of CMCase by *E. coli* JM109/LBH-10 was 576.8 U/mL, which was 1.80 times higher than that by *P. aquimaris* LBH-10.

Keywords: Carboxymethyl cellulase; *Escherichia coli* JM109; Gene cloning; Optimization, *Psychrobacter aquimaris*; Response surface methodology

Introduction

Cellulosic materials have great potential for use as cheap and renewable feedstocks for fermentable sugars [1]. The complete enzymatic hydrolysis of cellulosic materials need at least three different types of cellulases; endoglucanase (Carboxymethyl cellulase), exo-cellobiohydrolase (avicelase), and β-glucosidase [2]. The enzymatic saccharification of lignocellulosic materials for the production of ethanol was performed by commercial cellulases, in which the major cellulase was Carboxymethyl cellulase [3,4]. Carboxymethyl cellulases (CMCases) have been applied in the textile and detergent industries [5].

Most commercial cellulases are produced by solid state fermentations of fungal species [6]. Enzymes produced by marine microorganisms can provide numerous advantages over traditional enzymes due to the completely different regulatory mechanism for production [7]. A new psychrophilic marine bacterium had been isolated and identified as *Psychrobacter aquimaris* LBH-10 and characterization of its CMCase was reported [8]. The CMCase produced by this strain showed relatively higher activity at low temperature and acidic stability at pH 3.5. However, a major restriction in enzymatic saccharification of cellulosic materials is the cost of cellulases and low productivity [9]. One approach to increasing productivity of cellulases would be to isolate hyper-producers or constitutive mutants for cellulases. Another approach would be to clone genes coding for cellulases and then enhance expression of these genes by current molecular genetic techniques [10,11]. The gene encoding cold-active esterase of *Psychrobacter* sp. was cloned and expressed in *E. coli* [12].

We had reported identification of a psychrophilic marine bacterium, *Psychrobacter aquimaris* LBH-10 and characterization of the CMCase produced by this strain [8,13]. In this study, we described cloning of the CMCase gene of *P. aquimaris* LBH-10 and its expression in *E. coli* JM109. The optimal conditions for the production of CMCase by a recombinant *E. coli* JM109 were investigated using response surface methodology (RSM), which were compared with those for its wild strain, *P. aquimaris* LBH-10 [13,14].

Materials and Methods

Bacterial strain and medium

Psychrobacter aquimaris LBH-10 had been isolated from seashore of the Kyungsang province in Korea and identified in the previous study [8]. It utilized cellulosic materials such as Carboxymethyl cellulose (CMC), rice bran, and rice hulls and produced carboxymethl cellulase (CMCase). The strain was maintained on agar medium containing 20.0 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 $MgSO_4 \cdot 7H_2O$, 0.6 g/L $(NH_4)2SO_4$, and 15 g/L agar.

Cloning and sequence analysis of CMCase

The full-length CMCase gene of *P. aquimaris* LBH-10 was amplified by polymerase chain reaction (PCR) using two specific primers, 5'-AGGAGGAAAAGATCAGATATGAAACGGTCAATC-3' (forward) and 5'-TCCAGTATTTCATCCACAACGCAAACCTCC-3' (reverse). These primers were designed on the basis of DNA sequences of CMCase genes of *Bacillus* sp. previously cloned: CMCase genes of *B. subtilis* AY044252.1 (NCBI-gi: 15375077), *B. subtilis* Z29076.1 (gi: 509266), and *B. subtilis* X67044.1 (gi: 39776). The chromosomal DNA

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of *P. aquimaris* LBH-10 extracted using a Wizard Genomic DNA Prep. Kit (Promega Co., Madison, USA) was used as a template. PCR amplification was performed as described previously [15]. PCR for amplification 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 for 10 min at 72°C to ensure full extension of the products. The amplified PCR products were ligated with the T-tail site of pGEM-T Easy Vector System (Promega Co., Madison, USA) and constructed plasmids were transformed into *E. coli* JM109. The plasmid contained PCR product was isolated from transformed *E. coli* JM109 and was sequenced in both directions using ALF Red automated DNA sequencer (Pharmacia, Sweden). Analysis of sequence data and sequence similarity searches were performed using the BLAST (N) program of the National Center for Biotechnology Information (NCBI) [16]. The transformed *E. coli* JM109 harboring the CMCase gene of *P. aquimaris* LBH-10 was named as *E. coli* JM109/LBH-10.

Production of CMCase by *E. coli* JM109/LBH-10

E. coli JM109/LBH-10 was grown at 37°C in LB medium containing 100 μg/mL ampicillin. 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 72 h under aerobic conditions [8]. After cultivation, cells were removed from the culture broth by centrifugation at 12,000 x g for 20 min and supernatants were dialyzed against deionized water using dialysis tubing with a molecular weight cut-off of 12,000 to 14,000 Da.

Batch fermentations for the production of CMCase by *E. coli* JM109/LBH-10 were performed in 7 L bioreactors (Ko-Biotech Co., Korea). Working volume of a 7 L bioreactor was 5 L and inoculum size of batch fermentation for production of CMCase was 5% (v/v). Temperature for batch fermentations was maintained at 35°C. Agitation was provided by three six-flat-blade impellers in a 7 L bioreactor.

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 model constructed as a response function of the variables on cell growth and production of CMCase was a second-order polymial as follows (Equation 1):

$$Y = ß_0 + \Sigma ß_i X_i + \Sigma ß_{ii} X_{i2} + \Sigma ß_{ij} X_i X_j \quad (1)$$

Where, y is the measured response (cell growth as measured dry cells weight or production of CMCase), $ß_0$, $ß_i$, and $ß_{ij}$ 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 (Equation 2).

$$Y = ß_0 + ß_1X_1 + ß_2X_2 + ß_3X_3 + ß_{11}X_{12} + ß_{22}X_{22} + ß_{33}X_{32} + ß_{12}X_1X_2 + ß_{13}X_1X_3 + ß_{23}X_2X_3 \quad (2)$$

Regression analysis and estimation 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. 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-order polynomial equation. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA).

Analytical methods

CMCase activity was measured by the DNS (3,5-dinitrosalicylic acid) method, through the determination of the amount of reducing sugars liberated from CMC solubilized in 50 mM Tris-HCl buffer, pH 8.0 [17]. This mixture was incubated for 20 min at 50°C and the reaction was stopped by the addition of DNS solution. The treated samples were boiled for 10 min, cooled in water for color stabilization, and the optical density was measured at 550 nm. CMCase activity was determined by using a calibration curve for glucose (Sigma-Aldrich Co., UK). One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of glucose per min. Dry cells weight, as cell growth, was measured by directly weighing the biomass after drying to constant weight at 100 to 105°C after collection of cells by centrifugation at 12,000 x g for 10 min. Reducing sugars were determined by using the DNS method [18].

Results and Discussion

The full-length CMCase gene of *P. aquimaris* LBH-10 was cloned by PCR with primers based on DNA sequences of the CMCase gene of *Bacillus* species cloned previously [19]. As shown in Figure 1, the open reading frame (ORF) of the cloned gene consists of 1,497 nucleotides encoding a protein of 499 amino acids with a predicted molecular weight of 54,890 Da. Analysis of the putative cleavage site of signal peptide by using the Signal P program was located between Ala-29 and Ala-30 [20]. Comparison of the deduced amino acid sequence of the cloned gene with those of proteins registered in the BLAST database showed the highest identity to the CMCase of *Bacillus subtilis* (GenBank No. P10475) with 93% identity, as shown in Table 1.

Most CMCases like xylanase and β-1,3-1,4-glucanases are modular enzymes, which consist of two or more functional modules, such as catalytic and carbohydrate-binding modules (CBMs), connected to each other via a linker sequence [21]. On the basis of amino acid sequence similarities, as shown in Figure 2, catalytic modules, which are engaged in the hydrolysis of cellulose, are classified into 106 groups in glycoside hydrolase families and CBMs, which bind preferentially to cellulose, are also classified into 45 groups (http://cellwall.genomics.purdue.edu/families/4-3-2-2.html). Computer analysis with the deduced mature amino acid sequence of the CMCase from *P. aquimaris* LBH-10 using CDART program of NCBI revealed a modular enzyme composed of two discrete domains in the following order: a catalytic domain (Gly-31 through Asn-332) of glycoside hydrolase family 5 (GH5) and a family 3 (CBM3) (Glu-350 through His-499). Like the modular organization (GH5/CBM3) in many *Bacillus endoglucanases* (http://cellwall.genomics.purdue.edu/families/4-3-2-2.html), this enzyme has its catalytic domain in the N-terminal portion and CBM in the C-terminal portion [16].

Effect of carbon and nitrogen sources on cell growth and the production of CMCase by *E. coli* JM109/LBH-10 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. The best combination for cell growth of *E. coli* JM109/LBH-10 was found to be rice bran and

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   1 AGGAGGAAAAGATCAGATATGAAACGGTCAATCTCTATTTTTATTACGTGTTTATTGATT
                                 M  K  R  S  I  S  I  F  I  T  C  L  L  I
  61 ACAGTATTGACAATGGGCGGCTTGCAGGCTTCCCAGGCATCTGCAGCAGGGACAAAAACG
     T  V  L  T  M  G  G  L  Q  A  S  Q  A  S  A  A  G  T  K  T
 121 CCAGCAGCCAAGAATGGGCAGCTTAGCATAAAAGGAACACAGCTCGTAAACCGGGACGGC
     P  A  A  K  N  G  Q  L  S  I  K  G  T  Q  L  V  N  R  D  G
 181 AAAGCGGTACAATTGAAAGGGATCAGTTCACATGGATTGCAATGGTATGGCGATTTTGTC
     K  A  V  Q  L  K  G  I  S  S  H  G  L  Q  W  Y  G  D  F  V
 241 AATAAAGACAGCTTAAAATGGCTGAGAGACGATTGGGGCATAACCGTTTTCCGCGCGGCG
     N  K  D  S  L  K  W  L  R  D  D  W  G  I  T  V  F  R  A  A
 301 ATGTATACGGCAGATGGCGGTTATATTGATAATCCGTCCGTGAAAAATAAAGTAAAAGAA
     M  Y  T  A  D  G  G  Y  I  D  N  P  S  V  K  N  K  V  K  E
 361 GCGGTTGAAGCGGCAAAAGAACTCGGGATATATGTCATCATTGACTGGCATATCTTAAAT
     A  V  E  A  A  K  E  L  G  I  Y  V  I  I  D  W  H  I  L  N
 421 GACGGCAACCCAAACCAACATAAAGAGAAGGCAAAAGAATTTTTAAGGAAATGTCAAGT
     D  G  N  P  N  Q  H  K  E  K  A  K  E  F  F  K  E  M  S  S
 481 CTTTACGGAAACACGCCAAACGTCATTTATGAAATTGCAAACGAACCAAACGGTGATGTG
     L  Y  G  N  T  P  N  V  I  Y  E  I  A  N  E  P  N  G  D  V
 541 AACTGGAAGCGTGATATTAAACCGTATGCGGAAGAAGTGATTTCCGTTATCCGCAAAAAT
     N  W  K  R  D  I  K  P  Y  A  E  E  V  I  S  V  I  R  K  N
 601 GATCCAGACAACATCATCATTGTCGGAACCGGTACATGGAGCCAAGATGTGAATGATGCA
     D  P  D  N  I  I  I  V  G  T  G  T  W  S  Q  D  V  N  D  A
 661 GCCGATGATCAGCTAAAAGATGCAAACGTCATGTACGCGCTTCATTTTTATGCCGGCACA
     A  D  D  Q  L  K  D  A  N  V  M  Y  A  L  H  F  Y  A  G  T
 721 CACGGCCAATCTTTACGGGATAAAGCAAACTATGCACTCAGTAAAGGAGCGCCTATTTTC
     H  G  Q  S  L  R  D  K  A  N  Y  A  L  S  K  G  A  P  I  F
 781 GTGACGGAATGGGGAACAAGCGACGCGTCTGGAAATGGCGGTGTATTCCTTGACCAGTCG
     V  T  E  W  G  T  S  D  A  S  G  N  G  G  V  F  L  D  Q  S
 841 CGGGAATGGCTGAATTATCTCGACAGCAAGAACATCAGCTGGGTGAACTGGAATCTTTCT
     R  E  W  L  N  Y  L  D  S  K  N  I  S  W  V  N  W  N  L  S
 901 GATAAGCAGGAATCATCCTCAGCGTTAAAGCCGGGAGCATCTAAAACAGCCGGCTGGCCG
     D  K  Q  E  S  S  S  A  L  K  P  G  A  S  K  T  G  G  W  P
 961 CTTACAGATTTAACTGCTTCAGGAACATTCGTAAGAGAAAACATTCTCGGCAACAAAGAT
     L  T  D  L  T  A  S  G  T  F  V  R  E  N  I  L  G  N  K  D
1021 TCAACGAAAGAACGCCCTGAAACGCCAGCACAAGATAACCCCGCACAGGAAAACGGCATT
     S  T  K  E  R  P  E  T  P  A  Q  D  N  P  A  Q  E  N  G  I
1081 TCTGTACAATACAAAGCAGGGGATGGGGGTGTGAACAGCAACCAAATCCGCCCGCAGCTT
     S  V  Q  Y  K  A  G  D  G  G  V  N  S  N  Q  I  R  P  Q  L
1141 CACATAAAAAATAACGGCAATGCGACGGTTGATTTAAAAGATGTCACTGCCCGTTACTGG
     H  I  K  N  N  G  N  A  T  V  D  L  K  D  V  T  A  R  Y  W
1201 TATAACGCGAAAAACAAGGGCCAAAACTTTGACTGTGACTACGCGCAGATTGGATGCGGC
     Y  N  A  K  N  K  G  Q  N  F  D  C  D  Y  A  Q  I  G  C  G
1261 AATCTGACCCACAAATTTGTGACGCTGCATAAACCTAAGCAAGGTGCAGATACCTATCTG
     N  L  T  H  K  F  V  T  L  H  K  P  K  Q  G  A  D  T  Y  L
1321 GAACTGGGTTTTAAAACAGGAACGCTGTCACCGGGAGCAAGCACAGGGAATATTCAGCTT
     E  L  G  F  K  T  G  T  L  S  P  G  A  S  T  G  N  I  Q  L
1381 CGTCTTCACAATGATGACTGGAGTAGTTATGCACAAAGCGGCGATTATTCCTTTTTTCAA
     R  L  H  N  D  D  W  S  S  Y  A  Q  S  G  D  Y  S  F  F  Q
1441 TCAAATACGTTTAAAACAACGAAAAAAATTACATTATATCATCAAGGAAAACTGATTTGG
     S  N  T  F  K  T  T  K  K  I  T  L  Y  H  Q  G  K  L  I  W
1501 GGAACAGAACCCAATTAGTTAAGCTTTAGGCGGACATCAGCAACGATGTCCGCTTTTATT
     G  T  E  P  N  *
1561 ATCTTAAACAGCAATACATGGAGGTTTGCGTTGTGGATGAAATACTGAA
```

Figure 1: Complete nucleotide and deduced amino acid sequences of the CMCase gene of *P. aquimaris* LBH-10. Asterisk indicates the stop codon. The primers designed for gene cloning were showed under line.

LBH-10	159	T	P	N	V	I	Y	E	I	A	N	E	P	N	G	D	V	174
BACSU	159	T	P	N	V	I	Y	E	I	A	N	E	P	N	G	D	V	174
ERWCT	158	S	P	N	V	I	Y	E	I	A	N	E	P	N	G	G	V	173
BACS4	153	Y	P	N	V	I	Y	E	I	A	N	E	P	N	G	H	N	168
CLASA	782	D	T	I	I	A	F	D	L	K	N	E	P	H	G	K	P	797
DICD3	166	K	P	N	V	I	Y	E	I	Y	N	E	P	L	Q	V	S	181
RUMAL	125	K	T	N	V	I	Y	E	I	C	N	E	P	N	C	S	G	140
BACS6	363	N	H	Y	I	I	W	E	L	A	N	E	P	S	P	N	N	378
PSEFL	493	S	N	L	I	G	I	D	V	F	N	E	P	Y	D	Y	T	508
CLOLO	175	D	D	H	L	I	F	E	T	L	N	E	P	R	L	E	G	190
CLOSF	130	R	E	H	I	A	F	E	L	L	N	E	V	V	E	P	D	145
PAEBA	177	N	E	R	L	I	F	E	S	M	N	E	V	F	D	G	N	191
PAELA	167	P	S	K	L	M	F	E	S	V	N	E	P	R	F	T	D	182

Figure 2: Comparison of amino acid sequence alignment of the CMCase produced by *P. aquimaris* LBH-10 and other glycosyl hydrolasefamily 5 using Clustal W, a multiple alignment program. Amino acid sequence alignments of the CMCase produced by *E. coli* JM109/LBH-10 (LBH-10) from BACSU (P10475), ERWCT (Q59394), BACS4 (P06566), CALSA (P10474), DICD3 (P071030, RUMAL (Q079400, BACS6 (P19424), PDEFL (P270330), CLOLO (P549370, CLOSF (P23340), PAELA (P23550), and PAEBA (O08342).

yeast extract, whereas that for production of CMCase was fructose and yeast extract, as shown in Figure 3. The production of CMCase from 20.0 g/L fructose and 5.0 g g/L yeast extract was 381.5 U/mL. The best combination of carbon and nitrogen sources for cell growth of *E. coli* JM109/LBH-10 was different from that for production of CMCase.

The best combination of carbon and nitrogen source for cell growth of *P. aquimaris* LBH-10 was sucrose and malt extract, whereas that for production of CMCase was rice bran and peptone [13]. The best carbon and nitrogen sources for cell growth and well as those for production of CMCase by *E. coli* JM109/LBH-10 were found to be different from those for its wild type, *P. aquimaris* LBH-10. The best carbon and nitrogen sources for cell growth of *B. amyloliquefaciens* DL-3 were sucrose and malt extract, whereas those for production of CMCase were rice hulls and peptone [22]. However, the best carbon and nitrogen sources for cell growth of its recombinant *E. coli* JM109/DL-3 were fructose and ammonium chloride, whereas those for

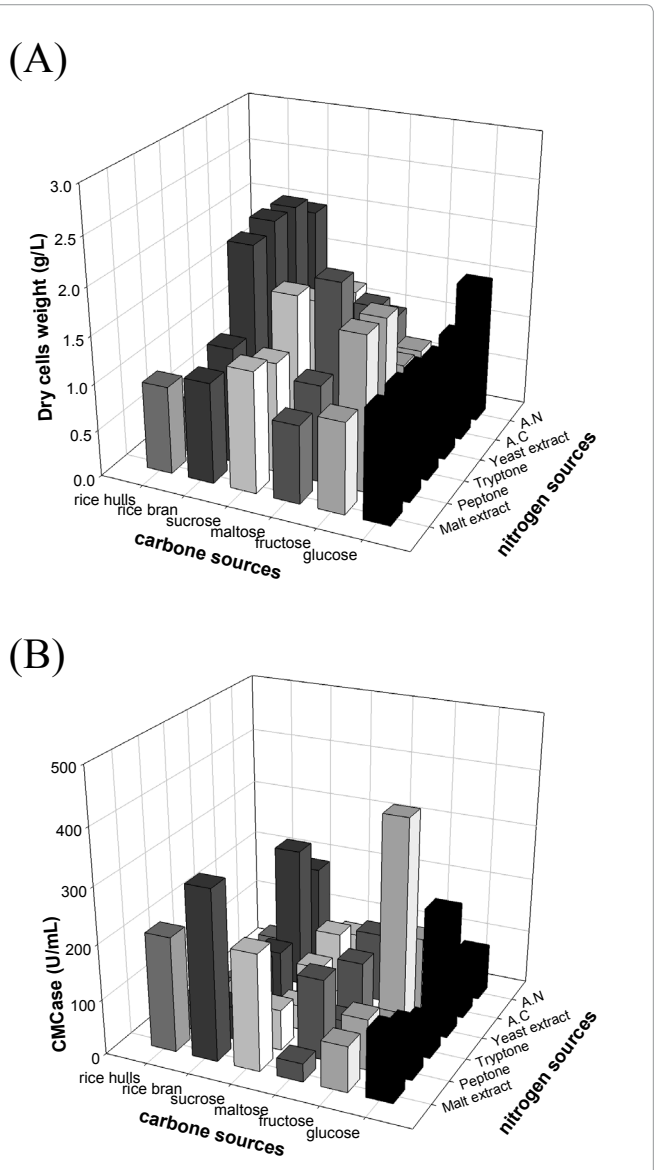

Figure 3: Effect of carbon and nitrogen sources on cell growth (A) and production of CMCase (B) by *E. coli* JM109/LBH-10.

production of CMCase were rice bran and tryptone [23]. The best carbon and nitrogen source for cell growth and production of CMCase by *B. subtilis* subsp. subtilis A-53 were also different from those for its recombinant *E. coli* JM109/A-53 [11,17]. Based on cost and availability of nitrogen sources, a combination of rice bran and ammonium chloride was chosen for next examination [24,25]. The production of CMCase by *E. coli* JM109/LBH-10 was 254.3 U/mL from 20.0 g/L rice bran and 5.0 g/L ammonium chloride.

The optimal conditions of rice bran, ammonium chloride, and initial pH of the medium for cell growth and the production of CMCase by *E. coli* JM109/LBH-10 were investigated using 'one-factor-at-a time' experiments. Composition of basic medium was 50.0 g/L rice bran, 7.5 g/L ammonium chloride, and initial pH of 7.0. The optimal conditions of rice bran, ammonium chloride, and initial pH for the cell growth of *E. coli* JM109/LBH-10 were 100.0 g/L, 7.5 g/L, and 7.0, respectively, whereas those for production of CMCase were 50.0 g/L, 7.5 g/L, and 7.0, as shown in Figure 4. The simultaneous effect of rice bran, ammonium chloride, and initial pH of the medium on cell growth and the production of CMCase by *E. coli* JM109/LBH-10 were also investigated using response surface methodology (RSM). The coded values of minimum and maximum ranges of rice bran (X_1), ammonium chloride (X_2), and initial pH of the medium (X_3) were 25.0 and 75.0 g g/L, 5.0 and 10.0 g/L, and 6.0 and 8.0, respectively. Cell growth, measured as dry cells weight (DCW), and production of CMCase from 20 different conditions ranged from 4.72 to 6.81 g/L and from 310.7 to 396.7 U/mL, as shown in Table 2. Multiple regression analysis of the experimental data gave the following second-order polynomial equation to represent cell growth of *E. coli* JM109/LBH-10 (Equation 3). The optimal conditions of rice bran, ammonium chloride, and initial pH of the medium for cell growth extracted by Design Expert Software were 73.4 g/L, 5.80 g/L, and 6.54, respectively. The maximum cell growth of 6.21 g/L was predicted by this model.

$$Y_1 = 5.69 + 0.55X_1 + 0.04X_2 + 0.01X_3 + 0.01X_1X_2 - 0.03X_1X_3 + 0.04X_2X_3 + 0.05X_{12} - 0.02X_{22} - 0.13X_{32} \quad (3)$$

Adequacy of the model was tested by the fisher's statistical test for the analysis of variance (ANOVA), as shown in Table 3. The model F-value of 26.29 from the analysis of variance (ANOVA) of cell growth implied that this model was significant. 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 and that of X_{32} ("probe > F" less 0.0500)

Homologous protein	Organism	Identity (%)	Positive (%)	GenBank
BACSU	*Bacillus subtilis*	93	96	P10475
ERWCT	*Erwinia carotovora* subsp. atroseptica	56	73	Q59394
BACS4	*Bacillus* sp. Strain N-4	56	71	P06566
CALSA	*Caldocellum saccharolyticum*	44	60	P10474
DICD3	*E. chrysanthemi* strain 3937	41	58	P07103
RUMAL	*Ruminococcus albus*	38	60	Q07940
BACS6	*Bacillus* sp. Strain KSM-635	35	52	P19424
PSEFL	*Pseudomonas fluorescens*	34	47	P27033
CLOLO	*Clostridium longisporum*	24	41	P54937
CLOSF	*Clostridium* sp. strain F1	22	48	P23340
PAELA	*Paenbacillus lautus*	21	42	P23550
PAEBA	*P. barcinonensis*	20	47	O08342

*The amino acid sequences were compared using the NCBI's BLAST search

Table 1: Comparisons of CMCase produced by *E. coli* JM109/LBH-10 with the glycosyl hydrolase family 5*.

Run	X_1 (g/L)	X_2 (g/L)	X_3	Y_1 (g/L)	Y_2 (U/mL)
1	75	5.0	8.0	5.99 ± 0.23	367.1 ± 25.2
2	50	7.5	7.0	5.71 ± 0.31	389.7 ± 33.6
3	50	7.5	7.0	5.82 ± 0.34	392.7 ± 28.4
4	50	7.5	7.0	5.86 ± 0.44	396.7 ± 35.8
5	25	10.0	8.0	5.21 ± 0.36	346.8 ± 29.7
6	75	10.0	8.0	6.19 ± 0.42	369.3 ± 30.2
7	8	7.5	7.0	4.72 ± 0.23	310.7 ± 28.7
8	50	3.3	7.0	5.49 ± 0.42	378.6 ± 33.9
9	25	10.0	6.0	5.15 ± 0.36	347.5 ± 30.8
10	25	5.0	6.0	5.06 ± 0.42	345.4 ± 28.7
11	50	7.5	8.7	5.29 ± 0.28	386.2 ± 35.2
12	75	10.0	6.0	6.12 ± 0.42	370.0 ± 25.6
13	50	7.5	7.0	5.66 ± 0.33	387.7 ± 29.5
14	50	7.5	5.3	5.22 ± 0.24	386.3 ± 36.3
15	50	7.5	7.0	5.60 ± 0.34	381.5 ± 42.5
16	92	7.5	7.0	6.81 ± 0.42	363.5 ± 33.8
17	25	5.0	8.0	5.12 ± 0.31	344.6 ± 29.7
18	50	7.5	7.0	5.52 ± 0.43	382.5 ± 28.2
19	75	5.0	6.0	6.21 ± 0.44	367.9 ± 32.7
20	50	11.7	7.0	5.65 ± 0.28	383.5 ± 33.6

Table 2: Central composite design (CCD) and determined response values.

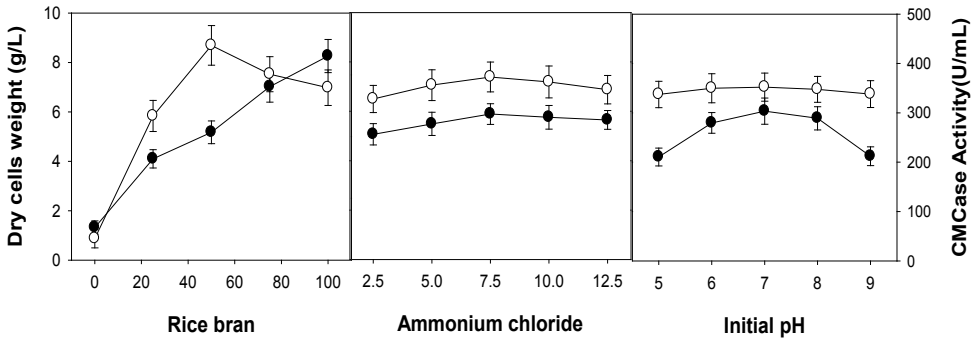

Figure 4: Effect of rice bran, ammonium chloride, and initial pH of the medium on cell growth and production of CMCase by *E. coli* JM109/LBH-10 (●, cell growth and ○, CMCase).

	Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*-value	Probe>F
Cell growth	Model	9	4.45	0.49	26.29	<0.0001
	X_1	1	4.10	4.10	217.87	<0.0001
	X_2	1	0.02	0.02	1.22	0.2961
	X_3	1	0.00	0.00	0.03	0.8661
	X_1^2	1	0.03	0.03	1.78	0.2117
	X_2^2	1	0.00	0.00	0.33	0.5792
	X_3^2	1	0.25	0.25	13.35	0.0044
	Error	5	0.08	0.02	-	-
	Total	19	4.64	-	-	-
CMCase	Model	9	8307.01	923.00	17.63	<0.0001
	X_1	1	2338.26	2338.26	44.66	<0.0001
	X_2	1	20.52	20.52	0.39	0.5453
	X_3	1	0.78	0.78	0.02	0.9051
	X_1^2	1	5844.92	5844.92	111.64	<0.0001
	X_2^2	1	304.99	304.99	5.83	0.0365
	X_3^2	1	109.93	109.93	2.10	0.1780
	Error	5	164.23	32.85	-	-
	Total	19	8830.57	-	-	-

Table 3: Parameter estimates and analysis of variance (ANOVA) of the design for cell growth and production of CMCase by *E. coli* JM109/LBH-10.

was significant for the cell growth of *E. coli* JM109/LBH-10. The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R_2 was 0.9594, which could explain 95.94% variation in the response. The value of the adjusted determination coefficient (Adj. R_2 = 0.9229) was very high to advocate for a high significance of this model [26,27]. The predicted determination of coefficient of 0.8001 was in reasonable agreement with the Adj. R_2 of 0.9229. From the statistical results obtained, it was shown that the above models were adequate to predict the cell growth of *E. coli* JM109/LBH-10 within the range of variables studied.

Multiple regression analysis of the experimental data also gave the following second-order polynomial equation to represent the production of CMCase by *E. coli* JM109/LBH-10 (Equation 4). The optimal conditions of rice bran, ammonium chloride, and initial pH of the medium for production of CMCase were 57.1 g/L, 6.40 g/L, and 6.70, respectively. The maximum production of CMCase of 388.9 U/mL was predicted by this model.

$$Y_2 = 388.66 + 13.08X_1 + 1.23X_2 - 0.24X_3 + 0.01X1X_2 + 0.01X_1X_3 + 0.04X_2X_3 - 20.14X_{12} - 4.60X_{22} - 2.76X_{32} \quad (4)$$

The model F-value of 17.63 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_{12} were highly significant. The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R_2 was 0.9407. The value of the adjusted determination coefficient (Adj. R_2 = 0.8874) was high to advocate for a high significance of this model. The predicted determination of coefficient of 0.6658 was also in reasonable agreement with the Adj. R_2 of 0.8874. From the statistical results obtained, it was shown that the above models were adequate to predict the production of CMCase by *E. coli* JM109/LBH-10 within the range of variables studied.

The optimal conditions for the production of CMCase by *P. aquimaris* LBH-10 were 50.0 g/L rice bran, 3.0 g/ peptone, and initial pH of 8.0 [13]. The percent participation of rice bran, peptone, and initial pH for production of CMCase were 52.0, 47.8, and 0.2%, respectively. The optimal conditions for the production of CMCase by *E. coli* JM109/LBH-10 were 57.1 g/L rice bran, 6.40 g/L ammonium chloride, and initial pH of 6.70. The rice bran was found to be highly significant for the production of CMCase by *E. coli* JM109/LBH-10. However, ammonium chloride and initial pH were not significant for production of CMCase. The significant factor for the cell growth of *E. coli* JM109/DL-3 was rice bran, whereas those for production of CMCase were rice bran and initial pH of the medium [10]. The

optimal conditions and significance of each factor for the production of CMCase by *E. coli* JM109/LBH-10 were different from those for *P. aquimaris* LBH-10 as previously reported [11,17].

The effect of temperature on cell growth and the production of CMCase by *E. coli* JM109/LBH-10 were investigated. The temperature for cell growth and production of CMC ranged from 25 to 45°C. The carbon and nitrogen source and initial pH of the medium were 57.1 g/L rice bran, 6.4 g/L ammonium chloride, and 6.7, respectively. The optimal temperatures for cell growth and the production of CMCase by *E. coli* JM109/LBH-10 were found to be 40 and 35°C, respectively, as shown in Figure 5. Cell growth and the production of CMCase by *E. coli* JM109/LBH under optimized conditions were 6.12 g/L and 523.2 U/mL. Significance of each value was analyzed by MYSTAT software (Systat Software, Inc., Chicago, USA).

The optimal temperatures for cell growth and the production of CMCase by *P. aquimaris* LBH-10 were 25 and 30°C, respectively [13]. Those for production of CMCases by bacterial and fungal stains ranged from 25 to 37°C, except for thermophilic microorganisms such as *Thermoascus aurantiacu*, which optimal temperature for production of CMCase is 50°C [23]. The optimal temperature for the cell growth of a psychrophilic marine bacterium, *P. aquimaris* LBH-10 was lower than that for production of CMCase. However, the optimal temperature for the cell growth of its recombinant *E. coli* JM109/LBH-10 was higher than that for production of CMCase. The optimal temperatures for cell growth and the production of CMCase by *B. subtilis* subsp. subtilis A-53 were 35 and 30°C, respectively, whereas those for its recombinant *E. coli* JM109/A-53 were 40 and 35°C [11,17]. The optimal temperatures for cell growth and the production of CMCase by *E. coli* JM109/LBH-10 were also different from those for *P. aquimaris* LBH-10 such as other recombinants, *E. coli* JM109/A-53 and *E. coli* JM109/DL-3.

The effect of agitation speed on cell growth and the production of CMCase by *E. coli* JM109/LBH-10 in a 7 L bioreactor were investigated using one-factor-at-a-time. Carbon and nitrogen sources for production of CMCase were 57.0 g/L rice bran and 6.4 g/L ammonium chloride. The initial pH of the medium and temperature were 6.7 and 35°C. Agitation speed ranged from 200 to 500 rpm and aeration rate was 1.0 vvm. Higher agitation speeds and aeration rates, which resulted in an increase of dissolved oxygen in the medium, enhanced cell growth as well as production of CMCase, as shown in Figure 6. The optimal agitation speeds for cell growth and production of CMCase were 500 rpm. The effect of aeration rate on cell growth and production of CMCase also was investigated. Aeration rate ranged from 0.5 to 2.0 vvm and the agitation speed was fixed to 500 rpm. The optimal aeration rates for cell growth and production of CMCase were 1.0 vvm. The optimal agitation speed and aeration rate for cell growth of *E. coli* JM109/LBH-10 were the same as those for its production of CMCase.

Based on the results from one-factor-at-a-time, the effect of agitation speed and aeration rate on cell growth and the production of CMCase by *E. coli* JM109/LBH-10 were investigated using RSM. The coded values of minimum and maximum ranges of agitation speed (X_1) and aeration rate (X_2) were 300 and 500 rpm and 0.5 and 1.5 vvm. Cell growth (Y_1') and production of CMCase (Y_2') from 13 different conditions ranged from 4.40 to 6.94 g/L and from 334.4 to 572.7 U/mL, as shown Table 4. Multiple regression analysis of the experimental data gave the following second-order polynomial equation to represent the cell growth of *E. coli* JM109/LBH-10 (Equation 5). The optimal agitation speed and aeration rate for cell growth were 480 rpm and 1.0 vvm. The maximum cell growth of 6.81 g/L was predicted by the model.

$$Y_1' = 6.59 + 0.50X_1 - 0.05X_2 - 0.46X_1X_2 - 0.24X_{12} - 0.90X_{22} \quad (5)$$

The model F-value of 18.07 from ANOVA of cell growth implied that this model was significant, as shown in Table 5. The ANOVA indicated that this model and the model terms of X_1, X_1X_2 and X_{12} were significant ("probe > F" less 0.0500) for cell growth. The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R_2 is 0.9281. The value of the adjusted determination coefficient (Adj. R_2 = 0.8767) is very high to advocate for a high significance of this model.

Multiple regression analysis of the experimental data also gave the following second-order polynomial equation to represent the production of CMCase by *E. coli* JM109/LBH-10 (Equation 6). The optimal agitation speed and aeration rate for production of CMCase were 480 rpm and 1.0 vvm, which were the same as those for cell growth. The maximum production of CMCase of 576.8 U/mL was predicted by the model.

$$Y_2' = 550.64 + 54.21X_1 + 8.40X_2 + 4.98X_1X_2 - 29.53X_{12} - 94.88X_{22} \quad (6)$$

The model F-value of 42.84 from ANOVA of production of CMCase implied that this model was also significant. The ANOVA indicated that this model and the model terms of X_1 and X_{22} were also highly significant model terms. The regression equation obtained from ANOVA indicated that the multiple correlation coefficient of R_2 was 0.9734. The value of the adjusted determination coefficient (Adj. R_2 = 0.9543) was very high to advocate for a high significance of this

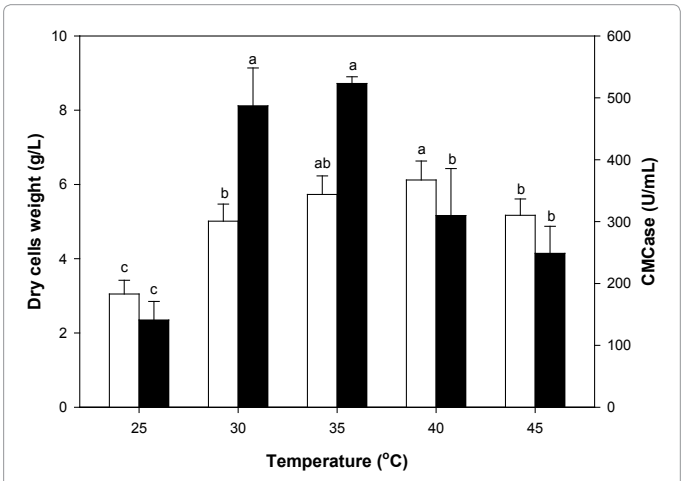

Figure 5: Effect of temperature on cell growth and production of CMCase by *E. coli* JM109/LBH-10 (■, cell growth and □, CMCase).

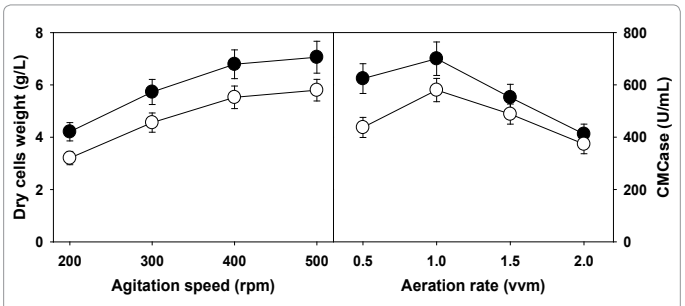

Figure 6: The optimal agitation speed and aeration rate for cell growth and production of PS-7 by *E. coli* JM109/LBH-10 in a 7L bioreactor (●, DCW and ○, CMCase).

model. The predicted determination of coefficient of 0.959 was also in reasonable agreement with the Adj. R_2 of 0.943.

The three-dimensional response surface plots were generated to investigate the interaction among agitation speed and aeration rate and to visualize the combined effects on the response of cell growth and the production of CMCase by *E. coli* JM109/LBH-10, as shown in Figure 7. The three-dimensional response surfaces and their respective contour plots are the graphical representation of Equations 5 and 6. In contrast to the circular shapes, the elliptical nature of curves indicates more significant mutual interactions between variables. The interactive effect of agitation speed and aeration rate on cell growth (Probe> F = 0.0209) was found to be higher than that on production of CMCase (Probe> F = 0.6101).

The concentration of dissolved oxygen in the medium is influenced by agitation speed, aeration rate, and the inner pressure of bioreactors [28]. Variation in agitation speed and 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 [29]. The optimal agitation speed and aeration rate for the cell growth of *P. aquimaris* LBH-10 had been reported to be different from those for production of CMCase like other *Bacillus* species [17,22]. However, the optimal agitation speed and aeration rate for cell growth of *E. coli* JM109/LBH-10 were exactly the same as those for production of CMCase. The optimal agitation speed for the cell growth of *E. coli* JM109/A-53 was the same as that for its production of CMCase, whereas its optimal aeration rate for cell growth was different from that for production of CMCase [11].

In this study, rice bran and ammonium chloride were developed as carbon and nitrogen sources for the production of CMCase by *E. coli* JM109/LBH-10. Rice bran from the rice processing industry is produced in large amounts in Korea, as well as other rice producing countries. Low-cost ammonium chloride as a nitrogen source is also available in large quantities. The optimal conditions for cell growth and production of CMCase by *E. coli* JM109/LBH-10 were found to be different from those for its wild type, *P. aquimaris* LBH-10, as shown in Table 6. The maximal production of CMCase by *E. coli* JM109/LBH-10 was 1.80 times higher than *P. aquimaris* LBH-10. The maximal productions of CMCase by *E. coli* JM109/A-53 and *E. coli* JM109/DL-3 were 3.5 and 2.4 times higher than their wild strains, *B. subtilis* subsp. subtilis A-53 and *B. amyloliquefaciens* DL-3, respectively [10,11]. A major restriction in enzymatic saccharification of cellulosic materials can be overcome by developing cheap and easy-available substrates,

Run	X_1 (rpm)	X_2 (vvm)	Y_1 (g/L)	Y_2 (U/mL)
1	400	1.0	6.54 ± 0.47	572.7 ± 41.0
2	400	1.0	6.94 ± 0.58	542.9 ± 36.2
3	500	0.5	6.24 ± 0.52	472.3 ± 38.9
4	541	1.0	6.94 ± 0.54	571.4 ± 39.2
5	400	0.3	4.98 ± 0.38	334.4 ± 30.7
6	400	1.7	4.40 ± 0.45	373.5 ± 29.3
7	500	1.5	5.52 ± 0.36	488.2 ± 33.4
8	400	1.0	6.62 ± 0.42	531.7 ± 42.1
9	300	1.5	5.76 ± 0.44	384.1 ± 32.7
10	259	1.0	5.08 ± 0.39	397.9 ± 36.5
11	400	1.0	6.52 ± 0.41	537.3 ± 39.8
12	300	0.5	4.65 ± 0.47	388.1 ± 32.0
13	400	1.0	6.32 ± 0.51	568.6 ± 41.3

Table 4: Central composite design (CCD) and determined response values.

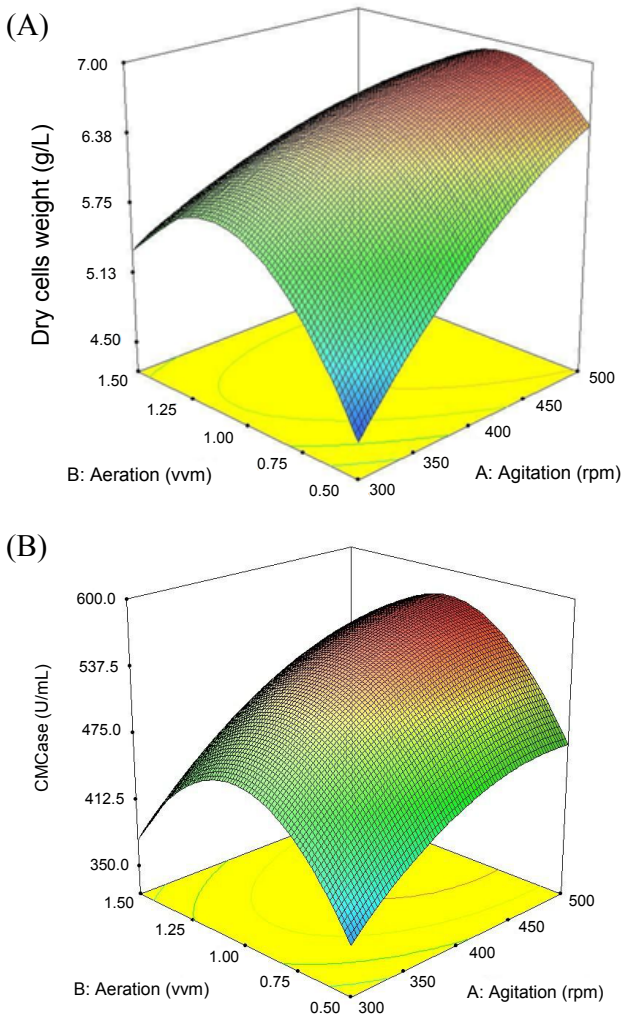

Figure 7: 3D response surface plots displaying combined effect of agitation speed and aeration rate on cell growth (A) and production of CMCase (B) by *E. coli* JM109/LBH-10.

	Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*-value	Probe>F
Cell growth	Model	5	8.59	1.72	18.07	0.0007
	X_1	1	1.98	1.98	20.82	0.0026
	X_2	1	0.02	0.02	0.24	0.6370
	$X_1 \cdot X_2$	1	0.84	0.84	8.80	0.0209
	X_1^2	1	0.40	0.40	4.24	0.0784
	X_2^2	1	5.65	5.65	59.36	0.0001
	Error	4	0.20	0.05	-	-
	Total	12	9.26	-	-	-
CMCase	Model	5	90416.0	18083.2	42.84	<0.0001
	X_1	1	23508.3	23508.3	55.70	<0.0001
	X_2	1	564.4	564.4	1.34	0.2433
	$X_1 \cdot X_2$	1	99.0	99.0	0.23	0.6101
	X_1^2	1	6482.5	6482.5	15.36	0.0042
	X_2^2	1	63946.1	63946.1	151.51	<0.0001
	Error	4	1927.0	481.8	-	-
	Total	12	93370.5	-	-	-

Table 5: Parameter estimates and analysis of variance (ANOVA) of the design for cell growth and production of CMCase by *E. coli* JM109/LBH-10.

Scale	Optimal condition	*E. coli* JM109/LBH-10 [This study]		*P. aquimaris* LBH-10 [13]	
		Cell growth	CMCase	Cell growth	CMCase
Flask scale-1	Rice bran (g/L)	73.4	57.1	-	50.0
	Ammonium chloride (g/L)	5.80	6.40	-	3.0*
	Initial pH	6.54	6.70	-	8.0
	Maximal production	6.21 g/L	388.9 U/mL	-	221.8 U/mL
Flask scale-2	Temperature (°C)	40	35	25	30
	Maximal production	6.12 g/L	523.3 U/mL	2.32 g/L	334.6 U/mL
Bioreactor	Agitation speed (rpm)	480	480	400	300
	Aeration rate (vvm)	1.0	1.0	1.5	1.0
	Maximal production	6.81 g/L	576.8 U/mL	3.36 g/L	320.3 U/mL

*Nitrogen source was peptone

Table 6: Comparison of optimal conditions for cell growth and production of CMCase by *P. aquimaris* LBH-10 and its recombinant *E. coli* JM109/LBH-10.

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