Korean J. Chem. Eng., **29**(3), 384-391 (2012)
DOI: 10.1007/s11814-011-0192-4

Optimization of salts in medium for production of carboxymethylcellulase by a psychrophilic marine bacterium, *Psychrobacter aquimaris* LBH-10 using two statistical methods

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(*Received 22 May 2011 • accepted 31 July 2011*)

Abstract−The concentration of four mineral salts in the medium for the production of carboxymethylcellulase (CMCase) by *Psychrobacter aquimaris* LBH-10 were optimized using orthogonal array method (OAM) and response surface method (RSM) and their results from two statistical methods were compared. The analysis of variance (ANOVA) of data from central composite design (CCD) based on OAM indicated that potassium phosphate gave the highest percentage participation for cell growth as well as production of CMCase. However, their relative participations of four salts for cell growth were different from those for production of CMCase. The ANOVA of results from RSM indicated that highly significant factors ("probe>F" less than 0.0001) for cell growth were K_2HPO_4 and $(NH_4)_2SO_4$, whereas those for production of CMCase were K_2HPO_4, NaCl, and $MgSO_4 \cdot 7H_2O$. The optimal concentration of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ for cell growth extracted by Design Expert Software based on RSM were 7.10, 0.84, 0.24, and 0.33 g/L, respectively, whereas those for production of CMCase were 3.00, 0.52, 0.34, and 0.45 g/L. The optimal concentrations of salts for cell growth and production of CMCase using RSM basically coincided with those using OAM as well as those from 'one-factor-at-a-time' method. The production of CMCase by *P. aquimaris* LBH-10 with optimized concentrations of salts was 273.0 U/mL, which was enhanced by 1.27 times higher than the previous report.

Key words: *Psychrobacter aquimaris*, Carboxymethylcellulase, Optimization, Salts, Orthogonal Array Method, Response Surface Method

INTRODUCTION

The complete enzymatic hydrolysis of cellulosic materials for production of fermentable sugars needs at least three different types of cellulases: endoglucanase (carboxymethylcellulase), exocellobiohydrolase (avicelase), and β-glucosidase [1]. The enzymatic saccharification of cellulosic materials such as rice hulls was performed by commercial cellulases, in which the major one was carboxymethylcellulase (CMCase) [2]. A major constraint in enzymatic saccharification of cellulosic materials is the cost of cellulases and low productivity [3]. Many studies including types of strains, culture conditions, and substrates on production of cellulases have been reported [4,5]. However, there have been few reports on optimization of mineral salts in the medium for production of cellulases.

Enzymes produced by marine microorganisms can provide numerous advantages over traditional enzymes due to the wide range of environments [6,7]. The CMCase produced by a marine psychrophilic bacterium, *P. aquimaris* LBH-10, showed thermal stability at low temperature and relatively low optimal pH of 3.5 [8]. Activities of enzymes from psychrophilic organisms are reported to be much higher at low temperature than those of its mesophilic and thermophilic counterparts [4,8]. Due to low thermal stability and acidophilic characteristics, the CMCase produced by *P. aquimaris* LBH-10 can be used in animal feed for improving the nutritional quality and digestibility [9]. It can be also applied in the textile and detergent industries [10].

The optimization of culture medium by the traditional 'one-factor-at-a-time' technique requires a considerable amount of work and time. An alternate strategy is a statistical approach, for example, orthogonal array method (OAM) based on factorial experimental design and response surface method (RSM,) involving a minimum number of experiments for a large number of factors, by which optimization of salts in the medium has been successfully demonstrated [11,12]. In this study, OAM and RSM were used to optimize concentrations of salts in medium and their influence on cell growth and production of CMCase was compared.

MATERIALS AND METHODS

1. Bacterial Strain and Medium

Psychrobacter aquimaris LBH-10 used in this study was isolated from sea-water and identified in the previous study [8]. 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 1.5% (w/v) agar.

2. Production of Carboxymethylcellulase (CMCase)

Starter cultures were prepared as described in the previous report

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[8]. The main culture was carried out in the medium containing 50.0 g/L rice bran, 3.0 g/L peptone, 5.0 g/L K_2HPO_4, 1.0 g/L NaCl, 0.2 g/L $MgSO_4 \cdot 7H_2O$, and 0.6 g/L $(NH_4)_2SO_4$ at 30 °C for 72 h under aerobic conditions. Batch fermentations for the production of CMCase by *P. aquimaris* LBH-10 were performed in a 7 bioreactor (Ko-Biotech Co., Korea). Agitation was provided by three six-flat-blade impellers in a 7 L bioreactor.

3. Experimental Design Using Orthogonal Array Method

Design of experiments (DOE) was performed based on the Taguchi method for investigating effects of salts in the medium on cell growth and the production of CMCase by *P. aquimaris* LBH-10 [13]. Qualitek-4 (W32b) software (Nutek, Inc., USA) was used for automatic design, analysis of experiments, and calculation of interactions among different factors. The $L_{16}(4^4)$ orthogonal array experiment had four factors - K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ - and each factor had four different levels. These trials were done in three replicates. The optimization for cell growth and production of CMCase was statistically analyzed using one-way analysis of variance (ANOVA).

4. Experimental Design Using Response Surface Method

The K_2HPO_4 (X_1), NaCl (X_2), $MgSO_4 \cdot 7H_2O$ (X_3), and $(NH_4)_2SO_4$ (X_4) were chosen as the independent variables and each variable was designated as −1, 0, and 1, respectively. Cell growth measured as DCW (Y_1) and CMCase (Y_2) was used as a dependent output variable. The total number of experiments was 30 ($=2^k+2k+6$), where k is the number of independent variables [14]. The interrelationships of the 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 three times on different occasions. 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 Eq. (1) that defines predicted response (Y) in terms of the independent variables (X_1, X_2, X_3, and X_4) was obtained:

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

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.

5. Analytical Methods

Dry cells weight was measured as described in the previous report [15]. Activity of CMCase produced by *P. aquimaris* LBH-10 was determined based on releases of reducing sugars from carboxymethylcellulose (CMC) using the 3,5-dinitrosalicylic acid (DNS) method, as described in the previous report [16]. Glucose (Sigma-Aldrich, UK) was used to prepare a calibration curve. One unit of CMCase was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to glucose per minute under the assay conditions.

RESULTS AND DISCUSSION

1. Optimization of Mineral Salts in Medium Using One-factor-at-a-time Experiments

The optimal concentrations of mineral salts in the medium for cell growth and the production of CMCase by *P. aquimaris* LBH-10 were investigated using 'one-factor-at-a-time' experiments. Concentrations of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ ranged from 0.0 to 10.0 g/L, from 0.0 to 2.0 g/L, from 0.0 to 0.8 g/L, and from 0.0 to 2.4 g/L, respectively. Carbon and nitrogen sources and initial pH of the medium were 50.0 g/L rice bran, 3.0 g/L peptone, and 8.0. Concentrations of four salts in the basic medium were 5.0, 1.0, 0.2, and 0.6 g/L, respectively. The optimal conditions of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ for cell growth were found to be 7.5, 1.0, 0.2, and 0.3, respectively, whereas those for production of CMCase were 2.5, 0.5, 0.4, and 0.3, as shown in Fig. 1. The optimal concentrations of mineral salts for cell growth were different form those for production of CMCase.

2. Optimization of Mineral Salts in Medium Using Orthogonal Array Method

Based on results from one-factor-at-a-time experiments, the optimal concentrations of four salts for cell growth and production of CMCase were investigated by a $L_{16}(4^4)$ orthogonal array method. The minimum and maximum levels of variables are shown in Table 1. Carbon and nitrogen sources and initial pH of the medium were 50.0 g/L rice bran, 3.0 g/L peptone, and 8.0. Cell growths, measured as dry cells weight (DCW), and productions of CMCase by *P. aquimaris* LBH-10 from 16 different conditions ranged from 1.71 to 2.01 g/L and from 244.4 to 280.2 U/mL, as shown in Table 2.

The last column in the analysis of variance (ANOVA) indicated the influence of each factor on cell growth and the production of CMCase by *P. aquimaris* LBH-10, as shown in Table 3. The factor of

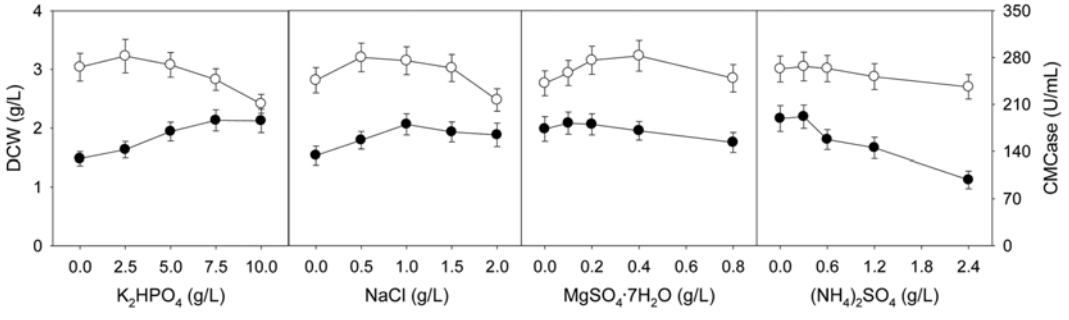


Fig. 1. Effect of salts in the medium on cell growth and the production of CMCase by *P. aquimaris* LBH-10 (●, DCW and ○, CMCase).

Table 1. Factors and their levels in the orthogonal array experiment based on Taguchi design using Qualitek-4 (W32b) software based on orthogonal array method

Factor	Level 1	Level 2	Level 3	Level 4
K_2HPO_4 (g/L)	0.0	2.5	5.0	10.0
NaCl (g/L)	0.0	0.5	1.0	1.5
$MgSO_4 \cdot 7H_2O$ (g/L)	0.1	0.2	0.4	0.8
$(NH_4)_2SO_4$ (g/L)	0.0	0.3	0.6	1.2

potassium phosphate (K_2HPO_4) gave maximum sum of squares (S) and percentage influence (33.0%) for cell growth of *P. aquimaris* LBH-10 followed by NaCl (27.5%), $(NH_4)_2SO_4$ (21.0%), and $MgSO_4 \cdot 7H_2O$ (15.5%). This factor also gave maximum sum of squares (S) and percentage influence (60.1%) for the production of CMCase by *P. aquimaris* LBH-10. However, the relative influences of four salts on cell growth were different from production of CMCase, as shown in Fig. 2. Based on calculated percent *P* (%), K_2HPO_4 was found to be the most important factor for cell growth as well as the production of cellulases by *P. aquimaris* LBH-10.

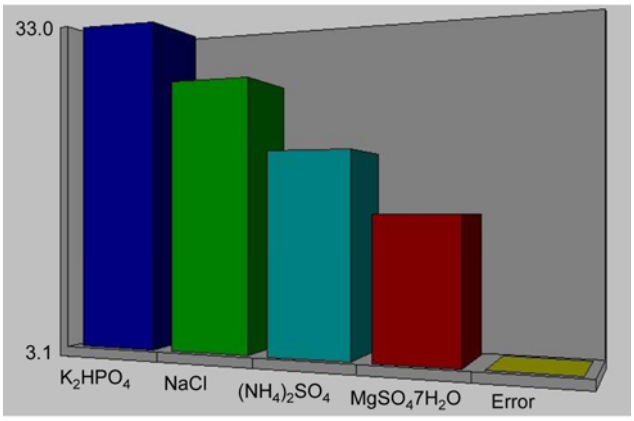
Three interactions between two factors were calculated by Qualitek-4 (W32b) software based on severity index (SI), and interaction pairs were shown in descending order of their SI, as shown in Table 4. With interaction of different factors, the factor of $MgSO_4 \cdot 7H_2O$ for cell growth perceived to be the least significant (at its individual levels), showed the highest SI percentage with the factor of $(NH_4)_2SO_4$ (SI=61.87%). The factor of K_2HPO_4 for the production of CMCase showed the highest SI percentage (SI=47.76%) with the factor of $MgSO_4 \cdot 7H_2O$. The optimal concentration of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ in the medium for cell growth were 10.0, 1.0, 0.2, and 0.3 g/L, respectively, whereas those for the production of CMCase were 2.5, 0.5, 0.4, and 0.3 g/L. The expected

Table 2. Simultaneous effect of mineral salts in the medium on cell growth and production of CMCase by *P. aquimaris* LBH-10 designed using Qualitexk-4 (W32b) software based on the L_{16} (4^4) orthogonal array method

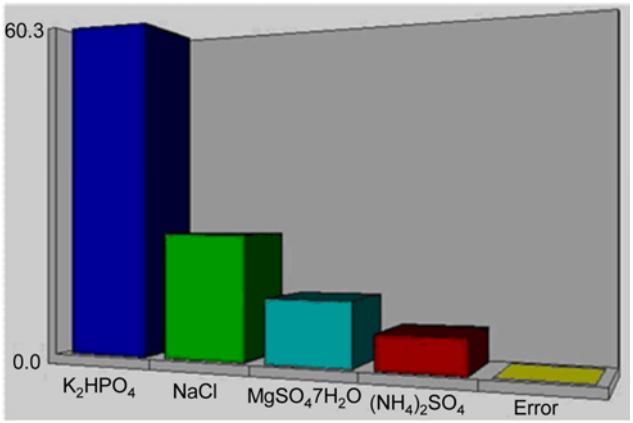
Run	K_2HPO_4 (g/L)	NaCl (g/L)	$MgSO_4 \cdot 7H_2O$ (g/L)	$(NH_4)_2SO_4$ (g/L)	DCW (g/L)	CMCase (U/mL)
1	0.0	0.0	0.1	0.0	1.71	250.4
2	0.0	0.5	0.2	0.3	1.92	273.6
3	0.0	1.0	0.4	0.6	1.80	269.3
4	0.0	1.5	0.8	1.2	1.71	260.1
5	2.5	0.0	0.2	0.6	1.77	267.0
6	2.5	0.5	0.1	1.2	1.72	278.0
7	2.5	1.0	0.8	0.0	1.87	264.9
8	2.5	1.5	0.4	0.3	1.90	280.2
9	5.0	0.0	0.4	1.2	1.75	259.8
10	5.0	0.5	0.8	0.6	1.82	265.7
11	5.0	1.0	0.1	0.3	2.00	258.3
12	5.0	1.5	0.3	0.0	2.00	253.1
13	10.0	0.0	0.8	0.3	1.87	244.4
14	10.0	0.5	0.4	0.0	1.92	259.4
15	10.0	1.0	0.2	1.2	2.01	248.4
16	10.0	1.5	0.1	0.6	1.93	246.4

Table 3. Analysis of variance (ANOVA) of cell growth and production of CMCase by *P. aquimaris* LBH-10 designed using qualitexk-4 (W32b) software based on the L_{16} (4^4) orthogonal array method

	Factor	DOF (f)	Sums of squares (S)	Variance (V)	F-ratio (F)	Pure sum (S′)	Percent of participation (P, %)
Cell growth	K_2HPO_4	3	0.06	0.02	54.50	0.05	33.00
	NaCl	3	0.05	0.02	45.54	0.05	27.46
	$MgSO_4 \cdot 7H_2O$	3	0.03	0.01	26.12	0.03	15.49
	$(NH_4)_2SO_4$	3	0.04	0.01	35.02	0.03	20.97
	Other/error	3	0.00	0.00	-	-	3.08
	Total	15	0.18	-	-	-	100.00
CMCase	K_2HPO_4	3	1080.84	360.28	10808434.72	1080.84	60.30
	NaCl	3	398.96	132.99	3989645.86	398.96	22.26
	$MgSO_4 \cdot 7H_2O$	3	202.32	67.44	2023236.30	202.32	11.29
	$(NH_4)_2SO_4$	3	110.31	36.77	1103122.34	110.31	6.15
	Other/error	3	0.00	0.00	-	-	0.00
	Total	15	1792.44	-	-	-	100.00



(a)



(b)

Fig. 2. The percentage contributions of each factor for cell growth (a) the production CMCase (b) by *P. aquimaris* LBH-10 analyzed using Qualitek-4 (W32b) software. X-axis shows factors and Y-axis shows percentage contributions of each factor.

cell growth and the production of CMCase by *P. aquimaris* LBH-10 under these optimized conditions were 2.13 g/L and 289.4 U/mL, respectively.

Table 5. Process variables used central composite design (CCD) with actual factor levels corresponding to coded factor levels using Design-Expert software based on response surface method

Variables	Symbol	Coded levels		
		−1	0	1
K_2HPO_4 (g/L)	X_1	2.5	5.0	7.5
NaCl (g/L)	X_2	0.5	1.0	1.5
$MgSO_4 \cdot 7H_2O$ (g/L)	X_3	0.2	0.4	0.6
$(NH_4)_2SO_4$ (g/L)	X_4	0.3	0.6	0.9

3. Optimization of Mineral Salts in the Medium Using Response Surface Method

Based on results from one-factor-at-a-time experiments, the optimal concentrations of four salts for cell growth and production of CMCase were also investigated using response surface methodology (RSM). The minimum and maximum ranges of variables and the full experimental plan with respect to their actual and coded values are shown in Table 5. The results of central composite design (CCD) experiments consisted of experimental and predicted values of four independent variables, as shown in Table 6. Cell growth and production of CMCase from 30 different conditions ranged from 1.75 to 2.09 g/L and from 249.2 to 278.2 U/mL.

The analysis of variance (ANOVA) of the design for cell growth indicated that the model *F*-value and Probe>F were 11.75 and <0.0001, which implied that this model was highly significant ("probe>F" less than 0.0001) and there was only a 0.01% chance that a "Model *F*-value" could occur to die due to noise, as shown in Table 7. The ANOVA also indicated that the model terms of X_1 and X_4 were highly significant and those of X_2, X_3, and X_2^2 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 the ANOVA indicated that the multiple correlation coefficient of R^2 is 0.9164. The model can explain 91.64% variation in the response. From the statistical results obtained, it was shown that this model was adequate to predict cell growth of *P. aquimaris* LBH-10 within the range of variables studied. Multiple regression analysis of the experimental data gave

Table 4. Estimated interactions of severity index (SI) for different parameters for cell growth and production of CMCase by *P. aquimaris* LBH-10 (The order is based on SI.)

	Interacting factor pairs	Columns	SI (%)	Col.	Opt.
Cell growth	$MgSO_4 \cdot 7H_2O \times (NH_4)_2SO_4$	3×4	61.87	7	[2,4]
	$K_2HPO_4 \times NaCl$	1×2	43.47	3	[4,3]
	$K_2HPO_4 \times (NH_4)_2SO_4$	1×4	30.10	5	[4,4]
	$K_2HPO_4 \times MgSO_4 \cdot 7H_2O$	1×3	26.75	2	[4,2]
	$NaCl \times (NH_4)_2SO_4$	2×4	26.75	6	[3,4]
	$NaCl \times MgSO_4 \cdot 7H_2O$	2×3	23.41	1	[3,2]
CMCase	$K_2HPO_4 \times MgSO_4 \cdot 7H_2O$	1×3	47.76	2	[2,3]
	$NaCl \times MgSO_4 \cdot 7H_2O$	2×3	29.32	1	[4,3]
	$NaCl \times (NH_4)_2SO_4$	2×4	28.21	6	[4,2]
	$MgSO_4 \cdot 7H_2O \times (NH_4)_2SO_4$	3×4	17.59	7	[3,2]
	$K_2HPO_4 \times NaCl$	1×2	17.03	3	[2,4]
	$K_2HPO_4 \times (NH_4)_2SO_4$	1×4	11.03	5	[2,2]

Table 6. Central composite design and determined response values using Design-Expert software

Run	X_1 (g/L)	X_2 (g/L)	X_3 (g/L)	X_4 (g/L)	Experimental		Predicted	
					Y_1 (g/L)	Y_2 (U/mL)	Y_1 (g/L)	Y_2 (U/mL)
1	7.5	0.5	0.6	0.3	1.98	263.2	1.96	263.1
2	5.0	1.0	0.4	0.6	1.95	264.4	1.92	264.3
3	7.5	1.5	0.6	0.9	1.91	253.3	1.91	253.2
4	2.5	1.5	0.2	0.3	1.97	265.6	1.95	264.7
5	2.5	1.5	0.6	0.9	1.79	268.3	1.80	267.4
6	5.0	1.0	0.4	0.6	1.93	263.1	1.92	264.3
7	7.5	1.5	0.6	0.3	2.01	254.7	2.00	254.4
8	2.5	0.5	0.2	0.9	1.83	272.7	1.82	271.9
9	2.5	1.5	0.6	0.3	1.89	269.7	1.89	268.3
10	2.5	0.5	0.2	0.3	1.93	274.1	1.91	272.9
11	5.0	2.0	0.4	0.6	1.87	252.9	1.88	253.7
12	5.0	1.0	0.4	0.0	2.01	263.9	2.05	265.4
13	2.5	1.5	0.2	0.9	1.87	265.2	1.86	264.0
14	0.0	1.0	0.4	0.6	1.80	276.3	1.79	279.7
15	2.5	0.5	0.6	0.3	1.85	278.2	1.85	276.7
16	5.0	1.0	0.4	0.6	1.91	263.2	1.92	264.3
17	2.5	0.5	0.6	0.9	1.75	276.8	1.76	264.3
18	5.0	1.0	0.4	0.6	1.94	265.2	1.92	264.3
19	7.5	0.5	0.2	0.3	2.06	259.1	2.02	259.0
20	5.0	1.0	0.0	0.6	1.95	256.2	2.00	257.0
21	5.0	1.0	0.4	0.6	1.91	264.6	1.92	264.3
22	5.0	0.0	0.4	0.6	1.78	268.8	1.81	270.3
23	5.0	1.0	0.4	0.6	1.89	265.2	1.92	264.3
24	5.0	1.0	0.8	0.6	1.89	262.8	1.87	264.4
25	7.5	1.5	0.2	0.3	2.09	250.6	2.06	250.6
26	7.5	1.5	0.2	0.9	1.99	249.2	1.97	249.6
27	7.5	0.5	0.6	0.9	1.88	261.8	1.87	261.6
28	5.0	1.0	0.4	1.2	1.88	262.5	1.87	263.2
29	10.0	1.0	0.4	0.6	1.96	252.8	2.01	251.7
30	7.5	0.5	0.2	0.9	1.96	257.7	1.93	257.8

Table 7. Parameter estimates and analysis of variance (ANOVA) of the design for cell growth of *P. aquimaris* LBH-10

Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*-value	Probe>F
Model	14	0.170	0.012	11.75	<0.0001
X_1	1	0.073	0.073	70.73	<0.0001
X_2	1	0.009	0.009	8.44	0.0109
X_3	1	0.024	0.024	23.08	0.0002
X_4	1	0.048	0.048	46.82	<0.0001
X_1X_2	1	0.000	0.000	0.000	1.0000
X_1X_3	1	0.000	0.000	0.000	1.0000
X_1X_4	1	0.000	0.000	0.000	1.0000
X_2X_3	1	0.000	0.000	0.000	1.0000
X_2X_4	1	0.000	0.000	0.000	1.0000
X_3X_4	1	0.000	0.000	0.000	1.0000
X_1^2	1	0.001	0.001	0.750	0.4007
X_2^2	1	0.010	0.010	9.440	0.0078
X_3^2	1	0.000	0.000	0.390	0.5403
X_4^2	1	0.003	0.003	2.85	0.1119
Error	5	0.002	0.001	-	-
Total	29	0.180	-	-	-

Table 8. Parameter estimates and analysis of variance (ANOVA) of the design for the production of CMCase by *P. aquimaris* LBH-10

Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*-value	Probe>F
Model	14	1712.440	122.320	49.21	<0.0001
X_1	1	1176.000	1176.000	473.15	<0.0001
X_2	1	410.030	410.030	164.97	<0.0001
X_3	1	82.880	82.880	33.35	<0.0001
X_4	1	7.040	7.040	2.83	0.1130
X_1X_2	1	0.062	0.062	0.03	0.8761
X_1X_3	1	0.063	0.063	0.03	0.8761
X_1X_4	1	0.062	0.062	0.03	0.8761
X_2X_3	1	0.062	0.062	0.03	0.8761
X_2X_4	1	0.063	0.063	0.03	0.8761
X_3X_4	1	0.062	0.062	0.03	0.8761
X_1^2	1	3.380	3.380	1.36	0.2615
X_2^2	1	8.720	8.720	3.51	0.0807
X_3^2	1	22.160	22160	8.91	0.0092
X_4^2	1	0.005	0.005	0.00	0.9642
Error	5	4.160	0.830	-	-
Total	29	1749.720	-	-	-

the following second-order polynomial equation in terms of coded factors (2) and the optimal conditions of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ for cell growth extracted by Design Expert Software were 7.10, 0.84, 0.24, and 0.33 g/L, respectively. The maximum cell growth of 2.03 g/L was predicted by this model.

$$Y_1 \text{ (cell growth)} = 1.920 + 0.055X_1 + 0.019X_2 - 0.031X_3 - 0.045X_4 - 0.005X_1^2 - 0.019X_2^2 + 0.004X_3^2 + 0.010X_4^2 \quad (2)$$

The ANOVA of the design for production of CMCase indicated that the model *F*-value and Probe>F was 49.21 and <0.0001, which also implied that this model was highly significant and there was only a 0.01%% chance that a "Model *F*-value" could occur to die due to noise, as shown in Table 8. The ANOVA also indicated that the model terms of X_1, X_2, and X_3 were highly significant and that of X_3^2 was significant for production of CMCase. The regression equation obtained from the ANOVA indicated that the multiple correlation coefficient of R^2 is 0.9787. The value of the adjusted determination coefficient (Adj. R^2=0.9588) is also very high to advocate for the high significance of this model [17]. From the statistical results obtained, it was shown that this model was adequate to predict the production of CMCase by *P. aquimaris* LBH-10 within the range of variables studied. Multiple regression analysis of the experimental data gave the following second-order polynomial Eq. (3) and the optimal conditions of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ for production of CMCase were 3.00, 0.52, 0.34, and 0.45 g/L, respectively. The maximum production of CMCase of 273.0 U/mL was predicted by this model.

$$Y_2 \text{ (CMCase)} = 264.26 - 7.00X_1 - 4.13X_2 + 1.86X_3 - 0.54X_4 - 0.06X_1X_2 + 0.06X_1X_3 - 0.06X_1X_4 - 0.06X_2X_3 + 0.06X_2X_4 - 0.06X_3X_4 + 0.35X_1^2 - 0.56X_2^2 - 0.90X_3^2 + 0.01X_4^2 \quad (3)$$

The significant factors on cell growth and production of CMCase based on data from orthogonal array method coincided with those from the response surface method. Moreover, two methods indicated that the optimal concentrations of four salts for cell growth were found to be different from those for production of CMCase. The optimal concentrations of salts in the medium for cell growth were different from those for the production of pullulan by *A. pullulan*. The optimal concentrations of salts for cell growth and production of pullulan varied with concentrations of carbon and nitrogen sources. Utilization rates of substrates were increased by the optimized concentration of salts, which results in enhanced cell growth and production of pullulan [18]. Two statistical methods also indicated that potassium phosphate was the most significant factor for cell growth and 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 [19,20]. Addition of potassium phosphate to the medium had a significant positive effect on both cell growth and the xanthan gum by *X. campestris* [21]. Sodium chloride was reported to be used as a physiological modulator of biosynthetic pathway of biopolymers [22]. Magnesium sulfate added to medium assisted with spore germination and initial growth of *A. fisheri*, which results in 1.9 fold increased production of xylanase [23]. Higher cell growth and production of key enzymes for the production of 1,3-propanediol were attained when ammonium chloride (NH_4Cl) was added in the medium [24]. Potassium phosphate seemed to act as a mineral salt for cell growth of *P. aquimaris* LBH-10 as well as a pH stabilizer in the medium, which can enhance the production of CMCase [25]. Ammonium sulfate used in this study seems to be mainly used as a nitrogen source for cell growth.

4. Production of CMCase with Optimized Concentrations of Salts

Batch fermentations for the production of CMCase by *P. aquimaris* LBH-10 were performed in a 7 bioreactor. Carbon and nitrogen sources and initial pH of the medium were 50.0 g/L rice bran, 3.0 g/L peptone, and 8.0. The concentrations of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ were 3.00, 0.52, 0.34, and 0.45 g/L, which were optimized for production of CMCase in this study. Working volumes of a 7 bioreactor were 5 L, and inoculum sizes of batch

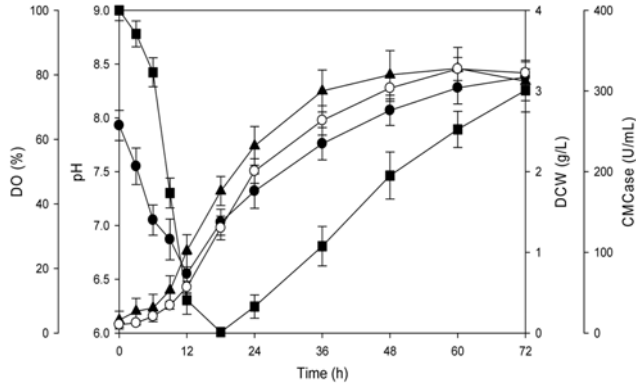


Fig. 3. Cell growth and production of CMCase by *P. aquimaris* LBH-10 in a 7 L bioreactor (●, pH; ■, DO; ▲, DCW; and ○, CMCase).

fermentations were 5% (v/v). Agitation speed and aeration rate were 300 rpm and 1.0 vvm. The pH in the medium rapidly decreased until 12 h after cultivation, and then increased until around 8.4, as shown in Fig. 3. The concentration of the dissolved oxygen in the medium dramatically decreased until 18 h, and the production of CMCase by *P. aquimaris* LBH-10 rapidly increased. Cell growth of *P. aquimaris* LBH-10 rapidly increased in the first 24 h of cultivation. The production of CMCase by *P. aquimaris* LBH-10 seemed to be parallel with cell growth of *P. aquimaris* LBH-10. The maximal production of CMCase in a 7 L bioreactor for 72 h under optimized conditions of salts was 322.4 U/mL.

The production of CMCase by *P. aquimaris* LBH-10 was enhanced by 1.27 times with optimized concentrations of salts in the medium using statistical methods. Moreover, time for production of CMCase was reduced using a bacterial strain with submerged fermentation, which resulted in increase in productivity of cellulases and decrease in their production cost. It normally takes more than seven days to produce fungal cellulases with solid-state fermentation [13]. The time for a batch culture of submerged fermentation for production of CMCase by this strain took about three days in this study.

CONCLUSION

The optimal concentrations of salts in the medium for cell growth and the production of CMCase by *P. aquimaris* LBH-10 were established using OAM and RSM and compared with previously used concentrations of salts, as shown in Table 9. The optimal concentrations of salts for cell growth and production of CMCase extracted by OAM basically coincided with those by RSM. However, each method gave its own detailed information on effects of salts on cell growth and the production of CMCase by *P. aquimaris* LBH-10. The number of combinations at an experiment can be minimized by reduced levels of factors, and the contribution percentage of each factor can be calculated using Qualitek-4 Software based on OAM, whereas the predicted values including the optimal concentrations can be extracted using Design Expert Software based on RSM.

ACKNOWLEDGEMENTS

This study was financially supported by the Dong-A University Research Fund.

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Table 9. Comparison of optimized concentrations of salts in the medium in this study and previous ones for cell growth and the production of CMCase by *P. aquimaris* LBH-10

Optimal condition	One-factor-at-a-time method		Orthogonal array method		Response surface method		Previous study	
	Cell growth	CMCase	Cell growth	CMCase	Cell growth	CMCase	Cell growth	CMCase
K_2HPO_4	7.5	2.5	10.0	2.5	7.10	3.00	5.0	5.0
NaCl (g/L)	1.0	0.5	1.0	0.5	0.84	0.52	1.0	1.0
$MgSO_4 \cdot 7H_2O$	0.3	0.4	0.2	0.4	0.24	0.34	0.2	0.2
$(NH_4)_2SO_4$	0.3	0.3	0.3	0.3	0.33	0.45	0.6	0.6
Maxi. production	2.19 g/L	282.5 U/mL	2.13 g/L	289.4 U/mL	2.03 g/L	273.0 U/mL	-	221.8 U/mL

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