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Enhanced production of carboxymethylcellulase of *Bacillus subtilis* subsp. *subtilis* A-53 by a recombinant *Escherichia coli* JM109/A-53 with pH and temperature shifts

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Abstract−Effect of shifts in pH of the medium and temperature on cell growth and production of carboxymethylcellulase (CMCase) by a recombinant *Escherichia coli* JM109/A-53 was investigated. Lower initial pH (pH 7.0) of the medium and higher temperature (40 °C) stimulated cell growth of *E. coli* JM109/A-53, whereas higher initial pH (pH 8.0) and lower temperature (35 °C) enhanced production of CMCase. The maximal production of CMCase was obtained when initial pH of 7.0 and temperature of 40 °C were simultaneously changed to 8.0 and 35 °C after 36 h. The production of CMCase with simple shifts in pH and temperature was 783.1 U/mL, which was 2.13 times higher than that under optimal conditions for cell growth. This indicated that a simple process with shifts in pH and temperature could be economically applied for enhanced production of CMCase by *E. coli* JM109/A-53 with industrial scales.

Keywords: Carboxymethylcellulase, *Escherichia coli* JM109, Optimization, Shifts in pH and Temperature

INTRODUCTION

Much effort has gone into the study of enzymes to hydrolysis of cellulosic biomass to obtain energy, chemicals, and single-cell proteins from an abundant and renewable resource, i.e., cellulosic biomass [1-3]. Enzymatic hydrolysis of cellulosic biomass required a complex reaction of three different types of enzymes: endoglucanase (carboxymethycellulase), exocellobiohydrolase (avicelase), and β-glucosidase [4,5]. The enzymatic saccharification of cellulosic materials such as rice hulls was performed by commercial cellulases, in which the major one was carboxymethylcellulase (CMCase) [6].

Many studies on types of strains, culture conditions, and substrates for production of cellulases have been reported [7-10]. Production of CMCase by wild and recombinant bacterial systems had been reported [4,11,12]. The optimal conditions for production of CMCase by *Bacillus amyloliquefaciens* DL-3 were different from those by its recombinant strain, *E. coli* JM109/DL-3 [4,13]. Moreover, their optimal conditions for cell growth were different from those for production of CMCase [8-10].

A gene encoding the CMCase of a marine bacterium, *B. subtilis* subsp. *subtilis* A-53, had been cloned in *E. coli* JM109 [14]. The optimal initial pH of medium and cultural temperature for cell growth and the production of CMCase by *E. coli* JM109/A-53 had been reported. Combination of two-stage controlled pH and temperature had been reported to enhance production of pullulan by *Aureobasidum pullulans* [15]. In this study, we investigated the effect of shifts in pH of the medium and temperature on cell growth and production of CMCase by *E. coli* JM109/A-53.

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MATERIALS AND METHODS

1. Bacterial Strain and Medium

A gene encoding the carboxymethylcellulase (CMCase) of a marine bacterium, *Bacillus subtilis* subsp. *subtilis* A-53, was cloned in *Escherichia coli* JM109 [11,14]. The full-length CMCase gene of *B. subtilis* subsp. *subtilis* A-53 was amplified by polymerase chain reaction (PCR) using two specific primers. Its 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 recombinant strain was named as *E. coli* JM109/A-53, which was used in this study. *E. coli* JM109/A-53 was grown at 37 °C in LB medium containing 100 μg/mL ampicillin.

2. Production of CMCase by *E. coli* JM109/A-53

Starter cultures were prepared by transferring cells from agar slants to 50 mL of the same medium except for agar in 250 mL Erlenmeyer flasks. The resulting cultures were incubated at 30 °C for two days under aerobic conditions. Each starter culture was used as an inoculum for 200 mL of medium in 500 mL Erlenmeyer flasks. The main culture for production of CMCase by *E. coli* JM109/A-53 was carried out in the medium containing 125.0 g/L CMC, 7.5 g/L ammonium chloride, 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 three days under aerobic conditions. Samples were periodically withdrawn from the cultures to examine cell growth and the production of CMCase.

Batch fermentations for the production of CMCase by *E. coli* JM109/A-53 were performed in 7 L bioreactors (Ko-Biotech Co., Korea). Working volumes of the 7 L bioreactors were 5 L, and inoculum size of batch was 5% (v/v). Agitation was provided by three six-flat-blade impellers in a 7 L bioreactor. Agitation speed and aeration rate were 400 rpm and 0.5 vvm [10].

3. Cultural Conditions for Shifts in pH and Temperature

Cultural conditions to investigate the effect of a shift in pH were 1) initial pH of 7.0 without shift in pH, 2) shift in initial pH of 7.0 to 8.0 after 12 h, 3) shift in initial pH of 7.0 to 8.0 after 24 h, 4) shift in initial pH of 7.0 to 8.0 after 36 h, and 5) initial pH of 8.0 without shift in pH. Cultural conditions to investigate the effect of shift in temperature were 1) temperature of 40 °C without shift in temperature, 2) shift in temperature from 40 °C to 35 °C after 12 h, 3) shift in temperature from 40 °C to 35 °C after 24 h, 4) shift in temperature from 40 °C to 35 °C after 36 h, and 5) initial temperature of 40 °C without shift in temperature. Cultural conditions to investigate the effect of shifts in pH and temperature were 1) initial pH of 7.0 and temperature of 40 °C without shifts, 2) shifts in initial pH of 7.0 to 8.0 and temperature from 40 to 35 °C after 12 h, 3) shifts in initial pH of 7.0 to 8.0 and temperature from 40 to 35 °C after 24 h, 4) shifts in initial pH of 7.0 to 8.0 and temperature from 40 to 35 °C after 36 h, and 5) initial pH of 8.0 and temperature of 35 °C without shifts.

4. Analytical Methods

Dry cells weight (DCW) was measured as described in the previous report [16]. Activities of the CMCase produced by *E. coli* JM109/A-53 were determined based on the release of reducing sugar from CMC using the 3,5-dinitrosalicylic acid (DNS) method, as described in the previous report [11]. Protein concentration was determined using the Bio-Rad protein assay kit according to the manufacturer's instructions with bovine serum albumin as the protein standard for the calibration curve [17]. Glucose (Sigma-Aldrich, UK) was used to prepare a calibration curve. One unit of each CMCase was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to glucose per minute under the assay condition.

RESUTS AND DISCUSSION

1. Effect of Shift in pH on Production of CMCase

Effect of shift in pH of the medium on cell growth of *E. coli* JM109/A-53 and production of CMCase was investigated in bioreactors. Carbon and nitrogen source for production of CMCase were 125.0 g/L rice bran and 7.5 g/L ammonium chloride. Cultural temperature was 35 °C, which had been optimized for the production of CMCase in the previous study [14]. Maximal cell growth was obtained at the initial pH of 7.0 without shift in pH, as shown in Fig. 1. However, maximal production of CMCase was obtained at shift in initial pH of 7.0 to 8.0 after 36 h. Production of CMCase with shift in initial pH of 7.0 to 8.0 after 36 h was 720.1 U/mL, which was 1.19 times higher than that without shift in initial pH of 8.0, which had been known to be the optimal pH for production of CMCase by *E. coli* JM109/A-53 [11]. The optimal initial pH of the medium for cell growth of *B. subtilis* subsp. *subtilis* A-53 and *E. coli* JM109/A-53 were 7.3 and 7.0, respectively, whereas those for production of CMCase were 6.8 and 8.0 [10,14]. The shift in pH from 7.0 to 8.0 after 36 h resulted in relatively higher cell growth of *E. coli* JM109/A-53, which seemed to enhance higher production of CMCase due to more cells to participate in production of CMCase.

2. Effect of Shift in Temperature on Production of CMCase

Effect of shift in temperature on cell growth of *E. coli* JM109/A-53 and production of CMCase was also investigated in bioreactors. Initial pH of the medium was 8.0, which had been also optimized for production of CMCase in the previous study [14]. Maximal cell growth was obtained at the culture temperature of 40 °C without shift in temperature, as shown in Fig. 2. However, maximal production of CMCase was obtained at shift temperature from 40 °C to 35 °C after 36 h. Production of CMCase with shift in temperature from 40 °C to 35 °C after 36 h was 743.1 U/mL, which was 1.26 times higher than that without shift in temperature at 35 °C, which had been also known to be the optimal temperature for production of CMCase. The optimal initial pH of the medium for cell growth of *B. subtilis* subsp. *subtilis* A-53 and *E. coli* JM109/A-53 was 35 and 40 °C, respectively, whereas that for production of CMCase was 30 and 35 °C [10,14]. The shift in temperature from 40 °C to 35 °C after 36 h also resulted in relatively higher cell growth. The shifts in temperature from the optimal condition for cell growth to that for production of CMCase seemed to enhance higher production of CMCase.

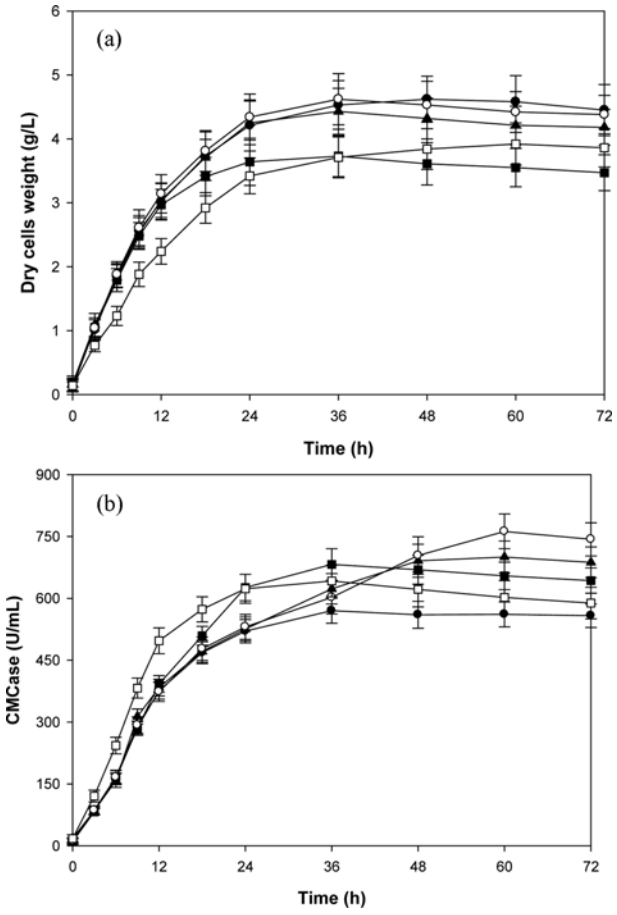

Fig. 1. Effect of shift in pH on cell growth (a) and production of CMCase (b) by *E. coli* JM109/A-53 (●, constant at pH 7.0; ■, shift in initial pH of 7.0 to 8.0 after 12 h; ▲, shift in initial pH of 7.0 to 8.0 after 24 h; ○, shift in initial pH of 7.0 to 8.0 after 36 h; and □, constant at 8.0).

3. Effect of Shifts in pH and Temperature on Production of CMCase

Effect of shifts in pH of the medium and temperature on cell growth of *E. coli* JM109/A-53 and production of CMCase was inves-

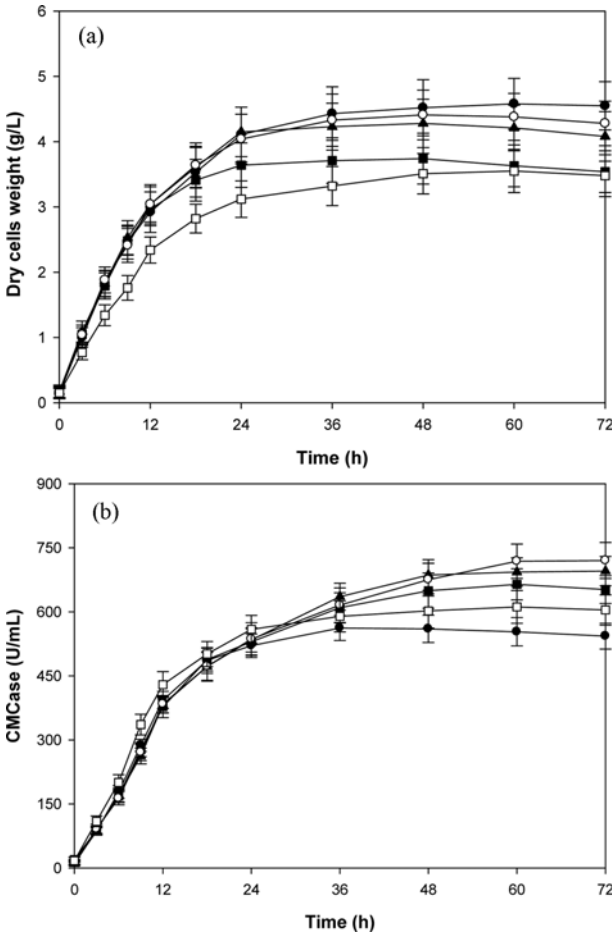

Fig. 2. Effect of shift in temperature on cell growth (a) and production of CMCase (b) by *E. coli* JM109/A-53 (●, constant at 40 °C; ■, shift in temperature from 40 to 35 °C after 12 h; ▲, shift in temperature from 40 to 35 °C after 24 h; ○, shift in temperature from 40 to 35 °C after 36 h; and □, constant at 35 °C).

tigated in 7 L bioreactors based on results from studies on effects of shifts in pH and temperature. Maximal cell growth was obtained at initial pH of 7.0 and temperature of 40 °C without shifts, as shown in Fig. 3. However, maximal production of CMCase was obtained at shifts in initial pH of 7.0 to 8.0 and temperature from 40 to 35 °C after 36 h. The maximal production of CMCase by *E. coli* JM109/A-53 with shifts in pH and temperature was 783.1 U/mL, which was 2.13 times higher than that under optimal conditions for cell growth, as shown in Table 1. Moreover, its production of CMCase was 1.27 times higher than those under optimal conditions for production of without shift. The optimal initial pH of medium and temperature for cell growth of *E. coli* JM109/A-53 were reported to be 7.0 and 40 °C, whereas those for production of CMCase were 8.0 and 35 °C.

Due to difference in optimal conditions for cell growth and production of CMCase, lower cell growth seemed to cause higher production of CMCase. However, more cells due to later shifts in pH and temperature for optimal conditions from cell growth to those for production of CMCase, could produce higher production of

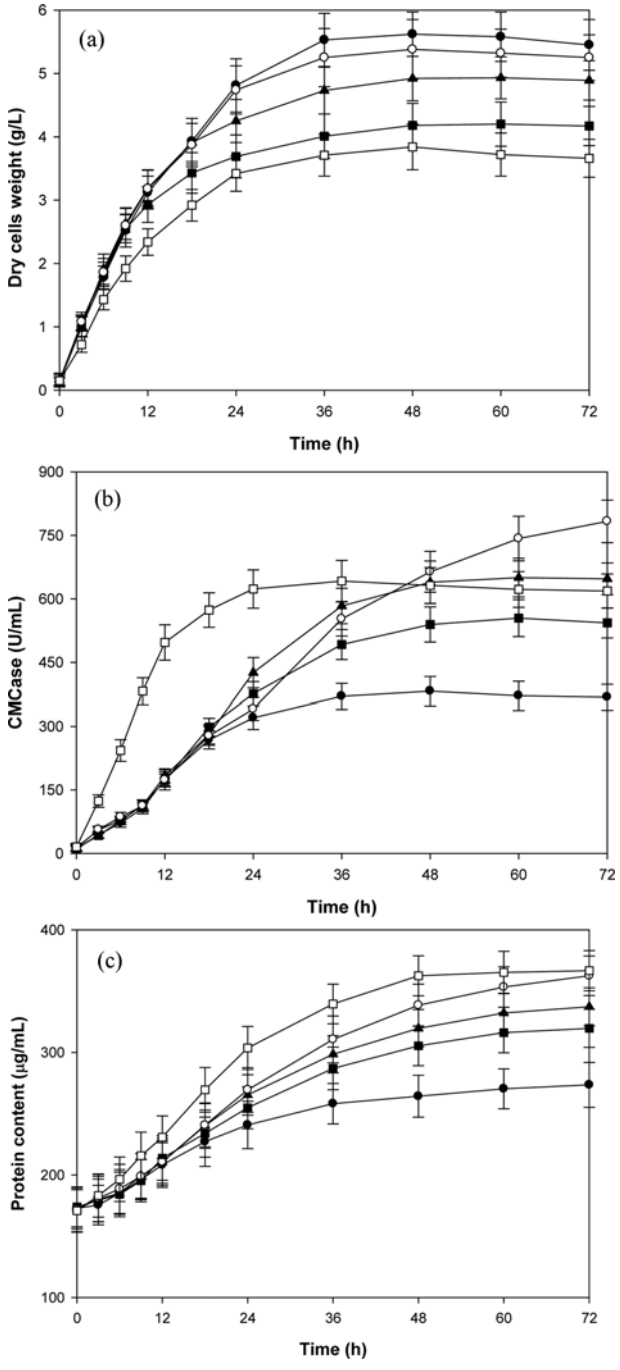

Fig. 3. Effect of shift in pH and temperature on cell growth (a), production of CMCase (b), and protein content (c) by *E. coli* JM109/A-53 (●, constant at pH 7.0 and 40 °C; ■, shift in initial pH of 7.0 to 8.0 and temperature from 40 to 35 °C after 12 h; ▲, shift in initial pH of 7.0 to 8.0 and temperature from 40 to 35 °C after 24 h; ○, shift in initial pH of 7.0 to 8.0 and temperature from 40 to 35 °C after 36 h; and □, constant at 35 °C and pH 8.0).

CMCase. Later shifts in pH and temperature also resulted in relatively higher total protein content, which was the similar tendency to production of CMCase. Shifts in optimal conditions from cell growth to those for production of CMCase after early stationary

Table 1. Effect of shifts in pH and temperature on cell growth and production of CMCase by *E. coli* JM109/A-53

	Conditions			DCW (g/L)	CMCase (U/mL)
	pH	Temperature (°C)	Time (h)		
Shift in pH	7.0	35	-	4.55	513.1
	7.0→8.0	35	12	3.54	652.2
	7.0→8.0	35	24	4.08	695.4
	7.0→8.0	35	36	4.28	720.1
	8.0	35	-	3.48	604.4
Shift in temperature	8.0	40	-	4.45	517.2
	8.0	40→35	12	3.47	643.1
	8.0	40→35	24	4.18	687.3
	8.0	40→35	36	4.38	743.1
	8.0	35	-	3.86	588.5
Shifts in pH and temperature	7.0	40	-	5.45	367.9
	7.0→8.0	40→35	12	4.17	543.1
	7.0→8.0	40→35	24	4.89	647.3
	7.0→8.0	40→35	36	5.25	783.1
	8.0	35	-	3.66	618.5

phase seemed to result in relatively higher cell growth and sequentially enhanced production of CMCase by *E. coli* JM109/A-53.

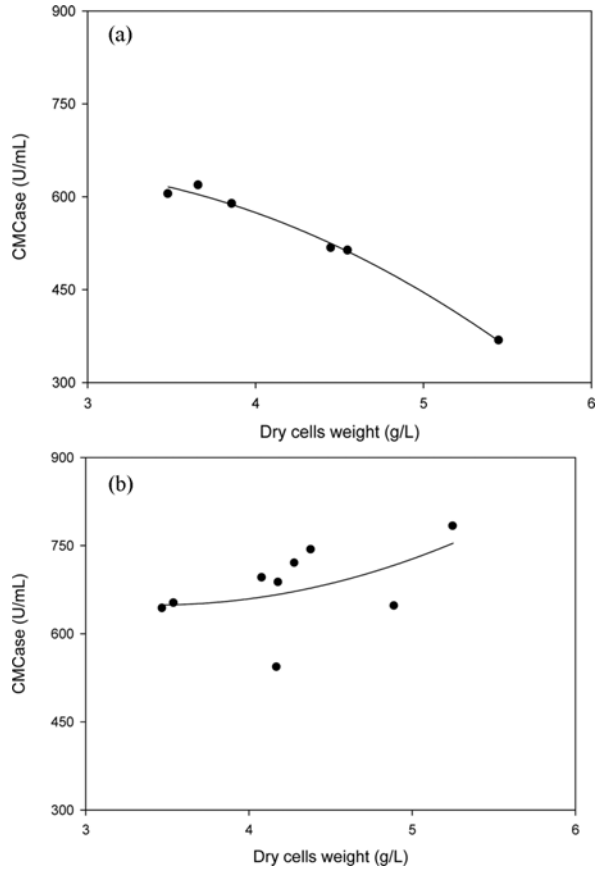

Fig. 4. Relationship between cell growth and production of CMCase by *E. coli* JM109/A-53 under fixed initial pH and temperature (a) and shifts in initial pH and temperature (b).

4. Relationship Between Cells Growth and its Production of CMCase

The relationship between dry cell growth and production of CMCase by *E. coli* JM109/A-53 was investigated using the statistical methodology (SigmaPlot for Windows Version 10.0, Systat Software Inc., San Jose, USA). As shown in Fig. 4(a), nonlinear regression analysis of the experimental data gave the following second-order polynomial equation to represent the relationship between dry cell weight (X) and production of CMCase (Y) obtained under the fixed initial pH of medium and cultural temperature (Eq. (1)):

$$Y=446.8++160.4X-32.1X^2 \quad (1)$$

The regression equation indicated that the multiple correlation coefficient of R^2 was 0.9904, which could explain 99.04% variation in the response. The value of the adjusted determination coefficient (Adj. R^2=0.9841) was very high to advocate for a high significance of this model [18]. From the statistical results obtained, it was shown that the above equation was adequate to predict production of CMCase by *E. coli* JM109/A-53 within the range of dry cells weight obtained under the fixed initial pH of medium and cultural temperature. However, the multiple correlation coefficient of R^2 from nonlinear regression analysis of dry cells weight and production of CMCase obtained under the shifts in initial pH of medium and cultural temperature was less than 0.5000, as shown in Fig. 4(b). A higher value indicates a better predictability of the dependent variable from the independent variables, which meant that production of CMCase could not be predicted by dry cells wegith obtained under under shifts in initial pH and temperature.

CONCLUSION

The optimal conditions for cell growth were different from those for production of CMCase by *E. coli* JM109/A-53. Multi-stage continuous culture was used for production of fuel ethanol to over-

come low productivity due to difference in optimal conditions for cell growth and production [19]. However, their major disadvantages are high cost for equipment and risk for contamination during cultivation [20]. A process with simple shifts in pH and temperature to enhance the production of CMCase by *E. coli* JM109/A-53 was developed in this study, which could be economically applied for enhanced production of CMCase with industrial scale.

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REFERENCES

1. R. K. Sukumaran, R. R. Singhania, G. M, Mathew and A. Pandey, *Renew. Energy*, **34**, 421 (2009).
2. G. Y. Wei, W. Gao, I. H. Jin, S. Y. Yoo, J. H. Lee, C. H. Chung and J. W. Lee, *Biotechnol. Bioprocess Eng.*, **14**, 828 (2009).
3. K. Y. Won, Y. S. Kim and K. K. Oh, *Korean J. Chem. Eng.*, **29**, 1341 (2012).
4. Y. J. Lee, H. J. Kim, W. Gao, C. H. Chung and J. W. Lee, *Biotechnol. Bioprocess Eng.*, **17**, 227 (2012).
5. H. Li, L. Yang, Y. Kim and S. J. Kim, *Korean J. Chem. Eng.*, **28**, 1085 (2011).
6. G. Y. Wei, Y. J. Lee, Y. J. Kim, I. H. Jin, J. H. Lee, C. H. Chung and J. W. Lee, *Appl. Biochem. Biotechnol.*, **162**, 1471 (2010).
7. W. Gao, E. J. Lee, S. U. Lee, J. Li, C. H. Chung and J. W. Lee, *J. Microbiol. Biotechnol.*, **22**, 1415 (2012).
8. H. J. Kim, Y. J. Lee, W. Gao, C. H. Chung, C. W. Son and J. W. Lee, *Biotechnol. Bioprocess Eng.*, **16**, 542 (2011).
9. H. J. Kim, Y. J. Lee, W. Gao, C. H. Chung and J. W. Lee, *Korean J. Chem. Eng.*, **29**, 384 (2012).
10. B. H. Lee, B. K. Kim, Y. J. Lee, C. H. Chung and J. W. Lee, *Enzyme Microb. Technol.*, **46**, 38 (2010).
11. B. K. Kim, B. H. Lee, Y. J. Lee, I. H. Jin, C. H. Chung and J. W. Lee, *Enzyme Microb. Technol.*, **44**, 411 (2009).
12. Y. J. Lee, B. K. Kim, B. H. Lee, K. I. Jo, N. K. Lee, C. H. Chung, Y. C. Lee and J. W. Lee, *Bioresour. Technol.*, **99**, 378 (2008).
13. K. I. Jo, Y. J. Lee, B. K. Kim, B. H. Lee, C. H. Chung, S. W. Nam, S. K. Kim and J. W. Lee, *Biotechnol. Bioprocess Eng.*, **13**, 182 (2008).
14. E. J. Lee, B. H. Lee, B. K. Kim, C. H. Chung and J. W. Lee, *Mol. Biol. Rep.*, **40**, 3609 (2013).
15. Z. Xia, S. Wu and S. Pan, *Carbohydr. Polym.*, **86**, 1814 (2011).
16. Y. J. Kim, W. Cao, S. U. Lee and J. W. Lee, *J. Life Sci.*, **22**, 1295 (2012).
17. H. J. Kim, W. Gao, C. H. Chung and J. W. Lee, *J. Life Sci.*, **21**, 1083 (2011).
18. A. Fernández, M. J. Ocio, P. S. Fernández, M. Rodrigo and A. Martinez, *Food Microbiol.*, **16**, 607 (1999).
19. S. Govindaswamy and L. M. Vane, *Bioresour. Technol.*, **101**, 1277 (2010).
20. H. W. Yen and J. T. Chang, *Bioprocess Biosyst. Eng.*, **36**, 193 (2013).