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Application of statistical experimental design for optimization of physiological factors and their influences on production of pullulan by *Aureobasidium pullulans* HP-2001 using an orthogonal array method

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Abstract−Physiological factors for the production of pullulan by *A. pullulans* HP-2001 were optimized using orthogonal array method and their influences were compared using Qualitek-4 software. The analysis of variance (ANOVA) indicated that the most important factor for cell growth was yeast extract, whereas that for production of pullulan was glucose. The optimal conditions for cell growth were found to be 100.0 g/L glucose, 10.0 g/L yeast extract, and initial pH of 6.0, whereas those for the production of pullulan were 100.0 g/L glucose, 2.5 g/L yeast extract, and initial pH of 5.5. Among four mineral salts in the medium, potassium phosphate (K_2HPO_4) was found to be the most important factor for cell growth as well as production of pullulan. Next important salt for cell growth was $(NH_4)_2SO_4$, whereas that for production of pullulan was NaCl. The optimal concentrations of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ for cell growth were 7.5, 1.00, 0.1, and 1.20 g/L, respectively, whereas those for production of pullulan were 2.5, 0.25, 0.8, and 0.30 g/L. The expected cell growth and the production of pullulan by *A. pullulans* HP-2001 under these optimized conditions were 12.61 and 11.49 g/L, respectively.

Key words: Pullulan, *Aureobasidium pullulans*, Optimization, Orthogonal Array Method

INTRODUCTION

Pullulan is a water-soluble microbial polysaccharide that can be produced in large quantities by fermentation [1]. Pullulan is a linear extracellular homopolysaccharide consisting of maltotriose and maltotetraose units interconnected by α-(1→6) and α-(1→4) linkages, which results in two characteristic properties of structural flexibility and enhanced solubility [2]. Consequently, pullulan provides highly viscous solutions at relatively low concentrations and can be utilized to make an oxygen-impermeable membrane [3]. Pullulan can be used as a coating and packing material, a sizing agent for paper, a starch replacer in low-calorie food formulation, cosmetic emulsions, and other industrial and medicinal applications [1].

Some important factors that affect production of pullulan are carbon and nitrogen sources [4,5], mineral salts in the medium [6], the initial pH of medium, temperature, and the oxygen supply [7]. Molecular weights of pullulan can also vary with carbon and nitrogen sources [8], initial pH of the medium [9], and oxygen supply [10]. Many studies on physiological factors for production of pullulan have been reported [11]. However, the comparative analysis and relative influence of each factor on cell growth and production of pullulan has not been reported.

The optimization of conditions for production of pullulan by classical methods involving the change of one variable at a time is extremely time-consuming and expensive when a large number of variables are considered [12]. The purpose of this study was to optimize major factors - carbon and nitrogen source and initial pH of the medium - as well as four salts in the medium - K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ - and their influences on cell growth and the production of pullulan by *A. pullulans* HP-2001 using an orthogonal array method.

MATERIALS AND METHODS

1. Bacterial Strain and Medium

Aureobasidium pullulans HP-2001 used in this study is a UV-induced mutant of *A. pullulans* ATCC 42023, which was isolated in the previous study [5]. The medium for the production of pullulan by *A. pullulan* HP-2001 contained 50 g/L glucose, 0.25 g/L yeast extract, 2.5 g/L K_2HPO_4, 0.25 g/L NaCl, 0.8 g/L $MgSO_4 \cdot 7H_2O$, and 0.3 g/L $(NH_4)_2SO_4$ [6]. Glucose was autoclaved separately and added to the medium in aseptic conditions.

2. Production of Pullulan

Starter cultures were prepared as described in the previous report [5]. Inoculated cultures were incubated for three days at 30 °C under aerobic conditions. Samples were periodically withdrawn from the cultures to examine cell growth and the production of pullulan by *A. pullulans* HP-2001. The culture broth after three days cultivation was centrifuged at 15,000 ×g for 15 min to remove cells. The supernatant was mixed with 2 vol of isopropyl alcohol and incubated at 4 °C for 24 h to precipitate the crude product, which was separated by centrifugation at 15,000 ×*g* for 20 min. The precipitated material was repeatedly washed with acetone and ether, dis-

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solved in deionized water (DW) and dialyzed against DW by using dialysis tubing with a molecular weight cut off at 14,000-12,000 Da. After dialysis for two to three days with four or five changes of DW, the solution was lyophilized.

3. Optimization for Production of Pullulan by *A. pullulans* HP-2001

Design of experiments (DOE) was performed based on Taguchi method for investigating effects of different variables containing carbon and nitrogen sources and initial pH of the medium on the production of pullulan by *A. pullulans* HP-2001. Qualitek-4 (W32b) software (Nutek, Inc., USA) was used for automatic design of experiments, analysis of results, and calculation of interactions among different factors [13]. The $L_{16}(4^3)$ orthogonal array experiment in this study had three factors - glucose, yeast extract, and initial pH of the medium - and each factor had four different levels. The $L_{16}(4^4)$ orthogonal array experiment had four factors - K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$. These trials were done in three replicates. Results from optimization of physiological factors for production of pullulan were statistically analyzed using one-way analysis of variance (ANOVA).

4. Analytical Methods

Dry cell weight and concentration of pullulan was measured as described in the previous report [14]. A standard curve for quantitation of pullulan was prepared from the authentic pullulan (Sigma-Aldrich, St. Louis, USA).

RESULTS AND DISCUSSION

1. Effect of Glucose, Yeast Extract, and Initial pH on Production of Pullulan

The simultaneous effect of glucose, yeast extract, and initial pH of the medium on cell growth and the production of pullulan by *A. pullulans* HP-2001 was investigated using the $L_{16}(4^3)$ orthogonal array method. Factors and levels of each factor are shown in Table 1. Cell growth, measured as dry cells weight (DCW), and production of pullulan from sixteen different conditions ranged from 9.13 to 12.23 g/L and from 7.10 to 12.17 g/L, respectively, as shown in Table 2. The last column in the analysis of variance (ANOVA) for the design of cell growth indicated the influence of each factor on cell growth of *A. pullulans* HP-2001, as shown in Table 3. Based on calculated percent of participation (P), the most important factor for cell growth was found to be yeast extract. This factor gave maximum sum of squares (S) and maximum percentage influence (58.90%) followed by glucose (27.64%), and initial pH of the medium (13.45%), as shown in Fig. 1(a). Explained variance of cell growth was calculated as 100.00% based on sums of squares. The ANOVA for the

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

Factor	Level 1	Level 2	Level 3	Level 4
Glucose (g/L)	25.0	50.0	75.0	100.0
Yeast extract (g/L)	1.0	2.5	5.0	10.0
Initial pH	5.5	6.0	6.5	7.0

Table 2. Simultaneous effect of glucose, yeast extract, and initial pH of the medium on cell growth and the production of pullulan by *A. pullulans* HP-2001 designed using Qualitexk-4 (W32b) software based on $L_{16}(4^3)$ orthogonal array experiment

Run	Glucose (g/L)	Yeast extract (g/L)	Initial pH	DCW (g/L)	Pullulan (g/L)
1	25	1.0	5.5	9.13	8.70
2	25	2.5	6.0	9.90	10.10
3	25	5.0	6.5	10.10	8.73
4	25	10.0	7.0	10.26	7.10
5	50	1.0	6.0	9.87	8.83
6	50	2.5	5.5	10.30	11.60
7	50	5.0	7.0	10.43	10.13
8	50	10.0	6.5	11.07	8.97
9	75	1.0	6.5	9.83	10.67
10	75	2.5	7.0	10.20	11.70
11	75	5.0	5.5	11.67	11.50
12	75	10.0	6.0	12.23	10.23
13	100	1.0	7.0	9.63	10.67
14	100	2.5	6.5	10.47	12.17
15	100	5.0	6.0	11.87	11.87
16	100	10.0	5.5	12.10	10.33

Table 3. Analysis of variance (ANOVA) of cell growth and the production of pullulan by *A. pullulans* HP-2001 analyzed using Qualitek-4 (W32b) software based on $L_{16}(4^3)$ orthogonal array experiment

	Factor	DOF (f)	Sums of squares (S)	Variance (V)	F-ratio (F)	Pure sum (S′)	Percent of participation (P, %)
DCW	Glucose	3	3.67	1.22	46717.48	3.66	27.64
	Yeast extract	3	7.80	2.60	99521.13	7.80	58.90
	Initial pH	3	1.78	0.59	22729.05	1.78	13.45
	Other/error	6	0.00	0.00	-	-	0.01
	Total	15	13.25	-	-	-	100.00
Pullulan	Glucose	3	17.14	5.71	33.97	16.63	54.61
	Yeast extract	3	11.48	3.83	22.75	10.97	36.03
	Initial pH	3	0.83	0.28	1.65	0.33	1.07
	Other/error	6	1.01	0.17	-	-	8.28
	Total	15	30.45	-	-	-	100.00

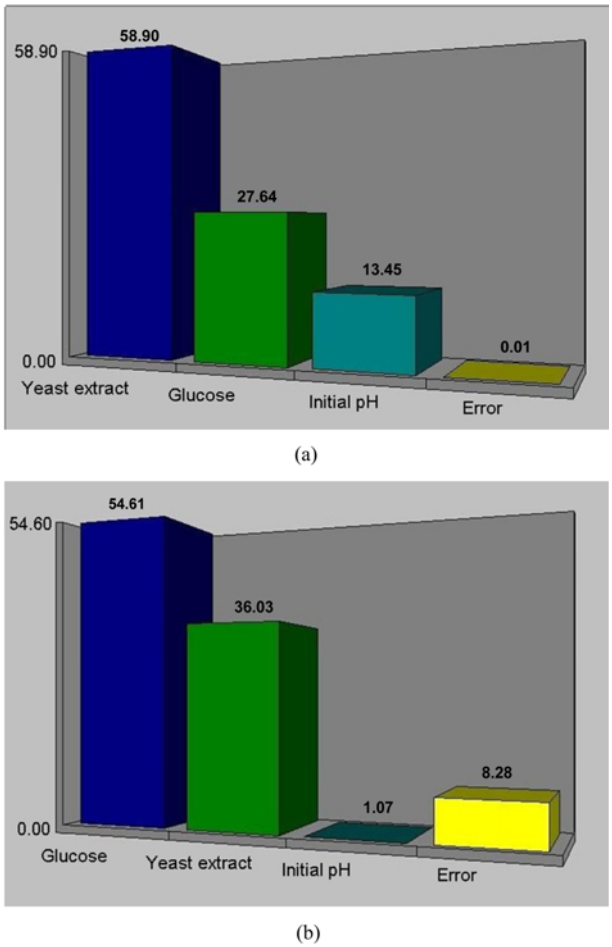


Fig. 1. The percentage contributions of glucose, yeast extract, and initial pH for cell growth (a) and the production of pullulans by *A. pullulans* HP-2001 (b) analyzed using Qualitek-4 (W32b) software. X-axis shows factors and Y-axis shows percentage contributions of each factor.

design of production of pullulan also indicated that the most important factor for production of pullulan was glucose. Glucose gave maximum percentage influence (54.61%) followed by yeast extract (36.03%), whereas initial pH of the medium had no significant effect, as shown in Fig. 1(b). Explained variance of pullulan produced by *A. pullulans* HP-2001 was also calculated as 96.71% based on sums of squares. The optimal conditions for cell growth were found to be 100.0 g/L glucose, 10.0 g/L yeast extract, and initial pH of 6.0, whereas those for production of pullulan were 100.0 g/L glucose, 2.5 g/L yeast extract, and initial pH of 5.5, as shown in Table 4. The expected cell growth and the production of pullulan by *A. pullulans* HP-2001 under these optimized conditions were 12.27 and 12.77 g/L, respectively.

The optimal conditions for cell growth of *A. pullulans* HP-2001 were different from those for production of pullulan as described in the previous report [7]. Moreover, relative influences of glucose, yeast extract, and initial pH of the medium on cell growth were found to be different from those on production of pullulan in this study. The fungus, *Aureobasidium pullulans*, can grow in various morphological forms including blastospore (yeast-like cells), hyphae, psuedohyphase, swollen cells, and chlamydospores [15]. The morphological content of *A. pullulans* is influenced by physiological factors such as carbon and nitrogen sources, pH of the medium, temperature, and dissolved oxygen level [16,17]. High production of pullulan has been found to correlate with high concentration of yeast-like cells in the culture [18]. With sufficient yeast extract added, the morphological form of *A. pullualns* was filamentous, which form could continue to grow [19]. It might be the reason why yeast extract was the most important factor for cell growth of *A. pullulans* HP-2001.

Production of pullulan was the highest when *A. pullulans* was grown in a medium containing glucose [20]. Monomeric carbon for biosynthesis of pullulan was known to be UDP-glucose, which was formed from UTP and glucose-1-phosphate by UDP-glucose pyrophosphorylase [2]. In certain ranges of C/N ratios, production of bacterial exopolymers increased with increased C/N ratio, whereas cell growth decreased [21]. Relative higher ratio of glucose to yeast extract in the medium seems to lead the morphological form of *A. pullualns* HP-2001 to yeast-like cells, which resulted in higher production of pullulan.

2. Effect of Mineral Salts in Medium on Production of Pullulan

The simultaneous effect of mineral salts in the medium on cell growth and the production of pullulan by *A. pullulans* HP-2001 was investigated using the L_{16} (4^4) orthogonal array method. Factors of four salts and levels of each factor were shown in Table 5. Carbon and nitrogen sources and initial pH of the medium were 50.0 g/L glucose, 2.5 g/L yeast extract, and 5.5 based on maximal production of pullulan as well as its conversion rate from glucose. Cell growth and production of pullulan from sixteen different conditions ranged from 11.89 to 12.50 g/L and from 9.06 to 11.28 g/L, respectively, as shown in Table 6. The last column in the ANOVA for the design of cell growth indicated that the most important factor for

Table 4. Optimal conditions for cell growth and production of pullulan by *A. pullulans* HP-2001 analyzed using Qualitek-4 (W32b) software based on L_{16} (4^3) orthogonal array experiment

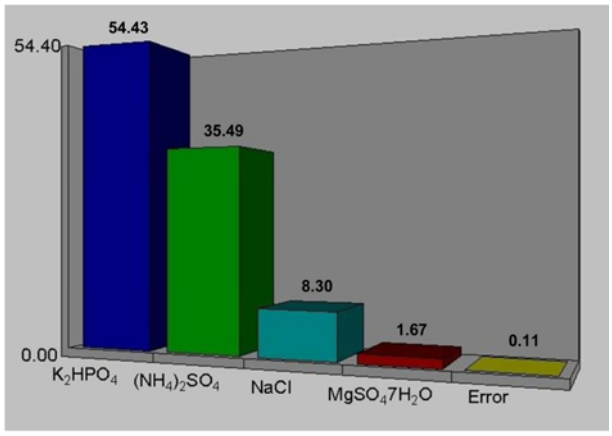
Factor	DCW			Pullulan		
	Optimized condition	Level	Contribution	Optimized condition	Level	Contribution
Glucose	100 g/L	4	0.45	100.0 g/L	4	1.05
Yeast extract	10.0 g/L	4	0.85	2.5 g/L	2	1.19
Initial pH	6.0	2	0.40	5.5	1	0.33
Total contribution		-	1.70	-	-	2.56
Current yield		-	10.57 g/L	-	-	10.21 g/L
Expected yield		-	12.27 g/L	-	-	12.77 g/L

Table 5. Factors and their level in the orthogonal array experiment based on Taguchi design using Qualitek-4 (W32b) software

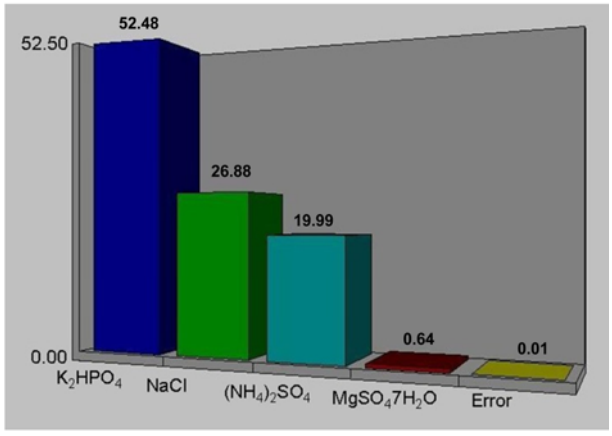
Factor	Level 1	Level 2	Level 3	Level 4
K_2HPO_4 (g/L)	1.0	2.5	5.0	7.5
NaCl (g/L)	0.10	0.25	0.50	1.00
$MgSO_4 \cdot 7H_2O$ (g/L)	0.1	0.2	0.4	0.8
$(NH_4)_2SO_4$ (g/L)	0.15	0.30	0.60	1.20

Table 6. Simultaneous effect of mineral salts in the medium on cell growth and the production of pullulan by *A. pullulans* HP-2001 designed using Qualitexk-4 (W32b) software based on L_{16} (4^4) orthogonal array experiment

	K_2HPO_4 (g/L)	NaCl (g/L)	$MgSO_4 \cdot 7H_2O$ (g/L)	$(NH_4)_2SO_4$ (g/L)	DCW (g/L)	Pullulan (g/L)
1	1.0	0.10	0.1	0.15	11.90	9.06
2	1.0	0.25	0.2	0.30	11.89	10.28
3	1.0	0.50	0.4	0.60	11.94	9.69
4	1.0	1.00	0.8	1.20	12.22	9.57
5	2.5	0.10	0.2	0.60	12.03	10.01
6	2.5	0.25	0.1	1.20	12.29	10.64
7	2.5	0.50	0.8	0.15	12.02	11.04
8	2.5	1.00	0.4	0.30	12.11	11.39
9	5.0	0.10	0.4	1.20	12.33	9.91
10	5.0	0.25	0.8	0.60	12.14	10.84
11	5.0	0.50	0.1	0.30	12.20	11.28
12	5.0	1.00	0.2	0.15	12.23	10.87
13	7.5	0.10	0.8	0.30	12.19	10.26
14	7.5	0.25	0.4	0.15	12.20	10.60
15	7.5	0.50	0.2	1.20	12.50	10.17
16	7.5	1.00	0.1	0.60	12.40	10.23



(a)



(b)

Fig. 2. The percentage contributions of salts in the medium for cell growth (a) and the production of pullulans by *A. pullulans* HP-2001 (b) analyzed using Qualitek-4 (W32b) software.

cell growth was potassium phosphate (K_2HPO_4), as shown in Table 7. This factor gave maximum sum of squares (S) and maximum percentage influence (54.43%) followed by $(NH_4)_2SO_4$ (35.49%), NaCl (8.30%), and $MgSO_4 \cdot 7H_2O$ (1.67%), as shown in Fig. 2(a). The ANOVA for the design of production of pullulan indicated that the most important factor for production of pullulan was also K_2HPO_4.

Table 7. Analysis of variance (ANOVA) for cell growth and the production of pullulan by *A. pullulans* HP-2001 analyzed using Qualitek-4 (W32b) software based on L_{16} (4^4) orthogonal array experiment

	Factor	DOF (f)	Sums of squares (S)	Variance (V)	F-ratio (F)	Pure sum (S′)	Percent of participation (P, %)
DCW	K_2HPO_4	3	0.25	0.08	2506.58	0.25	54.43
	NaCl	3	0.04	0.01	383.15	0.04	8.30
	$MgSO_4 \cdot 7H_2O$	3	0.01	0.00	77.65	0.01	1.67
	$(NH_4)_2SO_4$	3	0.16	0.05	1634.58	0.16	35.49
	Other/error	3	0.00	0.00	-	-	0.11
	Total	15	0.46	-	-	-	100.00
Pullulan	K_2HPO_4	3	3.23	1.08	32294.02	3.23	52.48
	NaCl	3	1.65	0.55	16541.23	1.65	26.88
	$MgSO_4 \cdot 7H_2O$	3	0.04	0.01	397.00	0.04	0.64
	$(NH_4)_2SO_4$	3	1.23	0.41	12298.98	1.23	19.99
	Other/error	3	0.00	0.00	-	-	0.01
	Total	15	6.15	-	-	-	100.00

Table 8. Optimal conditions for cell growth and production of pullulan by *A. pullulans* HP-2001 analyzed using Qualitek-4 (W32b) software based on L_{16} (4^4) orthogonal array experiment

Factor	DCW			Pullulan		
	Optimized condition	Level	Contribution	Optimized condition	Level	Contribution
K_2HPO_4	7.5 g/L	4	0.16	2.5 g/L	2	0.41
NaCl	1.00 g/L	4	0.08	0.25 g/L	2	0.23
$MgSO_4 \cdot 7H_2O$	0.1 g/L	1	0.04	0.8 g/L	4	0.07
$(NH_4)_2SO_4$	1.20 g/L	4	0.17	0.30 g/L	2	0.44
Total contribution	-	-	0.45	-	-	1.13
Current yield	-	-	12.16	-	-	10.36
Expected yield	-	-	12.61	-	-	11.49

It gave maximum percentage influence (52.48%) followed by NaCl (26.88%) and $(NH_4)_2SO_4$ (19.99%), whereas $MgSO_4 \cdot 7H_2O$ in the medium had no significant effect, as shown in Fig. 2(b). Explained variances of cell growth and production of pullulan were calculated as 100.00% based on sums of squares. The optimal concentrations of K_2HPO_4, NaCl, $MgSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ for cell growth were 7.5, 1.00, 0.1, and 1.20 g/L, respectively, whereas those for production of pullulan were 2.5, 0.25, 0.8, and 0.30 g/L, as shown in Table 8. The expected cell growth and the production of pullulan by *A. pullulans* HP-2001 under these optimized conditions were 12.61 and 11.49 g/L, respectively.

The optimal concentrations of mineral salts in the medium for cell growth of *A. pullulans* HP-2001 were different from those for production of pullulan. And relative influences of four mineral salts in the medium on cell growth were also found to be different from those on production of pullulan in this study. The optimal concentrations of salts for production of pullulan vary with concentrations of carbon and nitrogen sources [6]. In this study, K_2HPO_4 was found to be the most important factor for cell growth as well as the production of pullulan by *A. pullulans* HP-2001. Next significant factor for cell growth of *A. pullulans* HP-2001 was $(NH_4)_2SO_4$, whereas that for production of pullulan was NaCl. Potassium phosphate (K_2HPO_4) acted as a mineral salt for cells as well as a pH stabilizer, which resulted in enhanced cell growth and the production of bacterial exopolymers [22]. K_2HPO_4 is one of the major salts in the medium for the production of microbial polysaccharides and enzymes as well as a well-known ingredient in buffer solutions [23,24]. Ammonium sulfate seems to be used as a nitrogen source for cell growth, whereas sodium chloride could be used as a physiological modulator of biosynthetic pathway of pullulan [25]. Due to different metabolic pathways for cell growth and production of pullulan, relative influences of salts on cell growth seem to be different from those on production of pullulan.

CONCLUSION

Physiological factors for the production of pullulan by *A. pullulans* HP-2001 were optimized using orthogonal array method and their influences were compared by analysis of data using Qualitek-4 software based on Taguchi method. As described in previous reports, the optimal conditions for cell growth were different from those for production of pullulan. Influences of physiological factors on cell growth were also different from those on production of pullulan. The difference in optimal conditions and relative influence of each factor seems to be due to the difference in metabolic pathways of cell growth and production of pullulan.

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REFERENCES

1. R. S. Singh, G. K. Saini and J. F. Kennedy, *Carbohydr. Polym.*, **73**, 515 (2008).
2. K. I. Shingel, *Carbohydr. Res.*, **339**, 447 (2004).
3. T. D. Leather, *Appl. Microbiol. Biotechnol.*, **62**, 468 (2003).
4. J. W. Lee, W. G. Yeomans, A. L. Allen, F. Deng, R. A. Gross and D. L. Kaplan, *Appl. Environ. Microbiol.*, **12**, 5265 (1999).
5. H. P. Seo, C. H. Chung, S. K. Kim, R. A. Gross, D. L. Kaplan and J. W. Lee, *J. Microbiol. Biotechnol.*, **14**, 237 (2004).
6. W. Gao, Y. J. Kim, C. H. Chung, J. Li and J. W. Lee, *Biotechnol. Bioprocess Eng.*, **15**, 837 (2010).
7. W. Gao, Y. J. Kim, C. H. Chung, J. Li and J. W. Lee. *J. Life Sci.*, **20**, 1433 (2010).
8. H. P. Seo, C. W. Son, C. H. Chung, D. I. Jung, S. K. Kim, R. A. Gross, D. L. Kaplan and J. W. Lee, *Bioresour. Technol.*, **95**, 293 (2004).
9. J. H. Lee, J. H. Kim, I. H. Zhu, X. B. Zhan, J. W. Lee, D. H. Shin and S. K. Kim, *Biotechnol. Lett.*, **23**, 817 (2001).
10. J. H. Lee, J. H. Kim, M. R. Kim, S. M. Lim, S. W. Nam, J. W. Lee and S. K. Kim, *J. Microbiol. Biotechnol.*, **12**, 1 (2002).
11. Y. Lin, Z. Zhang and J. Thibault, *Process Biochem.*, **42**, 820 (2007).
12. R. S. Singh and H. Singh, *Appl. Biochem. Biotechnol.*, **152**, 42 (2009).
13. D. Shokri and G. Emitiazi, *Curr. Microbiol.*, **61**, 217 (2010).
14. H. P. Seo, K. I. Jo, C. W. Son, J. K. Yang, C. H. Chung, S. W. Nam, S. K. Kim and J. W. Lee, *J. Microbiol. Biotechnol.*, **16**, 374 (2006).
15. N. Madi, B. McNeil and L. M. Harvey, *Enzyme Microb. Technol.*, **21**, 102 (1997).
16. B. McNeil and B. Kristiansen, *Enzyme Microbiol. Technol.*, **12**,

521 (1990).
17. A. Wecker and U. Onken, *Biotechnol. Lett*., **13**, 155 (1991).
18. M. Ronen, H. Guterman and Y. Shabtai, *Biochem. Biophys. Methods*, **51**, 243 (2002).
19. M. Reeslev, J. C. Nielsen, J. Olsen, B. Jensen and T. Jacobsen, *Mycol. Res*., **95**, 220 (1991).
20. Z. Duan, Z. Chi, L. Wang and X. Wang, *Carbohydr. Polym*., **73**, 587 (2008).
21. H. P. Gandhi, R. M. Ray and R. M. Patel, *Carbohydr. Polym*., **34**, 323 (1997).
22. N. K. Lee, Y. B. Jo, I. H. Jin, C. W. Son and J. W. Lee, *J. Life Sci*., **19**, 1033 (2009).
23. H. Jin, J. K. Yang, K. I. Jo, C. H. Chung, S. K. Kim, S. W. Nam and J. W. Lee, *Process Biochem*., **41**, 270 (2006).
24. 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).
25. C. A. Jaleel, P. Manivannan, G. M. A. Lakshmanan, R. Sridharan and R. Panneerselvam, *Compt. Rend. Biol*., **330**, 806 (2007).