Appl Biochem Biotechnol (2012) 168:1394–1404
DOI 10.1007/s12010-012-9865-7

Evaluation of Metal Ions and Surfactants Effect on Cell Growth and Exopolysaccharide Production in Two-Stage Submerged Culture of *Cordyceps militaris*

Jian-Dong Cui · Ya-Nan Zhang

Received: 9 June 2012 / Accepted: 24 August 2012 /
Published online: 7 September 2012

Abstract During the two-stage submerged fermentation of medicinal mushroom *Cordyceps militaris*, it was found that K^+, Ca^{2+}, Mg^{2+}, and Mn^{2+} were favorable to the mycelial growth. The EPS production reached the highest levels in the media containing Mg^{2+} and Mn^{2+}. However, Ca^{2+} and K^+ almost failed to increase significantly exopolysaccharides (EPS) production. Sodium dodecyl sulfate (SDS) significantly enhanced EPS production compared with that of without adding SDS when SDS was added on static culture stage of two-stage cultivation process. The presence of Tween 80 in the medium not only simulated mycelial growth but also increased EPS production. By response surface methods **(**RSM), EPS production reached its peak value of 3.28 g/L under optimal combination of 27.6 mM Mg^{2+}, 11.1 mM Mn^{2+}, and 0.05 mM SDS, which was 3.76-fold compared with that of without metal ion and surfactant. The results obtained were useful in better understanding the regulation for efficient production of EPS of *C. militaris* in the two-stage submerged culture.

Keywords *Cordyceps militaris* · The two-stage submerged culture · Exopolysaccharides · Metal ion · Surfactant

Introduction

Cordyceps militaris, a famous traditional Chinese medicinal mushroom, belongs to the class Ascomycetes and DongChongXiaCao group in Chinese herbs [1, 2]. Some *Cordyceps* species have long been used for medicinal purposes in China, Japan, and Korea and other East Asia countries because of their various biological and pharmacological activities which

J.-D. Cui (✉)
Hebei Key Laboratory of Pharmaceutic Molecular Chemistry, College of Bioscience and Bioengineering, Hebei University of Science and Technology, 70 Yuhua East Road, Shijiazhang 050018, People's Republic of China
e-mail: cjd007cn@163.com

Y.-N. Zhang
Library, Hebei University of Science and Technology, 70 Yuhua East Road, Shijiazhang 050018, People's Republic of China

were generally attributed to the presence of the important bioactive ingredients such as adenosine, cordycepin, and exopolysaccharides (EPS) [3, 4]. In particular, EPS produced by *Cordyceps* species have been regarded as desired products with perceived health benefits. Thus, there is growing interest in improving EPS production. In order to improve EPS production, the influence of culture conditions and medium compositions for improving production of EPS from *C. militaris* has been reported [5–7]. Moreover, during the past decades, submerged fermentation technology also gives rise to potential advantages for obtaining higher EPS production in a compact space and shorter time with less chance of contamination [7–9]. However, the yields of EPS obtained are relatively low by a traditional submerged culture. Thus, there is a necessity to develop a novel process for EPS production. In general, metal ions play a significant role on the cell growth and metabolite biosynthesis. In particular, the regulatory effect of metal ions on microbial secondary metabolism has been recorded for a variety of species [10, 11]. It has been reported that external Ca^{2+} could change cell membrane permeability by controlling the internal Ca^{2+} gradient and the activity of some fungal enzymes involved in cell wall expansion [12, 13]. Mg^{2+} is essential to all fungi. It is a cofactor in enzymatic reactions, stabilizes the plasma membrane, and its uptake is ATP dependent [13]. Na^{+} was reported to influence the diversity of polysaccharide biosynthesis by medicinal mushroom *Phellinus linteus* [14]. In addition, some researchers had reported that fatty acid, oil, and surfactant promoted the production of fungal metabolites [8, 15]. The EPS production in an edible mushroom *Collybia maculata* was also substantially increased by supplementation of organic solvents into the medium [16]. In particular, recently, a two-stage submerged fermentation process was developed for production of *C. militaris* EPS in our lab [17]. However, the effects of metal ions and surfactant on cell growth and EPS production in two-stage submerged culture of *C. militaris* have not been reported yet. This study attempts to understand more of metal ion addition and surfactant addition in two-stage submerged culture for mycelial growth and EPS production by *C. militaris*. The information obtained in this work is helpful for the mycelial growth and EPS production by two-stage submerged cultivation of *C. militaris* on a large scale.

Materials and Methods

Microorganism and Grown Conditions

The strain of *C. militaris* (CICC 14015) was purchased from China Center of Industrial Culture Collection (CICC, Beijing, China). The stock culture was maintained on potato dextrose agar slants. Slants were incubated at 25 °C for 7 days and then stored at 4 °C. The slants were inoculated with mycelia and incubated at 25 °C for 7 days, and then used for seed culture inoculation. The seed culture medium consisted of the following components (g/L): glucose, 40; yeast extract, 10; KH_2PO_4, 1.0; and $MgSO_4{\cdot}7H_2O$, 0.5. The mycelia of *C. militaris* were transferred to the seed culture medium by punching out about 6 mm of the slants with a sterilized cutter. The seed culture was grown in a 250-ml shake flask containing 50 ml of liquid medium and incubated at 25 °C on a rotary shaker (150 rpm) for 5 days. Medium used for batch fermentation contained the following components (g/L): glucose 40; yeast extract, 10; KH_2PO_4, 1.0; peptone, 5; and $MgSO_4{\cdot}7H_2O$, 0.5. The pH was initially adjusted to 6. The flask culture experiments were performed in 250-ml flasks containing 50 ml of medium after inoculating with 10 % (*v*/*v*) of the seed culture. The culture was incubated at 25 °C on a rotary shaker incubator in two-stage (shake-flask fermentation followed by static culture) culture methods [17].

Metal Ion Experiments

The effects of metal ion on the cell growth and the production of EPS by *C. militaris* cells were investigated by using various mineral sources, i.e., $CaCl_2$, KCl, $MnCl_2$, and $MgCl_2$. The concentration of each mineral source was 1, 10, 20, 40, and 60 mM, respectively. The dynamic profiles of the cell growth, and EPS biosynthesis were monitored during the two-stage submerged fermentation process.

Surfactants Experiments

Effects of surfactants on the cell growth and the production of EPS by *C. militaris* cells were also studied during the two-stage submerged fermentation process. Surfactants such as Tween 80, sodium dodecyl sulfate (SDS), and cetyl trimethyl ammonium bromide (CTAB) were supplemented. The concentration of each surfactant was 0.01, 0.05, 0.1, and 0.5 mM, respectively. The other culture conditions were the same as above. The control experiment was conducted in the fermentation medium without surfactants.

Measurements of mycelial biomass and EPS

The mycelia dry cell weight (DCW) and crude EPS were determined by a method based on the protocol of Kim et al. [9]. For the measurement of mycelia DCW, samples collected from shake flasks were centrifuged at 10,000×*g* for 10 min, the sediment was washed twice with distilled water, and then dry cell weight was obtained by filtering culture samples through a pre-weighed filter paper and dried at 80 °C to constant weight. The resulting supernatant obtained as described above was mixed with four volumes of 99 % (*v*/*v*) ethanol, stirred vigorously, and left overnight at 4 °C. The precipitated crude EPS was recovered by centrifugation at 10,000×*g* for 10 min. The precipitated crude EPS was freeze–dried in a lyophilizer and the weight of the polymer was estimated.

Morphology Observations

During the submerged cultures, photographs of morphological changes in mycelium were obtained by using an XSP-2C microscope (BaTuo, Shanghai, China) with a 550D camera (Canon). The samples were fixed with an equal volume of fixative (13 mL of 40 % formaldehyde, 5 mL glacial acetic acid with 200 mL of 50 % ethanol). Each fixed sample (0.1 mL) was transferred to a slide, air-dried, and stained with methyl blue (0.3 g methyl blue, 30 mL 95 % ethanol in 100 mL distilled water) [16].

Experimental Design and Optimization

Based on the results obtained in preliminary experiments, Mg^{2+}, Mn^{2+}, and SDS were found to be the major variables in EPS production. Central composite design (CCD) was used to find the optimal concentrations of these three factors. Mg^{2+} (X_1), Mn^{2+} (X_2), and SDS (X_3) were chosen as the independent variables shown in Table 1. EPS production (Y) was used as dependent output variables. A set of 15 experiments consisting of four factorial points, six axial points (α=1.41), and five replicates at the center points were employed. All experiments were carried out in triplicates. A multiple regression analysis of the data was carried out with the statistical package (Stat-Ease Inc., Minneapolis, MN, USA) and the second-

Table 1 Process variables used central composite design with actual factor levels corresponding to coded factor levels

Variables	Symbol	Coded levels				
		−1.41	−1	0	1	1.41
Mg^{2+} (mM)	X_1	12.95	15	20	25	32.05
Mn^{2+} (mM)	X_2	7.18	8	10	12	12.82
SDS (mM)	X_3	0.015	0.025	0.05	0.075	0.085

order polynomial equation that defines predicted response (Y) in terms of the independent variables (X_1, X_2, and X_3) was obtained:

$$Y = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_{11}X^2{}_1 + B_{22}X^2{}_2 + B_{33}X^2{}_3 + B_{12}X_1X_2 + B_{23}X_2X_3 + B_{13}X_1X_3 \quad (1)$$

Where B_0 is constant; B_1, B_2, and B_2 are linear coefficients; B_{11}, B_{22}, and B_{33} are squared coefficients; and B_{12}, B_{23}, and B_{13} are interaction 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.

Results and Discussion

Effect of Metal Ion on Mycelial Growth and EPS Production by *C. militaris*

The effect of metal ions on mycelial growth and EPS production was examined by employing various mineral sources at a concentration of 10 mM. As shown in Table 2, among the various metal ions examined, K^+, Ca^{2+}, Mg^{2+}, and Mn^{2+} were favorable to the mycelial growth. The mycelial production was enhanced by 21.8, 40.3, 10.3, and 65.8 %, respectively. The EPS production reached the highest levels in the media containing Mg^{2+} and Mn^{2+}. The highest EPS production of 2.08 g/L was obtained when Mg^{2+} was added, which was enhanced by 133.2 % compared with the control. In contrast, Ca^{2+} and K^+ almost failed to increase significantly EPS production. Furthermore, there was significant effect of Mg^{2+} concentration on the cell growth of *C. militaris* (Fig. 1). The maximal cell density of 19.93 g/L was obtained at the level Mg^{2+} concentration of 20 mM. While an inhibition phenomenon on the cell growth was observed when concentration increased up to 40 mM. In contrast, Mg^{2+} concentration significantly affected EPS production, and 20 mM Mg^{2+} concentration were favorable for EPS production.

Table 2 Effect of metal ions on the cell growth and the production of EPS[a]

Surfactant	Biomass (g/L)	EPS (g/L)
Control	7.79±0.22	0.89±0.04
KCl	9.49±0.26	1.05±0.05
$CaCl_2$	10.93±0.31	0.75±0.03
$MgCl_2$	8.59±0.24	2.08±0.1
$MnCl_2$	12.92±0.36	1.92±0.09

[a]The standard deviation was calculated from three independent samples

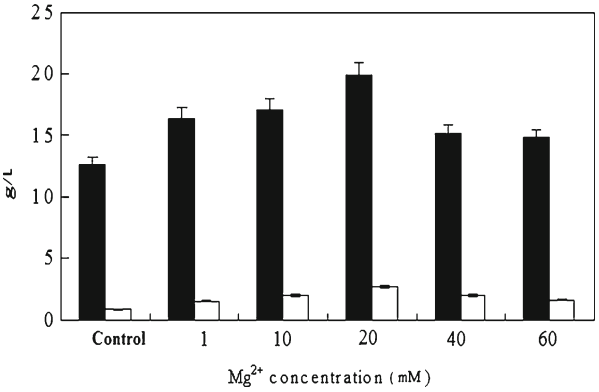

Fig. 1 Effect of Mg^{2+} concentration on mycelial growth and EPS production by *C. militaris*. *Unshaded bars* EPS, *shaded bars* DCW

EPS production of 2.69 g/L at 20 mM Mg^{2+} was enhanced by 3-fold compared with control. The results indicated that a relativity lower Mg^{2+} concentration led to a higher EPS production. For Mn^{2+} (Fig. 2), cell concentration increased with the increase of Mn^{2+} concentration, while an inhibition phenomenon on the cell growth was observed when Mn^{2+} concentration increased up to 20 mM. Moreover, cell concentration remained almost constant when Mn^{2+} concentration increased up to 40 mM. However, EPS concentration at the highest level Mn^{2+} of 10 mM was always higher than the other cases, and the lowest EPS accumulation was observed at its lowest level of 1 mM. EPS production of 2.53 g/L at 10 mM Mn^{2+} was enhance by 2.8-fold compared with control. This clearly demonstrated the Mn^{2+} concentration significantly affected EPS production. Some reports have shown that mineral ions are usually cognized as favorable bioelements for mycelial growth and production of secondary metabolites (like EPS) in several fungal traditional submerged fermentations [1, 12, 16, 18]. Chen et al. [10] observed a high level of mycelial growth and EPS production by *Fomes fomentarius* in media containing Ca^{2+} and Mg^{2+}. However, in the two-stage fermentation process, it was found that K^+, Ca^{2+}, Mg^{2+}, and Mn^{2+} were favorable to the mycelial growth. EPS production reached the highest levels in the media containing Mg^{2+} and Mn^{2+}. However, Ca^{2+} and K^+ were almost failed to increase significantly EPS production. These results are not in accordance with those obtained by Chen et al. [10]. The more likely explanation was that Mg^{2+} and Mn^{2+} are cofactors for the key enzyme which lead to EPS formation at the branch point of EPS biosynthesis. The addition of Mg^{2+} and Mn^{2+} may stimulate the key enzyme activity, which lead to more EPS accumulation.

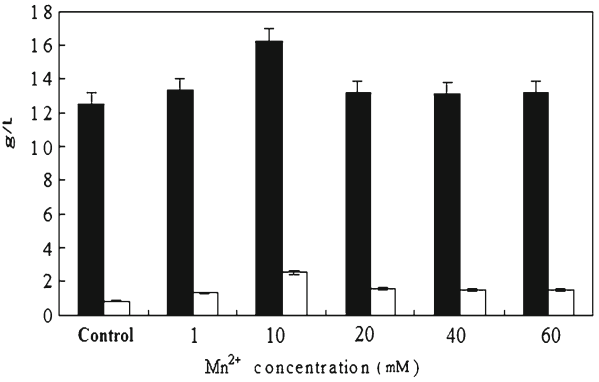

Fig. 2 Effect of Mn^{2+} concentration on mycelial growth and EPS production by *C. militaris*. *Unshaded bars* EPS, *shaded bars* DCW

Effect of Surfactants on Mycelial Growth and EPS Production by *C. militaris*

The influences of three different surfactants on mycelial growth and EPS production by *C. militaris* were studied by supplementing 0.1 mM of each into culture media on different culture stage (Table 3). The time of surfactants addition was determined by dividing the culture period into several phases: shake culture stage of two-stage cultivation process (early exponential phase) and static culture stage of two-stage cultivation process (stationary phase). The result revealed that SDS or CTAB significantly enhanced EPS production compared to control when SDS or CTAB was added on static culture stage of two-stage cultivation process. The maximum EPS production (1.96 g/L) was obtained when SDS was added on static culture stage. However, SDS or CTAB hardly influenced mycelial growth. In contrast, SDS or CTAB had a negative effect on mycelial growth when SDS or CTAB was supplemented into culture media on shake culture stage of two-stage cultivation process; as a result, EPS production was also significantly decreased. This result suggested that the addition of SDS or CTAB at the early growth phase had a detrimental effect on the sustenance of cell viability, thereby reducing the concentrations of both EPS and mycelial biomass. In addition, when Tween 80 was added to the medium on shake culture stage, the results showed that the mycelial growth was significantly increased. The highest cell concentration was 2-fold compared to control. Furthermore, EPS production was also increased 1.7-fold compared to control. Figure 3 showed the difference between the morphology of cells before and after SDS treatment. It can be clearly seen in the fresh cells that a complex of EPS exist in and around the pellet core region. However, this is not observed in SDS-treated cell, which implies that the amount of EPS entangled within the mycelial pellet is quite less than fresh cell, thereby contributing to the enhanced EPS concentration in the broth and making the morphology trimly. As depicted in Fig. 4, it was indicated that there was no significance of SDS concentration on mycelial growth of *C. militaris* when SDS was supplemented into culture media on static culture stage of two-stage cultivation process. However, SDS concentration significantly affected EPS production, and 0.05 mM SDS were favorable for EPS production. EPS production of 2.09 g/L at 0.05 mM SDS was enhance by 2.3-fold compared with control. For Tween 80 (Fig. 5), it was indicated there was no significance of Tween 80 concentration on EPS production of *C. militaris*. However, EPS production was increased partly compared to the control (without adding Tween 80). In contrast, compared with the control, a significant increase in mycelial growth was achieved when the Tween 80 was added into fermentation medium. The maximal cell density of 20.8, 22.4, 20.1, and 18.8 g/L was obtained at the Tween 80 level of 0.01, 0.05, 0.1, and 0.5 mM, respectively. The cell biomass was 1.22-, 1.32-, 1.18-, and 1.1-fold compared to the control, respectively. This clearly demonstrated the mycelial growth of *C. militaris* was significantly

Table 3 Effect of surfactant on the cell growth and the production of EPS

Surfactant	Biomass (g/L)	EPS (g/L)
Control	7.14±0.21	0.88±0.04
SDS[a]	10.32±0.28	0.25±0.01
SDS[b]	7.96±0.22	1.96±0.1
CTAB[a]	1.85±0.051	0.48±0.02
CTAB[b]	7.61±0.21	1.28±0.06
Tween-80[a]	15.28±0.43	1.48±0.07
Tween-80[b]	7.05±0.2	0.82±0.04

[a]Surfactant was added to medium when the shake culture (first stage of two-stage cultivation process) was started[b]Surfactant was added to medium when the static culture (second stage of two-stage cultivation process) were started

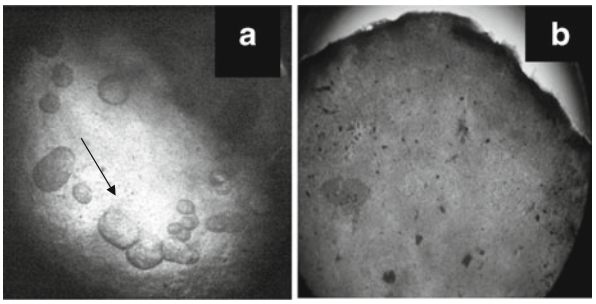

Fig. 3 Difference in morphology of *C. militaris* cell in the absence (**a**) and in the presence of SDS (**b**). Magnification ratios were 100. EPS within the mycelial pellet are indicated by the *arrow*

affected by Tween 80 concentration. Previous reports also showed that Tween 80 could stimulate mycelial growth of *Grifola frondosa* in traditional submerged fermentations, whereas, EPS production was found to be decreased [8]. In contrast, in the two-stage fermentation process, when Tween 80 was added to the medium on shake culture stage, the presence of Tween 80 in the medium not only simulated mycelial growth but also increased EPS production. Interestingly, Tween 80 did not result in remarkable increase in EPS production, nor in cell growth inhibition when Tween 80 was added to the medium on static culture stage. It is thought that cell growth has entered stationary phase when Tween 80 was added to the medium on static culture stage. In this phase, the cells are obviously not highly viable and show a slow hydrolysis rate on Tween 80. In contrast, cell growth just entered early exponential phase when Tween 80 was added to the medium on shake culture stage. The cells have higher viability, and Tween 80 was quickly hydrolyzed to release oleic acid. Some investigators have attempted to increase the production of microbial metabolites using some stimulating agents, such as surfactants, vegetable oils, and organic solvents [8, 13, 15, 19, 20]. These stimulation agents are known to mediate cell permeabilization by disorganizing the cell membrane or directly affecting the level of enzyme synthesis involved in product formation (Table 3).

Response Surface Analysis for the Optimization of Three Factors

Table 4 showed the actual EPS production in CCD. The regression coefficients and significance levels were given in Table 5. The analysis of variance (Table 5) indicated that the model terms of X_1, X_1^2, X_2^2, and X_3^2 were significant ("probe> F" less than 0.05), and the interactive effects of X_1X_3 and X_2X_3 were not significant, but the interactive effect of X_1X_2 was significant ("probe> F" less than 0.05). It means that Mg^{2+} and SDS have

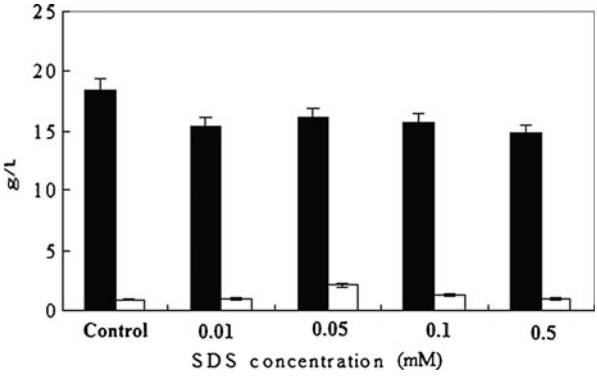

Fig. 4 Effect of SDS concentration on mycelial growth and EPS production by *C. militaris*. *Unshaded bars* EPS, *shaded bars* DCW

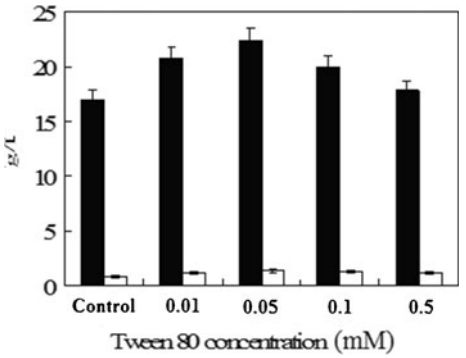

Fig. 5 Effect of Tween 80 concentration on mycelial growth and EPS production by *C. militaris*. *Unshaded bars* EPS, *shaded bars* DCW

important effects on EPS production, and the quadratic effects of Mg^{2+}, Mn^{2+}, and SDS are more significant than other factors. Multiple regression analysis of the experimental data gave the following second-order polynomial equation:

$$Y = 3.91 - 0.2262X_1 + 0.0212X_2 - 0.0883X_3 - 0.6925X^2{}_1 + 0.1483X_1X_2 - 0.0162X_1X_3 - 0.4025X^2{}_2 + 0.0362X_3X_2 - 0.755X_3^2 \quad (2)$$

where X_1, X_2 and X_3 are Mg^{2+}, Mn^{2+}, and SDS concentrations, respectively. The regression equation obtained from analysis of variance indicated that the multiple correlation coefficient of R^2 is 0.997. The value of the determination coefficient (R^2=0.997) indicates that the model can explain 99.7 % variation in the response, the value of adjusted R^2 (Adj. R^2= 0.992) is very high that indicated a high significance of the model. From the statistical results obtained, it was shown that the above models were adequate to predict EPS production within the range of variables studied. The 2D contour plots are generally the graphical

Table 4 Central composite design and response value

Run	X_1	X_2	X_3	EPS (g/L)
1	−1	−1	1	2.26
2	−1	1	−1	2.15
3	1	−1	−1	1.76
4	+1	+1	+1	1.89
5	−1.42	0	0	2.89
6	1.42	0	0	2.25
7	0	−1.42	0	3.12
8	0	1.42	0	3.18
9	0	0	−1.42	2.57
10	0	0	1.42	2.32
11	0	0	0	3.86
12	0	0	0	3.91
13	0	0	0	3.89
14	0	0	0	3.87
15	0	0	0	3.93

Table 5 Parameter estimates and analysis of variance

Source of variation	Degree of freedom (df)	Sum of squares (SS)	Mean squares (MS)	*F* value	Probe>*F*
X_1	1	0.2047	0.2047	40.716	0.0013[a]
X_2	1	0.0018	0.0018	0.3579	0.5757
X_3	1	0.0312	0.0312	6.2126	0.0549
X_1^2	1	3.6994	3.6994	735.512	0.0001[a]
X_1X_2	1	0.0440	0.0440	8.7552	0.0315[b]
X_1X_3	1	0.0005	0.0005	0.1045	0.7595
X_2^2	1	1.2497	1.2497	248.474	0.0001[a]
X_2X_3	1	0.0026	0.0026	0.5231	0.5019
X_3^2	1	4.3973	4.3973	874.268	0.0001[a]
Model	9	9.1767	1.0196	202.7233	0.0001
Error	5	0.0025	0.005		
Total	14	9.201			

[a]Highly significant[b]Significant

representations of the regression equation, and 2D contour plots are presented in Fig. 6. From the contour plots, it is easy and convenient to understand the interactions between two factors and also locate their optimum levels. The circular contour plots of response surfaces suggest that the interaction is negligible between the corresponding variables. An elliptical or saddle nature of the contour plots indicates the significance of the interactions between the corresponding variables. The contour plots in Fig. 6a showed that there was significant mutual interaction between Mg^{2+} and Mn^{2+}. However, there was almost no interaction between Mg^{2+} and SDS (Fig. 6b), Mn^{2+} and SDS (Fig. 6c), as was evident from the relatively circular nature of the contour curves. The optimal conditions were extracted by Design Expert Software with its optimization menus: X_1=1.53, X_2=0.56, X_3=0, The real values were Mg^{2+} concentration at 27.6 mM, Mn^{2+} concentration at 11.1 mM, and SDS concentration at 0.05 mM. The maximum EPS production obtained by using the above optimized concentrations of the variables is 3.35 g/L. The maximum EPS production obtained experimentally was found to be 3.28 g/L. This is obviously in close agreement with the model prediction. Previous studies on EPS production from various *Cordyceps* species were limited at the solid culture and conventional submerged culture [6–9]. However, the yield of some metabolites such as the EPS and cordycepin is very low. Shih and coworkers optimized culture conditions of mycelial growth and EPS production in the submerged culture of *C. militaris* strain. They found that the maximal EPS production was only 1.1, 1.1, 0.9, and 1.0 g/L for pH 4, 5, 6, and 7, respectively. Moreover, among organic sources, yeast extract yielded the best EPS production (1.5 g/L) [7]. In order to improve EPS production, recently, a new two-stage fermentation process was designed in our lab. The EPS production in the two-stage fermentation process is significantly higher than that in the conventional shake culture and static culture, the maximal EPS production reached 3.2 g/L [17]. In this study, the effects of metal ions and surfactant on cell growth and EPS production in two-stage submerged culture of *C. militaris* were further investigated, under optimized conditions, the maximum EPS production reached 3.35 g/L, and was about 3-fold in comparison with those of Shih et al. [7].

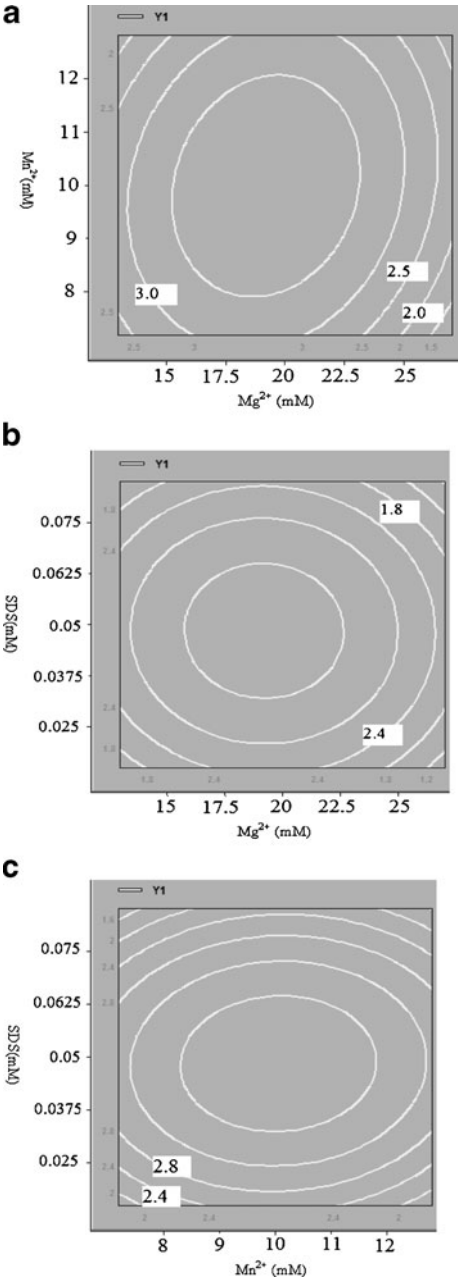

Fig. 6 Contour plots of EPS production: the effect of Mg^{2+} (X_1) and Mn^{2+} (X_2) (**a**), Mg^{2+} (X_1) and SDS (X_3) (**b**), Mn^{2+} (X_2) and SDS (X_3) (**c**), on EPS production

Conclusions

The effects of metal ions and surfactant on cell growth and EPS production in two-stage submerged culture of *C. militaris* were first reported in this study. The results from this study showed that the metal ions and surfactant likely play an important role on cell growth and EPS production of *C. militaris*. It can be concluded that metal ions such as K^+, Ca^{2+}, Mg^{2+},

and Mn^{2+} and surfactants such as Tween 80 could be favorable used as an additive for the mycelial growth of *C. militaris*. However, Ca^{2+} and K^{+} almost failed to increase significantly EPS production. A higher cell growth and EPS production were obtained with Tween 80. Meanwhile, Tween 80 added at the beginning of cultivation had resulted in the highest EPS production. On the contrary, SDS added on static culture stage of two-stage cultivation process significantly enhanced EPS production. The fundamental information obtained in this work is beneficial for further development of *C. militaris* cultivation process for high production of EPS and mycelial biomass on a large scale. Such work may also be helpful to other mushroom fermentation processes for enhanced production of mushroom EPS, particularly those with industrial potential.

Acknowledgments The project was partially supported by the National Natural Science Foundation of China (NSFC, project no. 21072041), open funding project of the National Key Laboratory of Biochemical Engineering (no. KF2010-12) and the Foundation (no. 2012IM004) of Ministry of Education Key Laboratory of Industrial Fermentation Microbiology (Tianjin University of Science and Technology), P. R. China, and Hebei Science Research Projects of Hebei Education Department (no. 2010128).

References

1. Kuo, Y. C., Tasi, W. J., Shiao, M. S., Chen, C. F., & Lin, C. Y. (1996). *The American Journal of Chinese Medicine, 24*, 111–115.
2. Mao, X. B., Eksriwong, T., Chauvatcharin, S., & Zhong, J. J. (2005). *Process Biochemistry, 40*, 1667–1672.
3. Song, C. H., Yang, B. K., Ra, K. S., Shon, D. H., Park, E. J., Go, G. I., et al. (1998). *Journal of Microbiology and Biotechnology, 8*, 277–279.
4. Gu, Y. X., Wang, Z. S., Li, S. X., & Yuan, Q. S. (2007). *Food Chemistry, 102*, 1304–1309.
5. Xu, C. P., Kim, S. W., Hwang, H. J., & Yun, J. W. (2002). *Biotechnology and Applied Biochemistry, 36*, 127–131.
6. Park, J. P., Kim, S. W., Hwang, H. J., & Yun, J. W. (2004). *Process Biochemistry, 39*, 2241–2247.
7. Shih, I. L., Tsai, K. L., & Hsieh, C. (2007). *Biochemical Engineering Journal, 33*, 193–201.
8. Hsieh, C. Y., Wang, L. H., Chen, C. C., Hsu, T. H., & Tseng, M. H. (2008). *Biochemical Engineering Journal, 38*, 198–205.
9. Kim, H. O., Lim, J. M., Joo, J. H., Kim, S. W., Hwang, H. J., Choi, J. W., et al. (2005). *Bioresource Technology, 96*, 1175–1182.
10. Chen, W., Zhao, Z., Chen, S. F., & Li, Y. Q. (2008). *Bioresource Technology, 99*, 3187–3194.
11. Raza, W., Yang, X. M., Wu, H. S., Huang, Q. W., Xu, Y. C., & Shen, Q. R. (2010). *Bioresource Technology, 101*, 9264–9271.
12. Kim, S. W., Hwang, H. J., Park, J. P., Cho, Y. J., Song, C. H., & Yun, J. W. (2002). *Letters in Applied Microbiology, 34*, 56–61.
13. Kim, H. O., & Yun, J. W. (2005). *Journal of Applied Microbiology, 99*, 728–738.
14. Zou, X., Sun, M., & Guo, X. (2006). *World Journal of Microbiology and Biotechnology, 22*, 1129–1133.
15. Nascimento, A. E., Sharia, A. E. N., Lima, M. A. B., & Takaki, G. M. C. T. A. (2000). *Brazilian Journal of Microbiology, 31*, 30–36.
16. Lim, J. L., & Yun, J. W. (2006). *Process Biochemistry, 41*, 1620–1626.
17. Cui, J. D., & Zhang, B. Z. (2011). *Letters in Applied Microbiology, 52*, 123–128.
18. Park, J. P., Kim, S. W., Hwang, H. J., & Yun, J. W. (2001). *Letters in Applied Microbiology, 33*, 76–81.
19. Breuil, C., Shindler, D., Sijher, B. J. S., & Kushner, D. J. (1978). *Journal of Bacteriology, 133*, 601–606.
20. Cui, J. D., Jia, S. R., & Sun, A. Y. (2008). *Letters in Applied Microbiology, 46*, 631–635.