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ORIGINAL PAPER

High cell density fermentation of *Gluconobacter oxydans* DSM 2003 for glycolic acid production

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Abstract *Gluconobacter oxydans* has a lower biomass yield. Uniform design (UD) was applied to determine the optimum composition of the critical media and their mutual interactions for increased biomass yield of *Gluconobacter oxydans* DSM 2003 in shake flasks. Fed-batch fermentation process for biomass was optimized in a 3.7-l fermentor. By undertaking a preliminary and improved fed-batch fermentation-process strategy, a cell density of 6.0 g/l (DCW) was achieved in 22 h and 14.1 g/l (DCW) in 35 h, which is the highest cell density of *G. oxydans* produced thus far in a 3.7-l bioreactor. The biomass production was increased by 135% compared with that using the original cultivation strategy. Bioconversion of ethylene glycol to glycolic acid was catalyzed by the resting cells of *G. oxydans* DSM 2003, and conversion rate reached 86.7% in 48 h. In summary, the approach including high-density fermentation of *G. oxydans* DSM 2003 and bioconversion process was established and proved to be an effective method for glycolic acid production.

Keywords *Gluconobacter oxydans* · Uniform design · Fed-batch · High-density fermentation · Bioconversion · Ethylene glycol · Glycolic acid

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Introduction

Gluconobacter oxydans strain is a Gram-negative, aerobic, rod-shaped acidophilic bacterium and is known for its incomplete oxidation of a wide range of carbohydrates and alcohols at low pH values by its membrane-bound dehydrogenases [1, 2]. The corresponding oxidative products are secreted almost completely into the medium [3–6]. The great advantage is that the substrates are regionly and stereoselectively oxidized. This combined chemical technology is employed in the synthesis of sugar derivatives that can only be obtained by complex protection group chemistry. This makes *G. oxydans* strains suitable objects for industrial application. But there is a bottleneck problem for industrial application, which is that *G. oxydans* has a lower biomass yield [7], leading to a higher production cost. To improve productivity for industrial applications, an increase in cell density would be advantageous.

Glycolic acid is one of the most important bulk fine chemicals, extensively used in adhesives, metal cleaning, textiles, leather processing [8, 9], biodegradable polymers [10], and as a component in personal-care products [11]. As the biological synthetic process is more advantageous for purification than the chemical process, which produces many by-products, biotransformation production of glycolic acid by a microbial process has been attracting a lot of attention.

Ethylene glycol is a cheap commercial product with great potential for microbial processes, since it is water-soluble and of low toxicity [12]. Herein, the production of glycolic acid with *G. oxydans* from ethylene glycol as the raw material is reported.

Uniform design (UD) is a useful and technically simple method, which was first proposed by Fang and co-workers [13, 14]. It is a powerful technique for testing multiple

process variables because fewer experimental trials are needed than if one variable is studied at a time. UD tables can be described as Un(q^m), where U stands for the UD, n for the number of experimental trials, q for the number of levels, and m for the maximum number of factors. Examples of successful applications of the UD method for improving technologies in various fields have been consistently reported [15–17].

In this paper, the medium composition and fermentation bioprocess for cell biomass production were investigated. The UD optimization technique was successfully applied for the medium optimization of biomass of *G. oxydans* DSM 2003. Optimization of fermentation and biomass formation of *G. oxydans* DSM 2003 led to a great enhancement in biomass yield and cell activity, which greatly reduced its cost as a catalyst. Furthermore, conversion of ethylene glycol to glycolic acid catalyzed by the resting cells of *G. oxydans* DSM 2003 was also studied.

Materials and methods

Chemicals

Glycolic acid was purchased from Sigma-Aldrich. Ethylene glycol was purchased from Shanghai Chemical Reaction. All other chemicals were of analytical grade and were commercially available.

Microorganisms and culture conditions

The strain *G. oxydans* DSM 2003 used for the biotransformation of ethylene glycol was grown in a liquid medium containing 80 g/l sorbitol, 20 g/l yeast extract, 5 g/l $(NH_4)_2SO_4$, 2 g/l KH_2PO_4, and 5 g/l $MgSO_4{\cdot}7H_2O$. All media were sterilized by autoclaving at 115°C for 20 min. Preculture was inoculated with a single cell colony from an Agar plate and incubated in an orbital shaker (250 rpm, 28°C) until late exponential growth phase (~22 h). The seed culture was repeated twice in order to adapt the culture to the fermentation environment. A 10% (v/v) inoculum of these cells was added to the fermentor and grown at 28°C for 22 h.

Experiment design

A UD $U_9(9^5)$ experimental design was used to determine the relationship existing between the response functions and variables as well as to suggest experimental trials able to optimize the culture-medium components. The concentrations of sorbitol (X_1), yeast extract (X_2), $(NH_4)_2SO_4$ (X_3), KH_2PO_4 (X_4), and $MgSO_4$ (X_5) were selected as the five independent variables, owing to their influence on biomass production. The cell density was chosen as the dependent variable or response. The experiments in shake flasks were carried out in 1,000-ml flasks with a volume of 100 ml at 28°C in a rotary shaker at 250 rpm.

A mathematical model, describing the relationships between biomass and the medium component contents in second-order polynomial equation, was developed by stepwise regression. The model was as follows:

$$Y = a_0 + \sum a_i X_i + \sum a_{ii} X_i^2 + \sum a_{ij} X_i X_j \quad (1)$$

where Y is the dependent variable (biomass production), a_0 is a constant representing the mean of the dependent variable obtained in each experiment, X_i and X_j ($i = 1, 5$; $j = 1, 5$; $i \neq j$) represent the independent variables, X_i^2 and X_iX_j are the interaction terms, and a_i, a_{ii}, and a_{ij} are the coefficients. The accuracy and general ability of the regression model equation were expressed by the coefficient of determination R^2. This regression model describes the interaction of the various independent variables on the values of biomass production. All the data analysis processes including data regression and determination of the best combination of factor values on the final regression were performed by DPS software (Data Processing System) [18].

Optimization of batch fermentation in 3.7-l bioreactor

To enhance *G. oxydans* DSM 2003 cell density, the process of fermentation was improved in a 3.7-l bioreactor (bioengineering AG) based on the optimized culture medium in shaking flasks with 2-L working volume. The pH was controlled by automatic addition of 2 M NaOH. Dissolved oxygen tension (DOT) was controlled by increasing agitation speed, airflow and fermentor pressure. The aeration flow, the fermentor pressure, and the agitation speed were variable and initially set at 200 l/h, 0.3 bar, and 400 rpm.

Optimization of fed-batch fermentation in 3.7-l bioreactor

Fed-batch fermentation was carried out in a 3.7-l bioreactor (Bioengineering AG). Initially, the reactor was filled with 1.8-l of medium. When the consumption of sorbitol in the batch medium was detected by an increase in dissolved oxygen tension, the fed-batch phase was initiated and the flow rate was increased stepwise.

Effect of different cultivations on resting-cell activity

G. oxydans DSM 2003 was cultured under different cultivation conditions. The cells were harvested and washed twice with physiological saline and resuspended in 0.1 M

buffer solution of potassium phosphate. The concentrations of the cells and ethylene glycol were 2.6 and 20 g/l, respectively. The bioconversion was conducted at 250 rpm, 28°C. After a 2-h reaction, the glycolic acid concentrations were determined by HPLC. The relative activity is the relative amount of glycolic acid produced under the same reaction conditions using different resting cells that were cultured under different conditions.

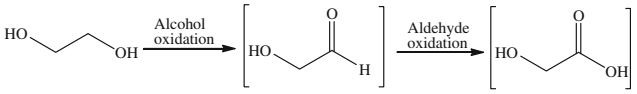
Conversion of ethylene glycol to glycolic acid using the resting cells of *G. oxydans* DSM 2003

The batch biotransformation of glycolic acid was carried out in a 3.7-l bioreactor (Bioengineering AG) with 2-l working volume. The pH was controlled at 5.5 by automatic addition of 4 M NaOH. The agitation speed was adjusted at 700 rpm. The fermentor pressure was controlled at 0.5 bars. The conversion was carried out with 7.8 g/l cell concentration (DCW), and the initial ethylene glycol concentration in the bioreactor was 70.0 g/l. Bioconversion of ethylene glycol to glycolic acid using the resting cells of *G. oxydans* DSM 2003 is presented in Scheme 1.

Analytical methods

Cell growth was monitored by optical density measurements at 600 nm using a UV/visible spectrophotometer. Samples were diluted to the appropriate concentration to keep the $OD_{600\ nm}$ value between 0.3 and 0.6.

After the reaction mixture was centrifuged, the concentration of glycolic acid in the supernatant was measured by HPLC (Agilent 1100) using a ZORBAX SB-Aq column (Agilent Technologies, USA) at 30°C, with an aqueous acidic solution (H_3PO_4, 0.1%) as eluent at a flow rate of 1.0 ml/min. When the absorbance of the eluate was monitored at 210 nm, glycolic acid was eluted at 4.0 min. The concentration of ethylene glycol was measured by gas chromatography (Agilent 6890), which was done with a flame ionization detector and a capillary DB-WAX column (0.25 mm × 25 m × 0.25 μm, Agilent Technologies, USA). The operation parameters were as follows: injection temperature, 250°C; detector temperature, 280°C; initial oven temperature was held at 100°C for 2 min, and then increased to 180°C at a rate of 30°C/min, maintained at 180°C for 1 min, and then increased to 220°C at a rate of 20°C/min. Ethylene glycol was observed at retention time of 4.6 min.



Scheme 1 Bioconversion of ethylene glycol to glycolic acid

Results

Optimization process of biomass production

The UD and the corresponding experimental data are shown in Table 1.

The polynomial model for biomass yield, $Y_{biomass}$, was regressed by mainly considering the significant terms and was as follows:

$$Y_{Biomass} = 4.0562 + 3.8783X_4 - 2.0544X_4^2 - 0.0039X_1X_3 + 0.0324X_1X_4 \quad (2)$$

The mathematical model was expressed in terms of the values of all the independent variables by neglecting the statistically insignificant terms. The determination coefficient of the model ($R^2 = 0.97179$) demonstrated a good correlation between the independent variables, indicating that 97.2% of the variability in the response could be explained by the model. The results of the F test of the model ($F = 16.9782$, where the F value is a ratio of the mean square from the regression to the mean square from the error between the experimental and corresponding predicted values) revealed that this regression was statistically significant ($P = 0.0089$) at the 97.2% confidence level.

Table 2 shows the analysis of variance for the experiment. The results of the regression analysis indicated that KH_2PO_4 was the important factor affecting the performance of the culture on biomass.

The optimal values of the test variables for maximum biomass production found in the final regression model

Table 1 Application of uniform design $U_9(9^5)$ for optimization of biomass production of *G. oxydans* DSM 2003

Runs	Variable level					Biomass (DCW, g/l)
	X_1 (g/l)	X_2 (g/l)	X_3 (g/l)	X_4 (g/l)	X_5 (g/l)	
1	10.0	10.0	3.0	1.75	0.8	1.260
2	20.0	15.0	5.0	1.25	0.7	1.644
3	30.0	20.0	2.5	0.75	0.6	1.675
4	40.0	25.0	4.5	0.25	0.5	1.099
5	50.0	7.5	2.0	2.0	0.4	1.730
6	60.0	12.5	4.0	1.5	0.3	1.790
7	70.0	17.5	1.5	1.0	0.2	1.977
8	80.0	22.5	3.5	0.5	0.1	1.574
9	90.0	27.5	5.5	2.25	0.9	1.842

Table 2 Results of the regression analysis for optimization of biomass production ($Y_{biomass}$) in the flasks

Term	Standard error	*t* value	*P* value
X_4	0.87444	3.60501	0.01546
X_4^2	0.92653	4.92561	0.00438
X_1X_3	0.68439	1.87733	0.11929
X_1X_4	0.93678	5.35417	0.00305

were as follows: sorbitol 73.0 g/l, yeast extract 18.4 g/l, $(NH_4)_2SO_4$ 1.5 g/l, KH_2PO_4 1.52 g/l, and $MgSO_4$ 0.47 g/l.

Verification of the model

The optimal medium obtained from the regression model for biomass was verified. The predicted maximum value of biomass was 2.4 g/l (DCW), whereas the corresponding experimental response was 2.5 g/l (DCW) in the flasks. The excellent correlation between predicted and measured values of these experiments justified the validity of the response model and supported the existence of an optimum point.

Optimization of batch fermentation in 3.7-l bioreactor

The influence of different constant pH values was investigated in the bioreactor. The optimum pH value for the biomass production was found to be pH 6.0. Optimization of the biomass production could be achieved by batch fermentation at a constant pH value of pH 6.0, $T = 28°C$, and inoculation volume 10% (v/v). With the optimal culture composition, maximum biomass production (11.5 g/l, DCW) was achieved within a total fermentation time of 22 h, which is nearly twice the original (6.0 g/l, DCW). Dissolved oxygen tension in the reaction system was found to play an important role in the production of biomass. When the oxygen tension was low, the production of biomass was also low and the fermentation time was longer. To guarantee oxygen supply to satisfy the requirement of cell growth, DOT was kept over 20% by increasing agitation speed (400–1,000 rpm), airflow (200–300 l/h), and fermentor pressure (0.3–0.6 bar).

Optimization of fed-batch fermentation in 3.7-l bioreactor

The sorbitol consumption rate changed in keeping with the cell growth pattern. *G. oxydans* cells entered log growth after a lag phase of about 2 h, and the growth time lasted 35 h. Sorbitol solution (200 g/l) was fed into culture medium when the DOT was above 25%. To better match the supply of sorbitol to cell requirements, a cell-dependent

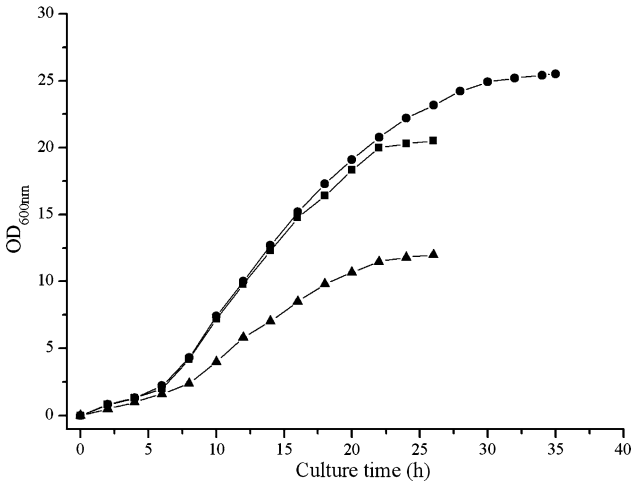


Fig. 1 Growth curves of *G. oxydans* DSM 2003 under different cultivation conditions. *Circles* indicate fed-batch, after optimization, *squares* represent batch, after optimization, and *triangles* indicate batch, before optimization

sorbitol feeding profile was created in our experiment. That is, sorbitol was fed into the bioreactor at an increasing rate according to the cell density, and the feeding rate was adjusted every 2 h. The sorbitol feeding rate started at 1.5 g l^{-1} h^{-1} and increased stepwise to 3.0 g l^{-1} h^{-1}. A biomass production of 14.1 g/l, DCW, was achieved in 35 h cultivation, which was the highest cell density of *G. oxydans* thus far.

The cell growth curve of *G. oxydans* DSM 2003 under different cultivation conditions in a 3.7-l scaled fermentor is shown in Fig. 1. As Table 3 and Fig. 1 show, this approach to high-density fermentation of *G. oxydans* DSM 2003 proved to be an effective method.

Effects of different cultivations on resting-cell activity

The studies were carried out under different cultivation conditions. The results obtained are given in Fig. 2. With improved cultivation, an increase in the activity of the resting cells was found. The optimum process was not only able to greatly enhance the biomass production, but also to improve the activity of the resting cells, indicating that the process of high cell density fermentation of *G. oxydans* for glycolic acid production was successful.

Batch bioconversion of ethylene glycol to glycolic acid using the resting cells of *G. oxydans* DSM 2003

The time course of glycolic acid production is presented in Fig. 3. A glycolic acid concentration of 70.2 g/l was achieved at 48 h with a molar conversion yield of 86.7%. The HPLC analysis of the reaction mixture after the reaction is shown in Fig. 4. Figure 4 shows that no detectable

Table 3 The results of culture-medium and fermentation-process optimization

Experiment	Biomass (DCW g/l)
Before optimization (in 1-l shake flask)	2.0
Before optimization (batch, in 3.7-l fermentor)	6.0
After optimization (in 1-l shake flask)	2.5
After optimization (batch, in 3.7-l fermentor)	11.5
After optimization (fed-batch, in 3.7-l fermentor)	14.1

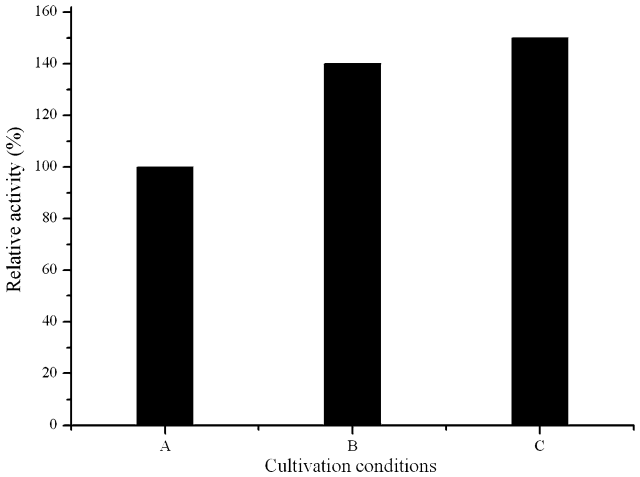


Fig. 2 The relative activity of the resting cells under different cultivations. *A* Batch, before optimization; *B* batch, after optimization; *C* fed-batch, after optimization

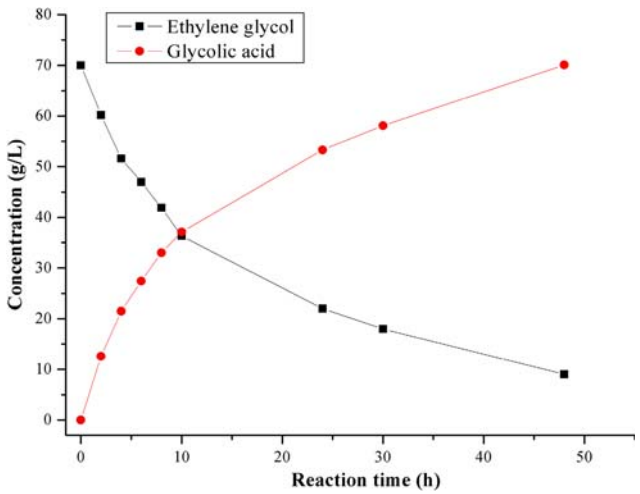


Fig. 3 Conversion of ethylene glycol to glycolic acid by the resting cells of *G. oxydans* DSM 2003

amounts of other compounds were observed. Microbial conversion of ethylene glycol to glycolic acid is expected to be an effective method for the production of glycolic acid with no by-product.

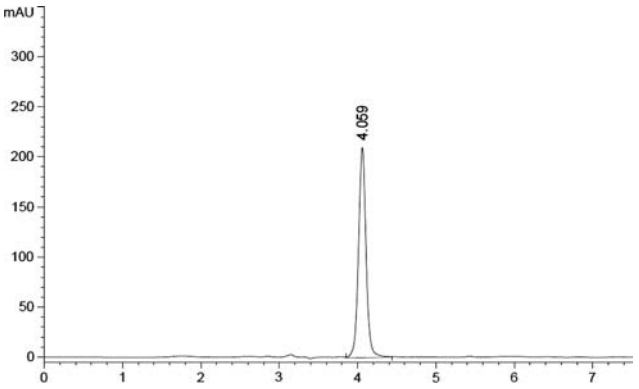


Fig. 4 HPLC spectrum of bioconversion sample at 210 nm

Discussion

Low biomass production has hampered the use of *Gluconobacter* strains for the manufacture of bulk products. To realize the industrialization of glycolic acid, it was necessary to achieve a large biomass production. In this paper, the optimization of culture medium composition and fermentation process for biomass production and bioconversion of ethylene glycol to glycolic acid by *G. oxydans* DSM 2003 was studied.

Using statistical optimization methods, we were able to overcome the limitations of classic empirical methods, and these methods appeared to be a powerful tool for optimization of biomass production of *G. oxydans* DSM 2003. In this study, the UD model was employed to study the combined effects of cultivation conditions on the production of biomass, followed by regression analysis to predict the maximum biomass in the flasks. Further experiments were carried out to verify the fitness of the model. The regression model seemed to fit reasonably well. The results of batch and fed-batch fermentation experiments in a 3.7-l fermentor showed that the biomass was increased greatly by using the optimized culture medium and fermentation process.

Albin reported the highest level of biomass achieved was 13.5 g/l (DCW) within a total feed fermentation time of 43 h [19]. The biomass yield obtained in our present study was 14.1 g/l (DCW) within a total fed-batch fermentation time of 35 h. To the best of our knowledge, this is the highest cell density reported thus far. Furthermore, the resting-cell activity was also improved.

In this study, a higher production of glycolic acid (70.2 g/l) was obtained in a batch biotransformation of *G. oxydans* DSM 2003 using ethylene glycol as a substrate. The higher molar conversion yield (86.7%) could make this process more economically feasible and was shown to be a promising process.

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References

1. De Ley J, Gillis M, Swings J (1984) The genus *Gluconobacter*. In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 1. Williams & Wilkins, Baltimore, pp 267–278
2. Deppenmeier U, Hoffmeister M, Prust C (2002) Biochemistry and biotechnological applications of *Gluconobacter* strains. Appl Microbiol Biotechnol 60:233–242. doi:10.1007/s00253-002-1114-5
3. Prust C, Hoffmeister M, Liesegang H, Wiezer A, Fricke WF, Ehrenreih A, Gottschalk G, Deppenmeier U (2005) Complete genome sequence of the acetic bacterium *Gluconobacter oxydans*. Nat Biotechnol 23:195–200. doi:10.1038/nbt1062
4. Matsushita K, Toyama H, Adachi O (1994) Respiratory chains and bioenergetics of acetic acid bacteria. Adv Microb Physiol 36:247–301. doi:10.1016/S0065-2911(08)60181-2
5. Tkac J, Navratil M, Sturdik E, Gemeiner P (2001) Monitoring of dihydroxyacetone production during oxidation of glycerol by immobilized *Gluconobacter oxydans* cells with an enzyme biosensor. Enzyme Microb Technol 28:383–388. doi:10.1016/S0141-0229(00)00328-8
6. Zigova J, Svitel J, Sturdik E (2000) Possibilities of butyric acid production by butanol oxidation with *Gluconobacter oxydans* coupled with extraction. Chem Biochem Eng Q 14:95–100
7. Gupta A, Singh VK, Qazi GN, Kumar A (2001) *Gluconobacter oxydans*: its biotechnological applications. J Mol Microbiol Biotechnol 3:445–456
8. Krochta JM, Tillin SJ, Hudson JS (1988) Thermochemical conversion of polysaccharides in concentrated alkali to glycolic acid. Appl Biochem Biotechnol 17:23–32. doi:10.1007/BF02779143
9. Kataoka M, Sasaki M, Hidalgo ARGD, Nakano M, Shimizu S (2001) Glycolic acid production using ethylene glycol-oxidizing microorganisms. Biosci Biotechnol Biochem 65:2265–2270. doi:10.1271/bbb.65.2265
10. Hayes MJ, Lauren MD (1994) Chemical stress relaxation of polyglycolic acid suture. J Appl Biomater 5:215–220. doi:10.1002/jab.770050306
11. Yamamoto Y, Uede K, Yonei N, Kishioka A, Ohtani T, Furukawa F (2006) Effects of alpha-hydroxy acids on the human skin of Japanese subjects: the rationale for chemical peeling. J Dermatol 33:16–22. doi:10.1111/j.1346-8138.2006.00003.x
12. Emiliani S, Van den Bergh M, Vannin AS, Biramane J (2000) Comparison of ethylene glycol, 1, 2-propanediol and glycerol for cryopreservation of slow-cooled mouse zygotes, 4-cell embryos and blastocysts. Hum Reprod 15:905–910. doi:10.1093/humrep/15.4.905
13. Fang KT (1980) The uniform design: application of number-theoretic methods in experimental design. Acta Math Appl Sin 3:363–372
14. Wang Y, Fang KT (1981) A note on uniform distribution and experimental design. Chin Sci Bull 26:485–489
15. Leung SYL, Chan WH, Leung CH, Luk CH (1998) Screening the fabrication conditions of ultrafiltration membranes by using the uniform design and regression analysis methods. Chemom Intell Lab Syst 40:203–213. doi:10.1016/S0169-7439(98)00019-7
16. Fang Q, Yeung HW, Leung HW, Huie CW (2000) Micelle-mediated extraction and preconcentration of ginsenosides from Chinese herbal medicine. J Chromatogr A 904:47–55. doi:10.1016/S0021-9673(00)00911-0
17. Xu CP, Yun JW (2003) Optimization of submerged-culture conditions for mycelial growth and exo-biopolymer production by *Auricularia polytricha* (wood ears fungus) using the methods of uniform design and regression analysis. Biotechnol Appl Biochem 38:193–199. doi:10.1042/BA20030020
18. Tang QY, Feng MG (2002) DPS Data Processing System for practical statistics. Science Press, Beijing, pp 308–313
19. Albin A, Bader J, Mast-Gerlach E, Stahl U (2007) Improving fermentation and biomass formation of *Gluconobacter oxydans*. J Biotechnol 131:160–161. doi:10.1016/j.jbiotec.2007.07.884