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Medium optimization of *Rhodococcus erythropolis* LSSE8-1 by Taguchi methodology for petroleum biodesulfurization

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Abstract–High production of *Rhodococcus erythropolis* LSSE8-1 and its application for the treatment of diesel oils was investigated. Culture conditions were optimized by Taguchi orthogonal array experimental design methodology. High cell density cultivation of biocatalyst with pH control and fed-batch feeding strategies was further validated in a fermentor with the optimal factors. Cell concentration of 23.9 g dry cells/L was obtained after 96 h cultivation. The resting cells and direct fermentation suspension were applied for deep desulfurization of hydrodesulfurized diesel oils. It was observed that the sulfur content of the diesel decreased from 248 to 51 μg/g by two consecutive biodesulfurizations. It implied that the biodesulfurization process can be simplified by directly mixing cell cultivation suspension with diesel oil. The biocatalyst developed with the Taguchi method has the potential to be applied to produce ultra-low-sulfur petroleum oils.

Key words: Biodesulfurization, High Cell Density Cultivation, *Rhodococcus erythropolis*, Taguchi Method

INTRODUCTION

The combustion of sulfur-containing fossil fuels produces environmentally hazardous SOx. Conventional hydrodesulfurization (HDS) of petroleum fractions involves an inorganic catalyst and hydrogen under high temperature and pressure conditions [1,2]. Furthermore, HDS is not effective for removing heterocyclic sulfur compounds such as dibenzothiophenes (DBTs) with substitutions adjacent to the sulfur moiety [3]. Over the past 20 years there has been considerable interest in developing new technologies to remove sulfur from petroleum distillates. Biotechnology may provide an alternative 'green' way to process fossil fuels [4,5]. Biocatalytic desulfurization (BDS) has the potential benefits of lower capital and operating costs and will produce substantially less greenhouse gases [6].

Many desulfurizing microorganisms, including *Rhodococcus rhodochrous* IGTS8 [7], *R. erythropolis* H-2 [8] and KA2-5-1 [9], *Mycobacterium* sp. [10], *Pseudomonas* sp. [11], *Nocardia* sp. [12], have been isolated. The genes related to DBT metabolism also have been cloned and characterized [13]. And, in the recent years, several papers have studied the influences of operational conditions on the cell growth and desulfurization rate [14-16]. Despite considerable progress in BDS, there are still some bottlenecks limiting the commercialization of the BDS process. Critical aspects of the process development include biocatalyst preparation, reactor design, product or by-product recovery, and oil-water separation [5,17,18]. Mass production of biocatalyst with high desulfurization activity, which is carried out by high cell density cultivation (HCDC), is an important technique for decreasing the cost of biocatalysts.

For efficient production of desulfurizing cells, it is highly essential to optimize all the culture conditions and composition for production media, which further facilitates economic design of the full-scale fermentation operation system. Conventional optimization procedures involve altering of one parameter at a time keeping all other parameters constant, which enables one to assess the impact of those particular parameters on the process performance. These procedures are time consuming. Unlike traditional one-at-a-time optimization strategy, Taguchi orthogonal array experimental design can handle any given system by a set of independent variables (factors) over a specific region of interest (levels). The Taguchi method facilitates identifying the influence of individual factors, establishing the relationship between variables and operational conditions and finally establishing the performance at the optimum levels obtained with a few well-defined experimental sets [19]. It has been increasingly used for various phases of an optimization process [20].

The aim of this work was to investigate the optimum culture conditions for achieving high cell density of *R. erythropolis* LSSE8-1 and to study the desulfurizing characteristics of the obtained cells. We found that both the resting cells and the direct fermentation suspension obtained from the HCDC showed high desulfurization activity. We therefore developed a simple BDS process by directly mixing cell cultivation suspension with diesel to yield ultra-low-sulfur petroleum oils.

MATERIALS AND METHODS

1. Bacterial Strain and Cultivation

Rhodococcus erythropolis LSSE8-1 (CGMCC 0643) was able to convert DBT to 2-hydroxybiphenyl (2-HBP) as the dead-end metabolite through a sulfur-specific pathway [21]. The bacterium was grown in minimal salt medium (MSM) containing (per liter) 2.44 g of KH_2PO_4, 12.03 g of $Na_2HPO_4 \cdot 12H_2O$, 2.0 g of NH_4Cl, 0.4 g of

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$MgCl_2 \cdot 6H_2O$, and 1% (v/v) of trace element solution. The trace element solution contained (per liter) 0.75 g of $CaCl_2$, 1 g of $FeCl_3 \cdot 6H_2O$ and 4 g of $MnCl_2 \cdot 4H_2O$. MSM supplemented with 1% (w/v) glycerol as the carbon source and 0.2 mmol/L DBT as a sulfur source was used in flask cultures. The initial pH value of the MSM medium was adjusted to about 7.1.

Flask cultures were carried out to select carbon, nitrogen and sulfur sources in 100 mL flasks which contained 20 mL of MSM for 48 h at 30 °C and 170 rpm (rotates per min) in a reciprocal shaking incubator. The inoculum size was 4% (v/v).

High cell density cultures were carried out at 30 °C in a 6.6 L fermentor (Bioflo 3000, New Brunswick Scientific Co. Inc., USA) containing initially 3 L of optimum culture selected in the flask experiments. The inoculum size was also 4% (v/v). The pH was maintained at 7.1 by using 10% (wt%) NaOH solution. The dissolved oxygen (DO) concentration was adjusted to 40% air saturation by manipulating the agitation speed from 50 to 650 rpm, and the aeration rate from 0.7 to 1.5 L/min. Both the pH and DO in the medium were monitored and controlled on-line with a computer controlled system. Dimethiconum antifoaming agent was added to prevent overflow.

2. Optimization Procedure and Experimental Design

A stepwise optimization was performed as follows: (1) employment of a one-at-a-time method to screen the optimal carbon, nitrogen and sulfur sources; and (2) optimization of culture components for cell growth and desulfurization activity by Taguchi methodology. The proposed submerged fermentation experiments were designed with five factors (carbon, nitrogen, sulfur source, magnesium chloride and trace element solution) at four levels with orthogonal array layout of L16 (4^5). All statistical experimental designs and results analyses were carried out by using Minitab 14 for Windows (Minitab Inc.).

3. Desulfurization of Diesel Oils

Two types of hydrodesulfurized diesel oil were obtained from SINOPEC Research Institute of Petroleum Processing. A-diesel contains 555 μg/g of total sulfur. The total sulfur is 248 μg/g in B-diesel which is used for deep BDS. Cells were harvested after centrifugation and then re-suspended in 0.1 mol/L phosphate buffer (pH 7.0) containing 10 g/L glycerol for determining the desulfurizing capability as resting cells. Biodesulfurization was performed by mixing cell culture suspension or resting cells with diesel oil in 100 mL flasks. The volume ratio of oil phase to aqueous phase was 1 : 2. The reactions were carried out in flasks at 30 °C on a rotary shaker at 170 rpm. All experiments were triplicated.

4. Analytical Methods

Cell growth was monitored by measuring optical density with a UV/Vis spectrometer (Lambda Bio40, Perkin Elmer Instruments, USA) at 600 nm. Calibration of the spectrophotometric response against cell dry weight (DCW) was performed: One OD_{600} unit corresponded to 0.395 g DCW/L.

During the course of bacterial growth, aliquots of the culture were acidified to pH=2 with HCl and extracted with equal volume of n-hexane for analysis. HPLC was performed on an Agilent 1100 liquid chromatograph equipped with an autosampler, a reversed phase Zorbax SB-C18 column (4.6×150 mm; 3.5 μm) and a diode array detector for the quantitative assay of DBT (retention time=5.49 min) and 2-hydroxybiphenyl (retention time=3.29 min). The mobile phase was 90% of methanol in water (v%) with flow rate of 1 $mL \cdot min^{-1}$, by using the external standard method at 254 nm. The specific desulfurization activity was assessed as the production rate of 2-hydroxybiphenyl (2-HBP), which is the end-product through DBT degradation pathway.

After the desulfurization reaction, total sulfur of diesel was measured according to previous reports [22,23]. The total sulfur content (by weight) was determined in triplicate for each sample by combustion of samples and measurement of the released sulfur dioxide with a microcoulomb analyzer (RPA-200, JiangSu JiangHuan Electroanalysis, China).

RESULTS AND DISCUSSION

1. Screening the Optimal Nitrogen, Carbon and Sulfur Sources

Eight kinds of carbon sources, five kinds of nitrogen and five kinds of sulfur sources were investigated with one-at-a time strategy. *R. erythropolis* LSSE8-1 was cultivated in MSM containing various carbon sources (see Table 1). 8 g/L of carbon source and 0.2 mmol/L of DBT were supplied, and cultures were carried out for 48 h. As shown in Table 1, glycerol showed the highest growth and desulfurization activity. The final optical cell density achieved 10.45 (OD_{600}). Sucrose, glycose, trisodium citrate dehydrate and ethanol showed a higher growth quantity than others.

The effects of nitrogen sources are shown in Table 2. Of all the nitrogen sources that were investigated, ammonium chloride, urea, ammonium nitrate, ammonium acetate showed similar growth rates and desulfurization activity. Ammonium chloride was used for all subsequent work.

R. erythropolis LSSE8-1 was grown in MSM which contained various sulfur sources (Table 3). DBT and dimethylsulfoxide (DMSO)

Table 1. Effect of carbon sources (8 g/L) on cell growth and specific desulfurization activity

Carbon sources	Growth (OD_{600})	Specific desulfurization activity ($\times 10^{-3}$ mmol/L/h)
Glycerol	10.45	3.85
Sucrose	8.72	3.35
Glycose	8.13	3.29
Ethanol	7.38	2.96
Trisodium citrate dihydrate	8.10	3.23
Sodium succinate	7.09	2.54
Potassium acetate	6.76	2.00
Paraffin	0.54	0.202

Table 2. Effect of nitrogen sources (0.04 mmol/L) on cell growth and desulfurization activity

Nitrogen sources	Growth (OD_{600})	Specific desulfurization activity ($\times 10^{-3}$ mmol/L/h)
Ammonium chloride	10.98	3.96
Urea	10.95	3.85
Ammonium nitrate	10.38	3.81
Ammonium acetate	10.66	3.85
Magnesium nitrate	6.56	1.98

Table 3. Effect of sulfur sources (0.2 mmol/L) on cell growth and desulfurization activity

Sulfur sources	Growth (OD_{600})	Specific desulfurization activity[a] ($\times 10^{-3}$ mmol/L/h)
DBT	8.85	7.46
DMSO	3.53	3.37
DMSO[b]	9.51	7.60
Sodium sulfate	5.81	0.83
Taurine	5.23	3.54

[a]After 48 h of growth, the cultures with sulfur sources were centrifuged and resuspended in saline solution containing 0.2 mmol/L DBT. Then desulfurization activities of these resting cell samples were determined after 24 hours reaction.
[b]DMSO (0.5 mmol/L) was used as the sulfur source in the experiment.

were appropriate sulfur sources for growth. As Gou et al. [21] reported, the cells of LSSE8-1 cultivated in MSM with DBT as the sole sulfur source showed a high growth and desulfurization activity. However, mass production of biocatalyst using DBT was considered to be impractical as a result of high price and the growth inhibition by 2-HBP. Moreover, there was a problem that residual DBT and DBT metabolites would contaminate oil products. As shown in Table 3, it appears to be the highest result in the case of the culture with 0.5 mmol/L DMSO, which had a different concentration level from others (0.2 mmol/L). Thus, DMSO was more suitable than DBT as a sulfur source.

2. Optimization of Culture Composition of LSSE8-1 Cells by Taguchi Methodology

As presented in Table 4, five factors, namely glycerol, ammonium chloride, DMSO, magnesium chloride and trace element solution at four levels with an orthogonal array layout of L16 (4^5), were selected for the Taguchi method. Submerged fermentation experiments were performed for cell production with *R. erythropolis* LSSE8-1 employing the 16 experimental trials and the result is shown in Table 5. The designed experimental condition showed significant variation in the growth. The growth levels were found to be dependent on the culture conditions.

The effects on the growth were determined by the analysis of variance technique to determine which factors were statistically significant. As shown in Table 6, the contribution percentage of DMSO is 73.3%. DMSO was statistically significant ($P<0.05$) related to the growth. The influence of these variables on the growth (48 h) was found to decline in the order: DMSO>magnesium chloride>ammonium chloride>trace element solution>glycerol.

The influence of each individual factor on the cell growth is shown in Fig. 1. The resulting optimum conditions were glycerol 10 g/L, ammonium chloride 3 g/L, DMSO 3 mmol/L, magnesium chloride 2 g/L and trace element solution 0.5%.

According to the optimum conditions, triplicate experiments were

Table 4. Selected culture condition factors and assigned levels

Serial no.	Factor	Level 1	Level 2	Level 3	Level 4
1	Glycerol (g/L)	5	10	20	30
2	Ammonium chloride (g/L)	1	2	3	4
3	DMSO (mmol/L)	0.2	0.8	1.5	3
4	Magnesium chloride (g/L)	0.2	0.5	1	2
5	Trace element solution (%)	0.5	1	2	3

Table 5. Design and experimental results of the L16 (4^5) orthogonal array experiment

Experiment no.	Column					Growth (OD_{600}) 48 h
	Glycerol	Ammonium chloride	DMSO	Magnesium chloride	Trace element solution	
1	5	1	0.2	0.2	0.5	3.36
2	5	2	0.8	0.5	1	5.05
3	5	3	1.5	1	2	10.08
4	5	4	3	2	3	10.73
5	10	1	0.8	1	3	9.83
6	10	2	0.2	2	2	3.51
7	10	3	3	0.2	1	10.81
8	10	4	1.5	0.5	0.5	7.80
9	20	1	1.5	2	1	9.99
10	20	2	3	1	0.5	11.72
11	20	3	0.2	0.5	3	2.13
12	20	4	0.8	0.2	2	4.87
13	30	1	3	0.5	2	8.40
14	30	2	1.5	0.2	3	6.59
15	30	3	0.8	2	0.5	11.11
16	30	4	0.2	1	1	0.04

Table 6. Analysis of variance (ANOVA)

Source of variance	Degree of freedom	Sum of square	Mean square	F value	P value	Percentage (%)
Glycerol	3	4.3	1.4	0.09	0.966	2.1
Ammonium chloride	3	17.1	5.7	0.37	0.776	8.5
DMSO	3	147.99	49.33	11.02	0.001	73.3
Magnesium chloride	3	22.6	7.5	0.50	0.687	11.2
Trace element solution	3	9.8	3.3	0.20	0.891	4.9
Total	15	201.79				100

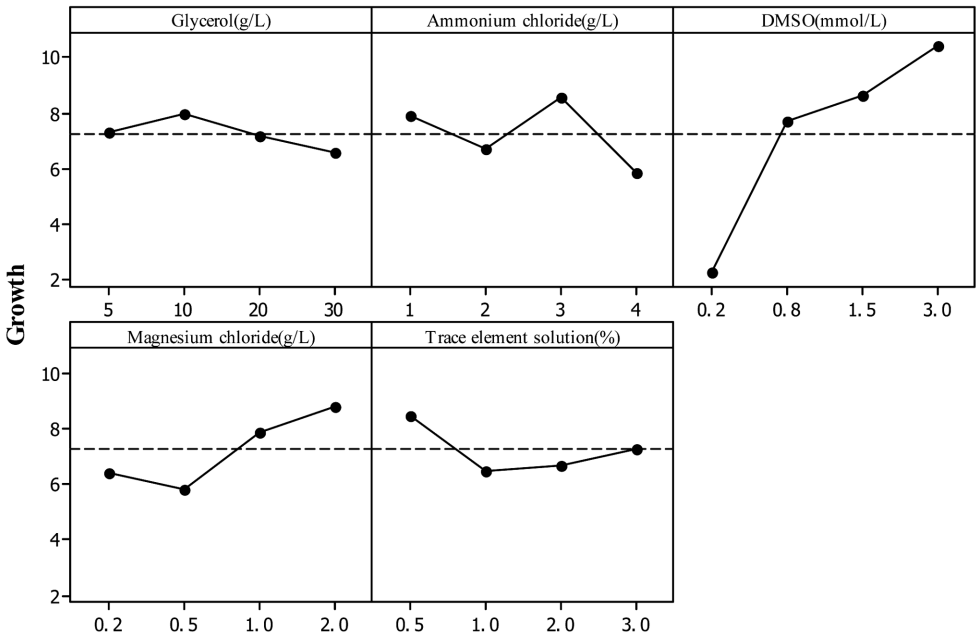

Fig. 1. Main effects plot of individual factors performance at different levels.

performed to confirm whether the optimum conditions were reproducible. The optimized culture conditions showed an enhanced cell production of 70% (from 3.40 to 5.78 g dry cells/L at 48 h cultivation). Application of Taguchi approach appears to have potential usage in fermentation optimization.

3. High Cell Density Culture in a Jar-fermentor

A batch culture was first operated without controlling the pH, with the result that the pH of the culture slowly decreased from 7.22 to 5.56 (data not shown). It was necessary to hold pH at proper value because lower pH was not appropriate to the cell growth. Thus, high cell density cultivation of *R. erythropolis* LSSE8-1 using pH control and fed-batch feeding strategies was further validated in the fermentor with the optimal factors. The pH was maintained at 7.1 during the whole fermentation process. The fed batch culture included an initial batch culture (3 L) followed by a feeding stage with constant flow of 0.3 mL/min. Initially, the batch culture contained glycerol 10 g/L, ammonium chloride 3 g/L, DMSO 3 mM, magnesium chloride 2 g/L, trace element solution 0.5% and 2-fold higher phosphate than the original MSM. 1 g/L of yeast extract was supplemented to enhance cell growth. After 44 h cultivation, 400 ml of feed culture was supplemented at a constant flow, which contained 0.3 mmol DBT, 9 mmol DMSO, 30 g glycerol, 45 ml ethanol, 30 g sucrose, 9 g NH_4Cl and 15 mL trace element solution. As

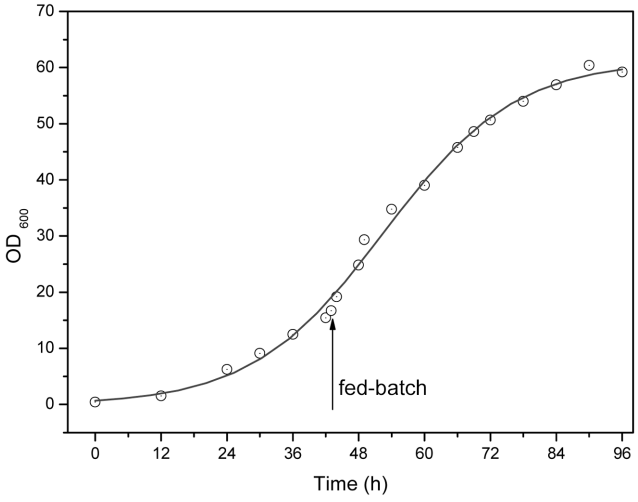

Fig. 2. Growth curves of fed-batch culture of LSSE8-1. Arrow indicates the feeding start point.

a result, a cell concentration of 23.9 g dry cells/L (OD_{600}=60.4) was obtained after 96 h cultivation as shown in Fig. 2.

Considering that the BDS process requires at least 20-40 g/L of

biocatalyst and the process must be achieved on industrial scale, the biocatalyst production rate is an important factor in the process development. At present, there are only few reports on the high density cultivation of cells for BDS. Wang et al. [24] reported a batch culture system to determine the kinetic parameters of culture for the strain *R. erythropolis* N1-36, accomplishing a maximum cellular concentration of 4.3 g/L (1×10^{10} cells/ml). Honda et al. [25] reported that about 30 g/L of *R. erythropolis* IGTS8 could be obtained by pH-stat feeding strategy in a jar-fermentor. Chang et al. [26] further reported production of desulfurization biocatalysts by two-step fermentation, in which the steps of cell growth and the desulfurization activity inducement are separated. Shan et al. [22] reported 25 g/L of cell density was obtained with *P. delafieldii* R-8 from high density culture. In our study, a new method was performed in HCDC by one step in which a multiple of DMSO and DBT was used. The function of the small quantity of DBT was the inducement of *Dsz* enzymes. Though cell density was less than that reported by Chang et al. [26], it results from different type and characteristic of microorganism.

4. Biodesulfurization for Hydrodesulfurized Diesel Oil

Forty milliliters of cell suspension (OD_{600}=34) was taken out from the fermentor and averagely divided into two portions. One part of suspension was collected by centrifugation and the upper solution was moved away. The collected cells were re-suspended into 20 ml of above-mentioned phosphate buffer, and mixed with 10 ml of A-diesel as resting cells desulfurization reaction. Another part of the suspension was mixed directly with 10 ml of A-diesel. These two processes had similar time courses of BDS and a similar BDS rate (see Fig. 3). With respect to cell suspension and resting cells, the desulfurization rate for the first 4 h was about 59.7 and 65.6 μmol-sulfur/g DCW/h, respectively, when the oil/water (O/W) phase ratio was 1 : 2. From a practical point of view, higher desulfurization rates and higher oil/water phase volumetric ratio are more convenient for BDS industrial applications [27,28]. In the literature, the desulfurization rates obtained with diesel oil were 10.8 [26] at 1 : 10 O/W ratio and 4 μmol/g DCW/h [29] at 1 : 9 O/W ratio. We achieved significantly higher desulfurization rates than others. And more importantly, we got the results at so high an O/W ratio. Although the highest desulfurization rates (250 μmol 2-HBP/g DCW/h) had been obtained by Konishi et al. [30], these rates were derived from experiments employing DBT in model systems rather than in the treatment of actual petroleum products.

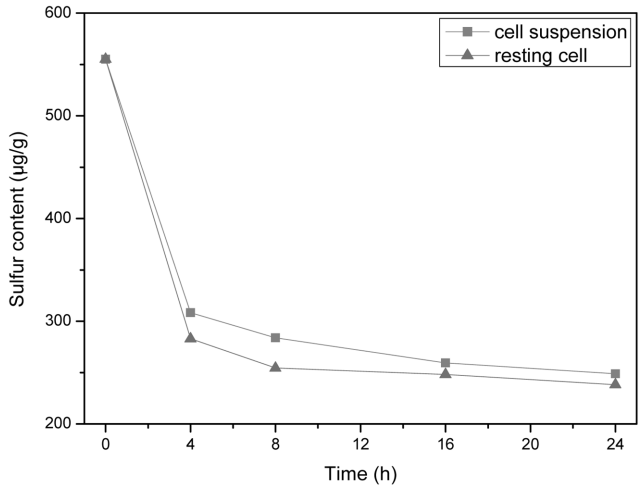

Fig. 3. Time courses of desulfurization of diesel oil using culture suspension and resting cells of *R. erythropolis* LSSE8-1. The experiments were carried out in 100 mL flasks and the oil/water phase ratio was 1 : 2.

After LSSE8-1 cells were cultivated for 72 h, 1.2 L of fermentation suspension (OD_{600} of cell suspension was 50) was directly reacted with 600 ml of B-diesel in the fermentor to produce low-sulfur oils. In order to further decrease the sulfur content, the treated oil was recovered after 24 h and reacted again with fresh fermentation suspension for another 24 h. The sulfur was reduced to 103 μg/g at a 1 : 2 oil/water ratio in the first BDS treatment. And finally it resulted in a 79.4% reduction of total sulfur from 248 to 51 μg/g in two consecutive BDS processes.

Thus it can be seen that both the resting cells and direct fermentation suspension can be absolutely applied for diesel desulfurization. According to these results, it is feasible that high cell density suspension containing the metabolites of sulfur source can be directly used for diesel biodesulfurization. The BDS process was apparently simplified, which had the advantage of cost saving. Thus, the simple process is convenient to treat a mass of petroleum fractions, and a continuous operation might be applied. The biocatalyst developed with the Taguchi method in this study has the potential for deep biodesulfurization process to yield ultra-low-sulfur petroleum oils.

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REFERENCES

1. R. Vazquez-Duhalt, E. Torres, B. Valderrama and S. Le Borgne, *Energy Fuels*, **16**, 1239 (2002).
2. C. S. Song, *Catal. Today*, **86**, 211 (2003).
3. B. L. McFarland, *Curr. Opin. Microbiol.*, **2**, 257 (1999).
4. D. J. Monticello, *Curr. Opin. Biotech.*, **11**, 540 (2000).
5. N. Gupta, P. K. Roychoudhury and J. K. Deb, *Appl. Microbiol. Biot.*, **66**, 356 (2005).
6. L. Linguist and M. Pacheco, *Oil Gas J.*, **97**, 45 (1999).
7. J. J. Kilbane and K. Jackowski, *Biotechnol. Bioeng.*, **40**, 1107 (1992).
8. T. Ohshiro, T. Hirata, I. Hashimoto and Y. Izumi, *J. Ferment. Bioeng.*, **82**, 610 (1996).
9. H. Yan, M. Kishimoto, T. Omasa, Y. Katakura, K. Suga, K. Okumura and O. Yoshikawa, *J. Biosci. Bioeng.*, **89**, 361 (2000).
10. T. Furuya, K. Kirimura, K. Kino and S. Usami, *FEMS Microbiol. Lett.*, **204**, 129 (2001).
11. M. F. Luo, J. M. Xing, Z. X. Gou, H. Z. Liu and J. Y. Chen, *Korean J. Chem. Eng.*, **20**, 702 (2003).
12. J. H. Chang, Y. K. Chang, H. W. Ryu and H. N. Chang, *FEMS Microbiol. Lett.*, **182**, 309 (2000).
13. K. A. Gray, O. S. Pogrebinsky, G. T. Mrachko, L. Xi, D. J. Monti-

cello and C. H. Squires, *Nat. Biotechnol.*, **14**, 1705 (1996).
14. C. H. del Olmo, V. E. Santos, A. Alcon and F. Garcia-Ochoa, *Biochem. Eng. J.*, **22**, 229 (2005).
15. S. Guchhait, D. Biswas, P. Bhattacharya and R. Chowdhury, *Chem. Eng. J.*, **112**, 145 (2005).
16. M. Rashtchi, G. H. Mohebali, M. M. Akbarnejad, J. Towfighi, B. Rasekh and A. Keytash, *Biochem. Eng. J.*, **29**, 169 (2006).
17. S. Le Borgne and R. Quintero, *Fuel Process Technol.*, **81**, 155 (2003).
18. J. D. Van Hamme, A. Singh and O. P. Ward, *Microbiol. Mol. Biol. R.*, **67**, 503 (2003).
19. K. K. Prasad, S. V. Mohan, R. S. Rao, B. R. Pati and P. N. Sarma, *Biochem. Eng. J.*, **24**, 17 (2005).
20. M. Y. Chang, G. J. Tsai and J. Y. Houng, *Enzyme Microb. Tech.*, **38**, 407 (2006).
21. Z. X. Gou, H. Z. Liu, M. F. Luo, S. Li, J. M. Xing and J. Y. Chen, *Sci. China Ser. B*, **45**, 521 (2002).
22. G. B. Shan, H. Y. Zhang, J. M. Xing, C. Guo, W. L. Li and H. Z. Liu, *Biochem. Eng. J.*, **27**, 305 (2006).
23. B. Yu, C. Ma, W. Zhou, Y. Wang, X. Cai, F. Tao, Q. Zhang, M. Tong, J. Qu and P. Xu, *FEMS Microbiol. Lett.*, **258**, 284 (2006a).
24. P. Wang and S. Krawiec, *Appl. Environ. Microb.*, **62**, 1670 (1996).
25. H. Honda, H. Sugiyama, I. Saito and T. Kobayashi, *J. Ferment. Bioeng.*, **85**, 334 (1998).
26. J. H. Chang, Y. J. Kim, B. H. Lee, K. S. Cho, H. W. Ryu, Y. K. Chang and H. N. Chang, *Biotechnol. Prog.*, **17**, 876 (2001).
27. L. Setti, G. Lanzarini and P. G. Pifferi, *Fuel Process Technol.*, **52**, 145 (1997).
28. J. J. Kilbane, *Curr. Opin. Biotechnol.*, **17**, 305 (2006).
29. B. Yu, P. Xu, Q. Shi and C. Q. Ma, *Appl. Environ. Microb.*, **72**, 54 (2006b).
30. M. Konishi, K. Kishimoto, T. Omasa, Y. Katakura, S. Shioya and H. Ohtake, *J. Biosci. Bioeng.*, **99**, 259 (2005).