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Optimum synthesis of esomeprazole catalyzed by *Rhodococcus rhodochrous* ATCC 4276 through response surface methodology

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Abstract−Enantiopure esomeprazole is an important drug in the treatment of gastric ulcer. The asymmetric sulfoxidation of omeprazole thioether was catalyzed by immobilized cells of a mutant of *Rhodococcus rhodocrous* ATCC 4276 to synthesize esomeprazole. The bioreaction was carried out in a biphasic system (chloroform-water), at a high substrate concentration (200 mM), and optimized using response surface methodology (RSM). The optimal yield of esomeprazole obtained was 94.8% with e.e. (>99%) without the formation of the sulfone form as a byproduct, under the optimal conditions: the concentration of immobilized cells, 283.5 g/L, the incubation temperature, 37.05 °C, and pH of phosphate buffer, 7.35, respectively. A quadratic polynomial model was developed with R^2 of 0.9998, which indicates that the model predicts the observed data with very high accuracy. The mutant exhibited a high enantioselective activity and substrate and product tolerance. The small size of immobilized cell beads (0.5-1 mm) creates a large reaction interface. The aerated flask provides enough oxygen for a high concentration of cells. The significant improvement of substrate tolerance may mainly be attributed to employing the chloroform-water biphasic system because organic substrates may be partitioned in the organic phase, eliminating potential damage and inhibition to cells. Based on the above, the asymmetric sulfoxidation catalyzed by immobilized bacterial cells is therefore more promising for efficient synthesis of chiral sulfoxides.

Keywords: Esomeprazole, Immobilized Cells, Response Surface Methodology, Organic-aqueous Biphasic Systems, Asymmetric Sulfoxidation

INTRODUCTION

Proton pump inhibitors (PPI), such as omeprazole and lansoprazole, have been used to treat stomach ulcers caused by hyperchlorhydria; however, only *S*-enantiomer of PPI has therapeutic effect due to its stereoselective pharmacokinetics [1,2]. Esomeprazole, with chiral sulfoxide structure, is an *S*-enantiomer of omeprazole. Chiral sulfoxides can be synthesized by the asymmetric sulfoxidation of sulfides with metal complexes [3-6] or enzymatic catalysts, such as monooxygenases [7,8], horseradish peroxidases [9-12], hemoglobin myoglobin [13] and a cytochrome C [14], which has many disadvantages such as environmental damage, requiring a cofactor cycling system and high cost. However, chiral sulfoxides can also be synthesized with whole cell bio-sulfoxidation of prochiral sulfides, which has many advantages including cost-effectiveness and no requiring expensive cofactor regeneration. Up to now, several whole cell bio-sulfoxidation of prochiral sulfides have been conducted in aqueous single-phase systems [15-21].

Specially, several syntheses of proton pump inhibitors were performed by whole cell bio-sulfoxidation of sulfides. The chiral sulfoxidation of the omeprazole sulfides was carried out using 15 strains with e.e. of 17% to 99% of the (R) configuration [22]. A fungal strain was used to form rabeprazole with 99% e.e. of the (S) configuration, and also form omeprazole and lansoprazole with the yield of 49% and 0.6%, respectively [23]. All those studies were also in aqueous single-phase systems and the substrate concentration was very low (0.08-1.5 g/L); conversions decreased significantly with the increase of the substrate concentration due to intense substrate and product inhibition on the cells [24].

In whole cell asymmetric oxidation, the immobilization of cells has been used to significantly improve the oxidation activity of cells [25], for example, up to approximately 2.5 fold [26] and the tolerance to substrate inhibition as compared with free cells [25,27,28]. In the synthesis of chiral sulfoxides by whole cell biooxidation of

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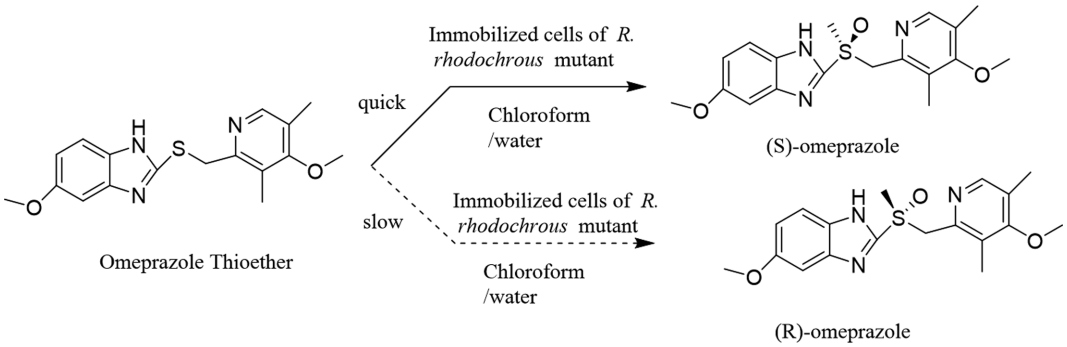

Scheme 1. Asymmetric bio-oxidation using immobilized cells of *Rhodococcus rhodochrous* ATCC 4276 mutant for preparation of (*S*)-omeprazole.

organic sulfides, organic-aqueous biphasic systems, such as the isooctane-aqueous biphasic system, were also employed to markedly elevate the tolerance to substrate inhibition of cells [29-31].

To the best of our knowledge, however, there are few reports for the asymmetric sulfoxidation of the omeprazole thioether in organic-aqueous biphasic systems using immobilized cells to form the chiral sulfoxide, esomeprazole. In the present study, both organic-aqueous biphasic systems and immobilized cells of a mutant of *Rhodococcus rhodochrou*s ATCC 4276 were thus employed to synthesize optically pure esomeprazole (Scheme 1). The concentration of the omeprazole thioether and the yield of esomeprazole was substantially improved by optimization of the bio-sulfoxidation using response surface methodology (RSM) with significantly fewer experiments.

MATERIALS AND METHODS

1. Chemicals and Microbial Strain

Esomeprazole, i.e. (*S*)-omeprazole, was obtained from Suzhou Vita Chemical Co., Ltd, omeprazole from Shandong Shouguang Fukang Pharmaceutical Co., Ltd, sodium alginate (SA), Qingdao Mingyue seaweed Group Co., Ltd, sodium alginate (SA), chloroform and acetonitrile, from Qingdao Hailitan Chemical Reagent Co., Ltd and omeprazole thioether (OT) from Jinan Wald Chemical Co., Ltd, respectively. All other chemical reagents were commercially available with analytical grade purity and used with no further purification. A mutant of *R. rhodochrous* ATCC 4276 used in the present study was obtained by complex mutagenesis induced by using 0.025 moL/L $NaNO_2$ for 20 min and UV irradiation with a distance of 20 cm for 30 seconds. The resultant mutant had a better bio-oxidation activity and tolerance to substrates than the wild-type strain.

2. Preparation of Immobilized Cell Beads

Strains of a mutant of *Rhodococcus rhodochro*us ATCC 4276 were inoculated 1-L Erlenmeyer flasks containing 200 mL of a medium composed of potassium nitrate (1 g), potassium dihydrogen phosphate (1 g), dipotassium hydrogen monophosphate (1 g), sodium chloride (1 g), magnesium sulfate heptahydrate (0.2 g), calcium chloride dihydrate (0.02 g), ferric chloride (0.001 g), yeast extract powder (1 g), n-hexadecane (1 ml) per liter of distilled water, adjusted pH to 6.8-7.0 with 1 mol/L sodium hydroxide solution. Incubation was carried out at 30 °C for 62 h on a rotary shaker at 160 rpm. The cells were then harvested by centrifugation at 5,000 rpm for 15 min and directly used to prepare immobilized cells.

Immobilized cell beads were prepared as follows. Harvested cells of a mutant of *R. rhodochrous* were suspended in an autoclaved 3.0% SA solution to result in the final concentration of 0.6 g wet cell/mL. The SA-cell mixture liquid was drawn into a syringe with a needle and then injected dropwise into a sterile solution of $CaCl_2$ (2%, w/v) with mechanical stirring, which resulted in beads of immobilized cells of the mutant of *R. rhodochrou*s (0.5-1 mm size) due to very small droplets ejected through the needle. The beads were washed twice with sterile deionized water and then preserved at 4 °C for 5 h for hardening and stored in a refrigerator until used.

3. Sulfoxidation of Omeprazole Thioether Catalyzed by Immobilized Cells in an Organic-aqueous Biphasic System to form Esomeprazole

The sulfoxidation was carried out in a 1 L Erlenmeyer flask placed on a bath shaker at 150 rpm and 34-40 °C for 95-115 h. To introduce clean air into the flask, a stainless steel pipe (inner diameter of 3 mm), which was connected to clean air source with silicone rubber hose, was assembled and clean air was ventilated in 0.2 v/v flow rate via the pipe. The organic-aqueous biphasic system was composed of chloroform and phosphate buffer (pH 6.3-8.3, glucose 2 g/L) with a ratio of 1/9. The omeprazole thioether was dissolved in chloroform, resulting in a final concentration of 100-200 mM (32.9-65.8 g/L), in which both the substrate and product concentrations were only based on the volume of organic phase unless otherwise specified. The final concentration was 10-20 mM (3.29-6.58 g/L), when the concentration was based on the total volume of reaction media including aqueous and organic phase. Phosphate buffer, the omeprazole thioether-chloroform solution and immobilized cell beads were added successively in a 1 L flask to make a final volume of 0.3-0.4 L, resulting in the final concentration of 200-300 g bead/L and 1/9 ratio of organic/aqueous phase. Samples were withdrawn from reaction mixture and filtered to regain immobilized cell beads, organic and aqueous phase, respectively. The aqueous phase was extracted twice with chloroform and then the resulting chloroform layer was combined with the previously obtained organic phase. The enantiomeric purity and conversion in the obtained organic phase were assayed by HPLC. The obtained immobilized cell beads were washed twice with sterile deionized

water for reuse.

4. HPLC Analysis

The concentrations of the omeprazole thioether and omeprazoles of both *S* and *R* form were analyzed with an HPLC system (Agilent 1200 LC) with a DAD detector at 302 nm and 30 °C, assembling a chiral column Amylose-SA (250×4.6 mm, 5 μm, YMC, Japan). The enantiomeric excess (e.e.) value of (*S*)-omeprazole was calculated as [(S−R)/(S+R)]×100%, where S and R were the concentration of (*S*)- and (*R*)-enantiomers, respectively. 20 μL of sample solution was used for injection using the mobile phase of a mixture of acetonitrile and phosphate buffer (pH 6.0) (15 : 85 v/v) at 1.3 mL min^{-1}. The retention times for omeprazole thioether, *S* and *R* form omeprazole were 9.6, 6.9 and 5.8 min, respectively.

5. NMR Spectrum

Both the ^{1}H and ^{13}C NMR spectra of (*S*)-omeprazole were recorded in DMSO-d_6 on Bruker AV-500, 500 MHz for ^{1}H and 125 MHz for ^{13}C, respectively. ^{1}H NMR: δ 8.10 (s, 1H), 7.51 (d, *J*= 7.5 Hz, 1H), 7.09 (d, *J*=1.3 Hz, 1H), 6.97 (dd, *J*=7.5, 1.3 Hz, 1H), 5.00 (s, 1H), 4.76 (s, 2H), 3.87 (s, 3H), 3.69 (s, 3H), 2.23 (s, 3H), 2.16 (s, 3H). ^{13}C NMR: δ 166.19 (s), 164.25 (s), 158.20 (s), 150.87 (s), 149.70 (s), 140.20 (s), 139.29 (s), 127.52 (s), 126.29 (s), 116.18 (s), 114.20 (s), 99.81 (s), 61.27 (s), 59.86 (s), 55.56 (s), 12.94 (s), 11.28 (s). Both the ^{1}H and ^{13}C NMR data obtained for (*S*)-omeprazole agree with the literature values [32].

6. Experimental Design and Statistical Analysis

The sulfoxidation of the omeprazole thioether catalyzed by the immobilized cells in an organic-aqueous biphasic system to form esomeprazole is a complex process in which many factors can affect the yield and e.e. of esomeprazole, such as organic solvents, the incubation time, ratio of organic to aqueous phase, the concentration of immobilized cell beads, the incubation temperature and pH of phosphate buffer. Based on the single factor preliminary tests, three independent factors, including the concentration of immobilized cell beads (A), the incubation temperature (B) and pH of phosphate buffer (C), were chosen as independent variables, while the yield of esomeprazole was determined as a response variable. For three factors and three levels all, 17 test points were thus designed by RSM Using Design Expert 8.0.5, and the central point test was repeated five times. These factors and levels of the experimental design are listed in Table 1.

Table 1. Factors and levels of Box-Behnken test design

Factors	Unit	Levels		
		−1	0	1
Concentration of immobilized cell bead (A)	g/L	200	250	300
Incubation temperature (B)	°C	34	37	40
Phosphate buffer pH (C)	-	6.3	7.3	8.3

RESULTS AND DISCUSSION

1. Synthesis of Esomeprazole via the Sulfoxidation of Omeprazole Thioether Catalyzed by Immobilized Cells of a Mutant of *R. rhodochrous*.

As shown in Table 2, the highest yield of esomeprazole reached 94.21% (entry 11) and all values of e.e. exceeded 99%; moreover, no sulfone product was detected during the biooxidation of omeprazole thioether. It is noteworthy that the concentration of the omeprazole thioether was 200 mM (65.8 g/L based on only the

Table 2. The experimental design and results of RSM[a]

Std	Run	Concentration of immobilized cell beads/g/L	Incubation temperature/°C	Phosphate buffer pH	Yield of esomeprazole (%)
		(A)	(B)	(C)	(Y)
17	1	0	0	0	94
1	2	−1	−1	0	75.1
6	3	1	0	−1	88.89
4	4	1	1	0	91.78
7	5	−1	0	1	74.64
8	6	1	0	1	90.53
12	7	0	1	1	84.55
11	8	0	−1	1	83.56
10	9	0	1	−1	84.72
13	10	0	0	0	94.12
15	11	0	0	0	94.21
3	12	−1	1	0	78.66
5	13	−1	0	−1	74.89
16	14	0	0	0	93.8
2	15	1	−1	0	92.02
14	16	0	0	0	93.88
9	17	0	−1	−1	82.4

[a]No sulfone product was detected during the oxidation of omeprazole thioether and all of e.e. >99%. The substrate concentration and the incubation time were 200 mM and 105 h, respectively.

volume of organic phase, or 5.22 g/L based on the total volume of reaction media) which is much higher than that in other research reports (0.5-1.5 g/L based on the total volume of reaction media) [23,25,27,33-36], suggesting that immobilized cells of a mutant of *R. rhodochrous* employed have very good substrate and product tolerance. Furthermore, the chloroform-water biphasic system, which greatly improves the tolerance, is also much better than the single aqueous phase system.

The following factors may contribute to the high yield, e.e. and tolerance to high substrate concentrations. Firstly, the mutant of ATCC 4276 is an excellent whole cell biocatalyst to synthetize esomeprazole due to its high enantioselectivity, activity and tolerance of substrate and product. Some microbial cells such as *Helminthosporium species*, *Rhizopus species*, *Trichaptum species*, *Trametes species*, *Mortierella. isabellina*, *Botrytis cinerea*, *Trichoderma viride* and *Eutypa lata* have been employed for the synthesis of chiral sulfoxides by biooxidation of sulfides with good enantiomeric purity [17,33,37, 38,89], However, their tolerance and yields obtained by those cells are less than that of the mutant of ATCC 4276 used in the present work. Secondly, the size of immobilized cell beads in the study are a range of from 0.5 to 1.0 mm that is much smaller than approx. 5 mm diameter [25,27] and 10 mm size [39] reported previously. While the smaller the bead diameter, the larger the reaction interface of cells with substrates, which is a great advantage for the reaction catalyzed by immobilized cells. The immobilization of whole cells can elevate the biooxidation activity at a higher level (from 0.2 up to 1.5 g/l) in a single water phase [25,26]. Elkin et al. performed a comparative study of sulfoxidation activity of free and immobilized *R. rhodochrous* IEGM 66 cells [25]. They found that free cells displayed maximal oxidative activity towards thioanisole (0.5 g/L), whereas immobilized cells provided for complete thioanisole transformation into (*S*)-thioanisole sulfoxide with e.e. of 82.1% at high (1.0-1.5 g/L) concentration of sulfide substrate. Porto et al. reported that the biotransformation of the precursor sulfides to sulfoxides using *Aspergillus terreus* CCT 3320 cells led to e.e. better than 95% [28]. The immobilized cells on chrysotile and cellulose/TiO_2 showed a similar biocatalytic behavior in the conversion rate and in the sulfoxide enantiomeric excess. Obviously, the sulfide substrate concentration and e.e. the above reported are much lower than ours (e.e. >99% at 5.22 g/L of substrate). Third, the flask used for the sulfoxidation of the omeprazole thioether catalyzed by immobilized cells was aerated to provide enough oxygen for a high concentration of cells (optimal, 284 g bead/L), which is very advantageous to the biooxidation reaction. For small incubation the cell concentration is usually not too high because of the limitation of oxygen supply, such as asymmetric oxidation of sulfide with resting cells of *Rhodococcus* sp. in a biphasic system at 20-40 wet cell g/L [24], enantioselective oxidation of phenyl methyl sulfide and its derivatives into optically pure (*S*)-sulfoxides with *Rhodococcus* sp. CCZU10-1 in an n-octane–water biphasic system at 70-100 wet cell g/L [40], and lyophilized *Rhodotorula* yeast as all-in-one redox biocatalyst for catalyzing the reduction of prochiral arylketones at 6.6 lyophilized yeast g/L [35]. Finally, the significant improvement of substrate tolerance may mainly be attributed to employing the organic-aqueous biphasic system. Both the substrate tolerance and enantioselectivity can be improved significantly when organic-aqueous biphasic systems are employed [23,29-31]. For the aqueous-organic biphasic system, the ratio of two phases affects not only the interfacial areas of cells to substrate, but also the organic solvent influence on cells, thus exerting significant effect on the catalytic activity of cells [24,29,40,41]. It is generally believed that the enzyme activity can be significantly affected by organic solvents [31,41], and oxidation activity is positively correlated with log P of solvent, for example, relative activity was 33.5% for ethyl acetate (log P 0.68) and 161% for chloroform (log P 1.97), respectively [40]. The polar solvent (log P<1.0) may damage cell membranes, leading to impaired cell activity [23].

An organic solvent with good biocompatibility may significantly improve the catalytic activity of cells. Chloroform was thus selected as an organic phase, and the optimal volumetric ratio of water to chloroform was 90 : 10 (v/v) according to the single factor preliminary tests. Because the solubility of omeprazole thioether in chloroform is greater than 0.15 g/mL, for the chloroform-water biphasic system, almost all the substrate omeprazole thioether and product esomeprazole partition in the chloroform phase while the cells are in the water phase. We speculate that the substrate and product partitioned in chloroform contribute little or no inhibition to the catalytic activity of cells. Li et al. found that the asymmetric reduction of 2-octanone with Baker's yeast FD-12 in the aqueous medium was inhibited severely by the substrate and the product at higher concentration in the aqueous medium, while FD-12 showed good tolerance, catalytic activity and enantioselectivity in the water/n-dodecane system where the metabolic activity retention and viability of FD-12 were 98% and 91.6%, respectively [42]. Lou et al. used two typical ionic liquids (ILs), hydrophobic (BMIM·PF_6) and hydrophilic (BMIM·BF_4) as solvents in the asymmetric reduction of acetyltrimethylsilane to enantiopure (*S*)-1-trimethylsilylethanol catalyzed by immobilized *Saccharomyces cerevisiae* cells [43]. It was observed that BMIM·PF_6 and BMIM·BF_4 can markedly boost the activity and the stability of the immobilized cells. The reason may be that substrates in a single aqueous system are absorbed by cells or substrates are enriched on cells; however, only a part of the adsorbed substrates will be subject to oxidation to prepare sulfoxides catalyzed by cells, while other adsorbed substrates may damage the cell and inhibit the catalytic activity of cells. On the contrary, substrates in an organic-aqueous biphasic system, are primarily partitioned in the organic phase, not enriched in cells. The substrate in contact with cells is greatly reduced, and the substrate adsorbed by cells right now may mainly participate in sulfoxidation and not inhibit the catalytic activity of cells. He et al. also noticed cells adsorbing substrates and the cells were soaked in ethyl acetate to extract the adsorbed substrate [40].

2. RSM Optimization of Asymmetric Biooxidation of Omeprazole Thioether Catalyzed by Immobilized Cells of a Mutant of *R. rhodochrous*

RSM is widely employed to design and analyze the effects of independent variables on test results, obtaining optimum experimental conditions and exploring the interactions between experimental variables; the optimal fitting model was thus acquired. Table 2 shows the experimental results of bio oxidation of the omeprazole thioether for synthesis of esomeprazole catalyzed by immobilized cells of a mutant of *R. rhodochrou*s. As shown in Table 3, regres-

Table 3. Significance test and results for regression coefficients of model

Source	Coefficient	Sum of squares	df	Mean square	F-value	Prob>F
Model	94	860.89	9	95.65	4,934.09	<0.0001**
A	7.49	448.95	1	448.95	23,157.98	<0.0001**
B	0.83	5.49	1	5.49	283.43	<0.0001**
C	0.3	0.71	1	0.71	36.52	0.0005**
AB	−0.95	3.61	1	3.61	186.21	<0.0001**
AC	0.47	0.89	1	0.89	46.06	0.0003**
BC	−0.33	0.44	1	0.44	22.81	0.0020*
A^2	−5.59	131.62	1	131.62	6,789.18	<0.0001**
B^2	−4.02	68.08	1	68.08	3,511.61	<0.0001**
C^2	−6.17	160.47	1	160.47	8,277.54	<0.0001**
Residual		0.14	7	0.019		
Lack of fit		0.023	3	7.608E-003	0.27	0.845
Pure error		0.11	4	0.028		
Cor total		1,972.81	16			

C.V.%=0.16 R^2 0.9998 R^2_{Adj} 0.9996 R^2_{Pred} 0.9994

*Significant at $p<0.05$. **Significant at $p<0.01$.

sion analysis was performed with the Design Expert 8.0.5 package to estimate the effects of the concentration of immobilized cell beads (A), the incubation temperature (B) and pH of phosphate buffer (C), on the yield of esomeprazole (Y). Using the Design Expert 8.0.5 package, the quadratic polynomial equation was established as follows:

$$Y=94+7.49A+0.83B+0.3C-0.95AB+0.47AC-0.33BC-5.59A^2-4.02B^2-6.17C^2 \quad (1)$$

A positive effect will impose to the yield when the coefficient in Eq. (1) is positive; on the contrary, a negative coefficient exerts a negative influence on the yield of esomeprazole. As shown in Eq. (1), A, B, C and AC possess positive coefficients, indicating that these factors raise the yield. Whereas interaction terms AB and BC, and all quadratic terms, reduce the yield due to their negative coefficients.

Table 3 shows the analysis results of variances, the measurement coefficient R^2 is 0.9998, meaning that 99.98% of the variation of the yield can be expressed by the model and the correlation of estimated data with experimental is excellent. Moreover, the adjusted R^2 is 0.9996, indicating that the experimental data are in good correlation with the estimated. Generally, the smaller the *p*-value (<0.001), the greater the significance of the corresponding coefficient. Conversely, the larger the F-value, the greater the significance of the corresponding coefficient. In this study, the F ratio of the quadratic regression model was 4,934.09 with a lower probability value ($p<0.0001$), showing that the model is a very accurate prediction of the yield. With the Prob>F value of the lack-of-fit ($p\geq0.05$), the insignificant lack-of-Fit F-value of 0.27 further demonstrates that the data in the experimental domain are well predicted by the model, quadratic polynomial Eq. (1), therefore, which is sufficient for simulating the yield of esomeprazole within the range of experimental variables. As shown in Table 3 and Eq. (1), based on the F value, all of the linear, interaction and quadratic terms have a very significant effect on the yield of esomeprazole ($p<0.0001$-0.002), especially A, B and C ($p<0.0001$), i.e., the concentration of immobilized *R. rhodochrous* cell beads (A), the incubation temperature (B) and buffer pH (C) have a greater effect on the biooxidation of prochiral sulfides to form esomeprazole. As shown in Figs. 1 and 2, the yield of esomeprazole increased rapidly with the increase in the concentration of immobilized cell

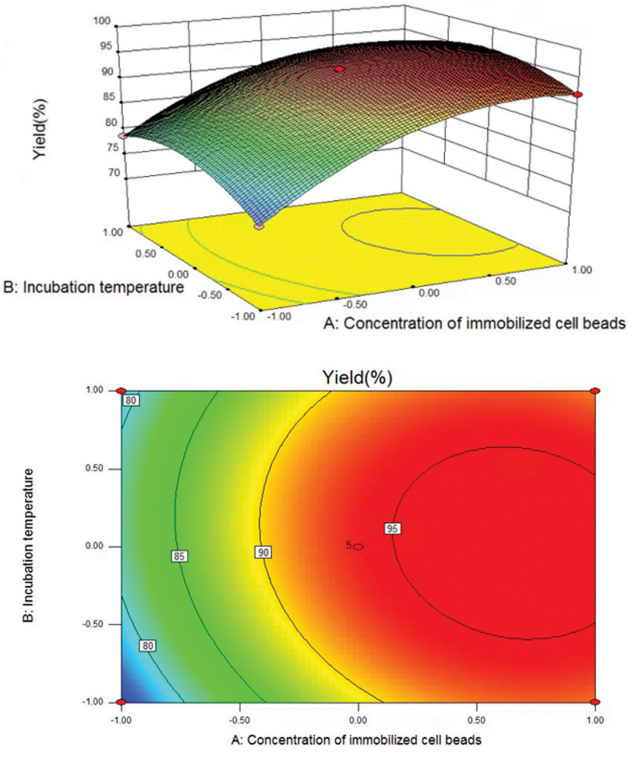

Fig. 1. Contour map and response surface of Y=f (A, B).

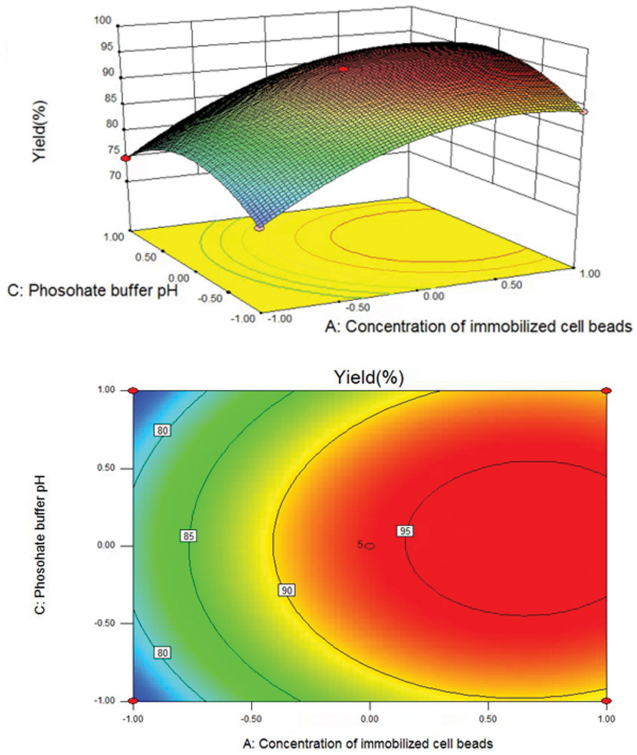

Fig. 2. Contour map and response surface of Y=f (A, C).

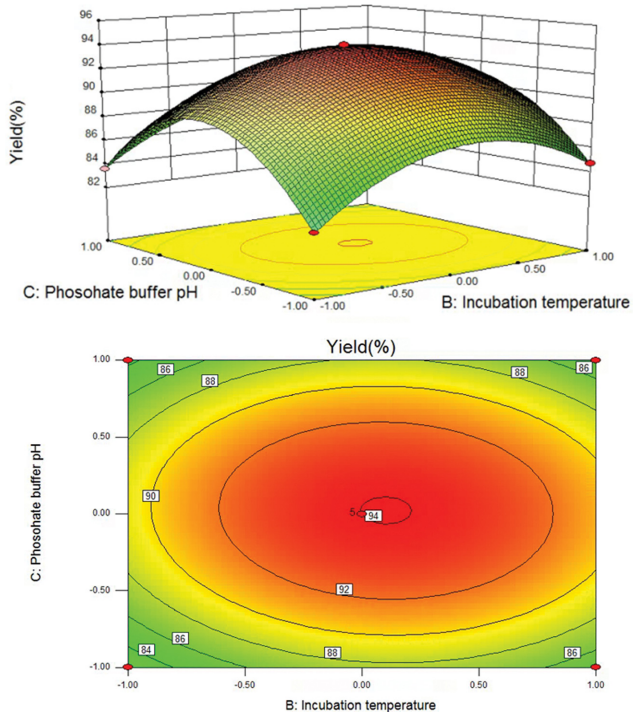

Fig. 3. Contour map and response surface of Y=f (B, C).

beads until about 280 g/L, and then the yield improved little although the concentration of immobilized cell beads continued to increase up to 300 g/L. With the concentration increase, the corresponding catalytic activity of cells also increased; however, because the concentration was too high, mass transfer restrictions appeared caused by a high cell concentration, limiting the catalytic activity [34]. The conversion was actually reduced when the concentration of cells was too high [24,35,40]. As shown in Figs. 1 and 3, the yield of esomeprazole increased with the temperature increasing from 34 to about 37 °C; however, the yield decreased with further increase of the temperature from 37 to 40 °C, indicating that the optimum temperature for the *R. rhodochrous* cell was 37 °C, and if the temperature deviates from this optimal temperature, the catalytic activity of cells will be reduced. The incubation temperature is an important variable that has a significant effect on the activity and stability of both enzymes and cells [44-47]. As shown in Figs. 2 and 3, the yield of esomeprazole increased with the increase in buffer pH from 6.3 up to about 7.3; however, the yield became decreasing with further increase of buffer pH up to 8.3. The catalytic activity of cells is affected by buffer pH, which can affect the ionic state of substrates and enzymes and thus affect both the yield and e.e. of the product catalyzed by enzymes [48] and whole cells [34].

3. Interactive Effects of Independent Variables on the Asymmetric Biooxidation of the Omeprazole Thioether Catalyzed by Immobilized Cells of a Mutant of *R. rhodochrous*

The interactive effects of independent variables including A, Band C on the biooxidation of the omeprazolethioether catalyzed by immobilized cells of a mutant of *R. rhodochrous* were investigated based on both response surface plots and contour plots, which intuitively reveal the effect of the interaction between independent variables on the yield of esomeprazole. Generally, elliptical contour plots demonstrate that the mutual interaction between factors is significant; conversely, circular contour plots imply that the interaction is not significant. The contour plots in Figs. 1-3 are elliptical with $p<0.0001$, 0.0003 and 0.002, respectively, indicating that the mutual interactions between A, B and C are significant. Eq. (1) shows that the interaction of A with C due to its positive coefficient is synergistic, while the interactions AB and BC with negative coefficients are antagonistic. Among those interaction terms, AB with $p<0.0001$ in comparison with AC and BC has the highest influence on the yield of esomeprazole. Fig. 1 exhibits the effects of A and B on the yield of esomeprazole at constant C of 7.3, in which the yield of esomeprazole is sensitive to a minor variation of the experimental variables A and B.

4. Determining and Verifying of Optimal Conditions for the Asymmetric Biooxidation of the Omeprazole Thioether Catalyzed by Immobilized Cells of a Mutant of *R. rhodochrous*

Optimal asymmetric biooxidation conditions in an organic-aqueous biphasic system were determined using RSM. The effect of the concentration of immobilized cell beads (A), the incubation temperature (B) and pH of phosphate buffer (C) on the yield of esomeprazole exhibited well by both the regression equation and the response surface analysis and contour plots. The optimal parameters determined were as follows: the concentration of immobilized cells of a mutant of *R. rhodochrous* 283.5 g/L, the incubation temperature, 37.05 °C, and pH of phosphate buffer, 7.35, respectively. With three repeated tests at the omeprazole thioether concentration of 200 mM for 105 h, the optimal yield of esomeprazole obtained was 94.8%, which is in very good agreement with the

estimated results (95.6%), and the corresponding e.e. was >99%, while no sulfone product was detected. This result indicates that in the chloroform-water biphasic system, the asymmetric sulfoxidation of the omeprazole thioether catalyzed by immobilized *R. rhodochrou*s cells is therefore more promising for the efficient asymmetric biooxidation for synthesis of chiral sulfoxides.

CONCLUSIONS

In the chloroform-water biphasic system, the asymmetric sulfoxidation of the omeprazole thioether to synthesize esomeprazole catalyzed by immobilized cells of the mutant of *R. rhodochrou*s ATCC 4276 was optimized using RSM at a high substrate concentration (200 mM). The optimal conditions were a concentration of immobilized cells 283.5 g/L, incubation temperature 37.05 °C, and pH of phosphate buffer 7.35. Under these conditions, the yield of esomeprazole obtained was 94.8% with e.e. (>99%) without the formation of the sulfone form. Using RSM, a quadratic polynomial model was developed with R^2 of 0.9998, which predicts the observed data with very high accuracy. The significant improvement of substrate tolerance may mainly be attributed to employing the chloroform-water biphasic system because almost all substrates may be distributed in the organic phase, which results in little damage and inhibition to cells. For the efficient synthesis of chiral sulfoxides, the sulfoxidation catalyzed by immobilized cells of the mutant of ATCC 4276 is therefore more promising because of its high enantioselectivity, activity and tolerance to substrate and product.

CONFLICTS OF INTEREST

There are no conflicts to declare.

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