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Optimization of the asymmetric synthesis of (*S*)-1-phenylethanol using Ispir bean as whole-cell biocatalyst

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Abstract: In this study, enantiomerically pure (*S*)-1-phenylethanol was produced via asymmetric bioreduction of acetophenone. Ispir bean *(Phaseolus vulgaris*) was used as an alcohol dehydrogenase (ADH) source since whole cells are cheaper than isolated enzymes. Acetone powder methodology was applied for biocatalyst. Glucose was used as a cosubstrate in-order to regenerate cofactor (NADPH). The reactions were carried out in an orbital shaker whose temperature and agitation rate can be controlled. (*S*)-1-phenylethanol concentration was analyzed by HPLC using a Chiralcel OB column. Effects of the reaction time, substrate concentration, cosubstrate concentration and biocatalyst concentration on the (*S*)-1-phenylethanol production were investigated using Response Surface Methodology (RSM). 36 h bioreduction time, 6 mM acetophenone concentration, 25.15 mM glucose concentration, and 175 mg/mL biocatalyst concentration were determined as optimum values. In these conditions, 2.4 mM (*S*)-1-phenylethanol was obtained in phosphate buffer (pH=7.0) at 30°C with >99% enantiomeric excess.

Keywords: (*S*)-1-phenylethanol; response surface methodology; asymmetric bioreduction; acetone powder; enantioselectivity

List of abbreviations

ADH	alcohol dehydrogenase
GDH	glucose dehydrogenase
NADPH	nicotinamide adenine dinucleotide
C_b	biocatalyst concentration, mg/mL
$C_{(s)\text{-}1\text{-}pe}$	(*S*)-1-phenylethanol concentration, mM
C_g	glucose concentration, mM
C_R	(*R*)-enantiomer concentration, mM
C_S	(*S*)-enantiomer concentration, mM
C_{So}	initial substrate concentration, mM
C	conversion
ee	enantiomeric excess
N	agitation rate, rpm
T	temperature, °C
t	reaction time, h
HPLC	high pressure liquid chromotography
RSM	response surface methodolgy

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1 Introduction

The mechanism of drug interactions as well as the significance of chirality is well understood. Often only one stereoisomer exhibits therapeutic biological activity, the other isomer does not have such an effect. Therefore, enantiopurity has the foremost significance of the production [1]. Chiral alcohols are valuable and helpful starting materials for the synthesis of several modern pharmaceuticals and agrochemicals. Thus it is important to provide enantiopure compounds in biotechnology [2-6]. In a kinetic resolution, the theoretical yield is 50% of the starting material [6]. Therefore, asymmetric reduction of prochiral ketones into chiral alcohols is a widely used process [7,8]. Since, it can give 100% theoretical yield [9].

(*R*)- and (*S*)- phenylethanols are useful building blocks for the synthesis of complex molecules and are attractive compounds for a wide range of potential application in drug industry [10-12].

Biocatalysts have many advantages over all other catalysts. Enzymes are one kind of biocatalyst and they specifically catalyze chemical reactions. Because they can actively work under moderate condition, enzymes are an important alternative in terms of environmental factors in chemical processes industry [6]. Instead of using an

isolated enzyme as a biocatalyst in asymmetric reduction reactions, whole-cell is a preferable alternative with the main advantage of the elimination of costly enzyme purification process. At the same time, enzymes are most stable due to the presence of their natural environment inside the cells [13,14]. Also with the use of whole cells, may not necessitate the addition of expensive cofactors [13-15]. When whole-cell biocatalysts were used, internal cofactor regeneration is possible by adding cosubstrate or glucose [14]. Plant cells are a potential enzyme source for the asymmetric reduction reactions. The use of locally accessible plant cells may offer an alternative to isolated enzymes in recent years [5,9,13,15-17].

There are many asymmetric reduction reactions with various fruits and vegetables, for example, carrot, potato, sweet potato, apple, cucumber, onion, radish, grape, garlic, tomato, peach, orange, apple, bean, turnip are reported in the literature [5,7-9,13,15-25]. In these studies, the conversion and enantiomeric excess values range from 30% to 100% and 33% to >99% respectively.

Also, different biological whole-cell catalyst can be used as an enzyme source for the asymmetric reduction reactions of different substrates. For example, *Lactobacillus kefir* reduced the acetophenone to (*R*)-1-phenylethanol with >%99 enantiomeric excess and 79% conversion [26], *Kluyveromyces marxianus* reduced different aryl ketones to (*S*)-alcohols in the under mild reaction conditions with nearly 96% enantiomeric excess [27], *Lactobacillus reuteri* whole cells could reduce various aryl ketones with high conversion and enantiomeric excess [28], and baker's yeast reduced the different aryl-containing ketones generally up to 90% enantiomeric excess and conversion [29].

The acetone powder methodology, which is a preparation process applied to biocatalyst, has become a preferred method in recent years. Under favor of this pretreatment, biocatalyst size can be minimized, mass transfer limitations are reduced and enzyme-substrate interaction is increased. Asymmetric reduction reactions are catalyzed by ADH. ADH catalyze the enantioselective reduction of ketones with the help of nicotinamide cofactors (NADH or NADPH). However, cofactors are highly expensive and at the end of the reaction nicotinamide cofactors run out. With ADH-GDH enzyme couple, cofactor regeneration was carried out successfully.

Only a few acetone powder biocatalyst studies have been reported in the literature. Nakamura et al. [30] reported acetone powder of *Geotrichum candidum* to catalyze the reduction of ketones. They compared resting cell and acetone powder on the reduction of Methyl 3-Oxobutanoate. For 30 mM substrate concentration, resting cell and acetone powder showed that 97% yield, 39% enantiomeric excess and 99% yield and 99% enantiomeric excess. They performed the reduction reaction presence of 2-hexanol and a small amount of a coenzyme, NAD^+ and as a result the enantiomeric excess increased from 39% to 99%. On the other hand, they showed that acetone powder of *G. candidum* reduced acetophenone with 89% yield and 99% enantiomeric excess. Hamada et al. [31] reported acetone powder of *G. candidum* reduced (R)-2-chloro-1-(m-chlorophenyl) ethanol with 94% yield and 98% enantiomeric excess for 8.3 mM initial substrate concentration. They used NAD^+ for coenzyme and 2-propanol for reducing agent. Xie et al. [9] reported that acetone powder of adzuki bean could reduce various aromatic ketones at high concentrations. Their study showed that the adzuki bean could reduce 100 mM acetophenone, exhibiting 98.6% enantiomeric excess and 90.5% conversion. Nakamura et al. [32] reported that when biocatalyst changed from wet whole-cell to powdered, no reduction was observed because of the loss of the necessary co-enzyme(s) and/or co-enzyme regeneration systems during treatment the cells with acetone. They recommended to the addition of cosubstrate for improvement the enantioselectivity. Moreover, they mentioned that the addition of both cosubstrate and NAD^+ enormously advanced both chemical yield and enantiomeric excess.

In this study, (*S*)-1-phenylethanol which is the precursor of many pharmacological products was produced by asymmetric bioreduction of acetophenone. Aceton powder of Ispir beans have been used as ADH source. Optimum conditions for reaction time, initial substrate concentration, cosubstrate concentration and, biocatalyst concentration to produce (*S*)-1-phenylethanol was defined using Response Surface Methodology.

2 Materials and methods

2.1 Chemicals

Acetophenone, (*R*)-1-phenylethanol, (*S*)-1-phenylethanol, and other chemicals were purchased from Sigma Aldrich (Sigma-Aldrich Corporate, St. Louis, MO USA).

2.2 Biocatalyst

Ispir beans *(Phaseolus vulgaris*) were obtained from a local producer in Ispir-Erzurum-Turkey. A metal mortar was used to grind the Ispir beans.

2.3 Biocatalyst preparation: Acetone powder

Ispir bean particulates were kept in water (the mass ratio of particulate to water was 1/5) for 5-6 h at room temperature with 100 rpm stirring rate. The supernatant after steeping was centrifuged at 7500 rpm for 20 min and then filtrated. The supernatant was slowly mixed with chilled acetone (–20°C, v/v = 1/1). The suspension was centrifuged again at 7500 rpm for 20 min and the precipitate was collected and stored at 4°C [9].

2.4 Bioreduction experiments in a batch bioreactor

Biotransformation of acetophenone (Figure 1) by asymmetric reduction was carried out in a batch bioreactor. Experiments performed in the batch system were carried out in 50 mL mouth-capped bottles with 10 mL working volume. Desired amount of biocatalyst was added to the buffer medium. Acetophenone dissolved in the dimethyl sulfoxide solvent and glucose used for the cofactor regeneration was added to the buffer medium (pH=7.0) to initiate the reaction. The reaction was carried out 30°C and 150 rpm on an orbital shaker.

All of the reactions were carried out as double-repetitive. In bioreduction reactions, reaction time, substrate concentration, cosubstrate concentration and, biocatalyst concentration were optimized to maximize the produced (*S*)-1-phenylethanol using Response Surface Methodology (RSM).

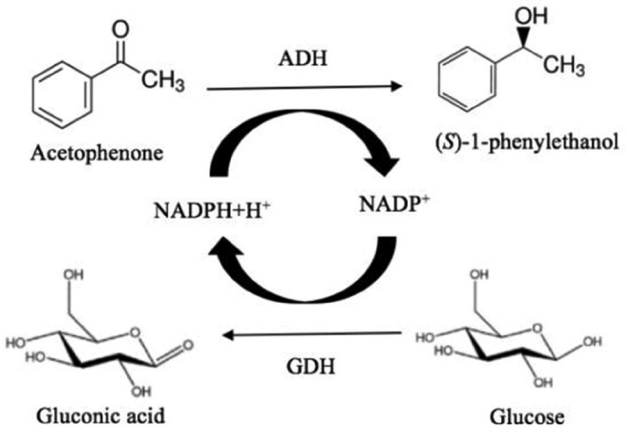

Figure 1: Asymmetric bioreduction of acetophenone catalyzed by Ispir bean.

2.5 Analytical methods

At the end of the reaction, the organic and aqueous phases were separated and the organic phase was analyzed by HPLC. (*R*)- and (*S*)- phenylethanols analysis was performed Chiralcel OB column (4.6 mm × 50 mm, Daicel Chemical Ind. Ltd. France) at 30°C, 10 microliters of injection volume, 0.90 mL/min flow rate of hexane/2-propanol (95/05) eluent, with a 254 nm UV detector [26]. The enantiomeric excess (ee) and conversion rate (C) were calculated using Eq. 1 and Eq. 2, respectively.

$$ee\% = \frac{C_S - C_R}{C_S + C_R} \times 100 \quad (1)$$

$$C\% = \frac{C_{So} - C_S}{C_{SO}} \quad (2)$$

2.6 Response surface methodology

RSM is a mathematical and statistical method used to optimize the operation conditions. It is also used to define the optimum operating conditions. In RSM, the quadratic model is widely used. Since the it is more flexible and in it is easy to estimate the parameters. Also, quadratic models are good at solving real-response surface problems [33].

Quadratic model can be shown as:

$$Y = B_0 + \sum_{i=1}^{n} B_i x_i + \sum_{i=1}^{n} B_{ii} x_i^2 + \sum_{i=1}^{n} \sum_{j=1}^{n} B_{ij} x_i x_{ij} + \lambda \quad (3)$$

where $x_1 - x_n$ are the input variables, Y is a response, B_i and B_{ij} are unknown parameters and λ is an error [34].

For the quadratic model used in this study, the range and levels of independent variables and the numerical values of quantities are displayed in Table 1.

Table 1: Experimental range and levels of independent variables.

Independent variables	Range and levels				
	–2	–1	0	1	2
Reaction time (h), X_1	24	36	48	60	72
Substrate concentration (mM), X_2	2	6	10	14	18
Glucose concentration (mM), X_3	20	25	30	35	40
Biocatalyst concentration (mg/mL), X_4	100	125	150	175	200

In the regression equation, the test variables were coded according to the following equation:

$$x_i = \frac{\left(X_i - X_i^*\right)}{\Delta X_i} \tag{4}$$

where x_i is the coded value of the *i*-th independent variable, X_i is the uncoded value of the *i*-th independent variable, X_i^* is the uncoded *i*-th independent variable at the center point and ΔX_i is the step change value [34]. 2^4 full factorial center composite design for four independent variables was used in this study. The full factorial composite design consists of a complete 2^k factorial design, where k is the number of test variables, n_0 center points ($n_0 \geq 1$), and two axial points on the axis of each design variable at a distance of α (=$2^{k/4}$, (=2 for k=4) from the design center. The total number of design points is $N=2^k+2k+n_0$; thus for this procedure, 30 experiments were required for 4 independent variables [35].

Pre-experimental studies on asymmetric reduction of acetophenone show that the input minimum and maximum values of substrate concentration, cosubstrate concentration, and biocatalyst concentration will be as listed in Table 1.

Reaction time, substrate concentration, glucose concentration, and biocatalyst concentration were chosen as the independent output variables and response variable is determined as (*S*)-1-phenylethanol concentration obtained as the product. The 'Design Expert' software (Version 6.01, Stat-Ease, Inc., Minneapolis, USA) was used for regression and graphical analysis of the data obtained.

The statistical importance of the quadratic model was determined by the analysis of variance (ANOVA). The effect of each parameter was evaluated by the F-value and the p-value. The characteristic of the fit of the quadratic model was identified by the R^2 value.

3 Results

In this study, enantiopure (*S*)-1-phenylethanol with high enantioselectivity and chemical yield was produced with asymmetric bioreduction of acetophenone. The asymmetric synthesis of alcohol is significantly influenced by the initial substrate concentration, glucose concentration, reaction time and biocatalyst concentration [36].

The RSM experiments performed and the results obtained under the operational conditions are listed in Table 2. The quadratic model equation is given below for the actual value (Eq. 5).

$$\begin{aligned} Y = {} & 0,45 - 0,18x_1 - 0,20x_2 - 0,41x_3 - 0,07x_4 \\ & + 0,26x_1^2 + 0,46x_2^2 + 0,30x_3^2 + 0,09x_4^2 \\ & + 0,17x_1x_2 - 0,05x_1x_3 - 0,22x_1x_4 \\ & - 0,25x_2x_3 - 0,28x_2x_4 - 0,30x_3x_4 \end{aligned} \tag{5}$$

where Y is the concentration of (*S*)-1-phenylethanol ($C_{(s)\text{-1-PE}}$). Enantiomeric excess values were obtained at > 99% in all the experiments.

In Table 3, the model F-value of 3.67 implies the model is significant. Probe values indicate the importance of each coefficient. These design variables are important parameters when the probe> F value is less than 0.05. For the model, Probe> F (<0.0088) less than 0.05 indicates that the model represents the system well. The "Lack of Fit F-value" of 45.44 implies

Table 2: The actual values of the experimental conditions and response variable.

Run	t (h)	C_{So} (mM)	C_g (mM)	C_b (mg/mL)	$C_{(s)\text{-1-PE}}$ (mM)
1	36	6	25	125	1.30
2	60	6	25	125	1.70
3	36	14	25	125	2.76
4	60	14	25	125	2.73
5	36	6	35	125	1.48
6	60	6	35	125	1.42
7	36	14	35	125	1.93
8	60	14	35	125	2.52
9	36	6	25	175	2.37
10	60	6	25	175	1.95
11	36	14	25	175	2.98
12	60	14	25	175	2.65
13	36	6	35	175	2.53
14	60	6	35	175	0.39
15	36	14	35	175	0.41
16	60	14	35	175	0.69
17	24	10	30	150	1.67
18	72	10	30	150	0.27
19	48	2	30	150	1.44
20	48	18	30	150	2.07
21	48	10	20	150	1.86
22	48	10	40	150	0.42
23	48	10	30	100	0.26
24	48	10	30	200	0.33
25	48	10	30	150	0.50
26	48	10	30	150	0.50
27	48	10	30	150	0.46
28	48	10	30	150	0.59
29	48	10	30	150	0.27
30	48	10	30	150	0.38

Table 3: ANOVA for quadratic model.

Source	Sum of Squares	DF	Mean Square	F-value	p-value Prob > F
Model	19.50	14	1.39	3.67	0.0088
x_1	0.85	1	0.85	2.23	0.1560
x_2	0.95	1	0.96	2.52	0.1335
x_3	4.13	1	4.12	10.86	0.0049
x_4	0.12	1	0.12	0.32	0.5751
x_1^2	1.96	1	1.96	5.15	0.0383
x_2^2	5.89	1	5.89	15.51	0.0013
x_3^2	2.63	1	2.63	6.92	0.0189
x_4^2	0.26	1	0.26	0.69	0.4160
$x_1 x_2$	0.46	1	0.46	1.23	0.2855
$x_1 x_3$	0.06	1	0.06	0.15	0.7054
$x_1 x_4$	0.77	1	0.77	2.03	0.1749
$x_2 x_3$	1.04	1	1.04	2.73	0.1195
$x_2 x_4$	1.29	1	1.29	3.41	0.0848
$x_3 x_4$	1.43	1	1.43	3.78	0.0710
Residual	5.69	15	0.38		
Lack of Fit	5.63	10	0.56	45.44	0.0003
Pure Error	0.062	5	0.01		
Cor Total	25.19	29			

R^2=0.77
Adeq Precision=5.986

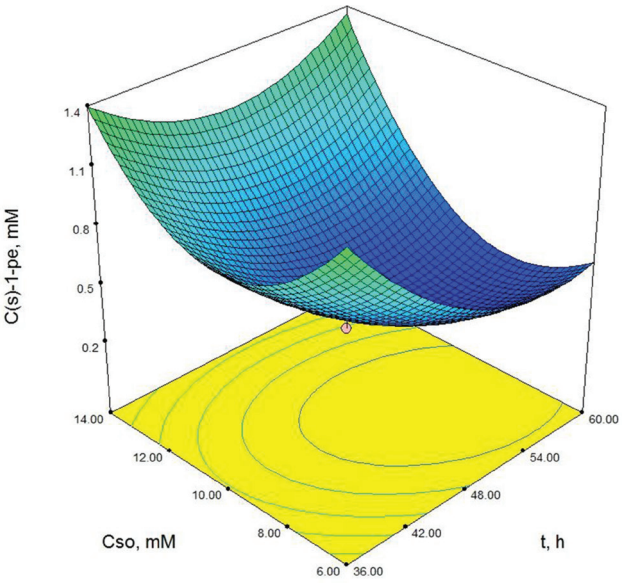

Figure 2: Contour-plot of (*S*)-1-phenylethanol concentration: The effect of time and initial substrate concentration. Other variable is held at zero level. C_g=30 mM, C_b=150 mg/mL.

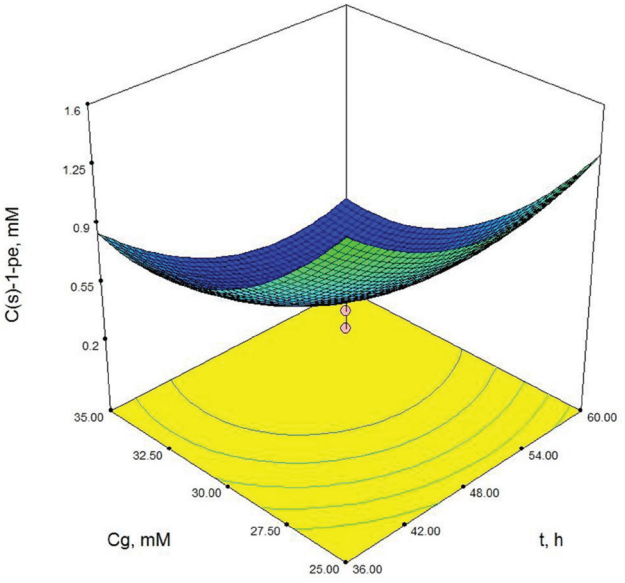

Figure 3: Contour-plot of (*S*)-1-phenylethanol concentration: The effect of time and glucose concentration. Other variable is held at zero level. C_{so}=10 mM, C_b=150 mg/mL.

the Lack of Fit is significant. Adeq Precision measures the signal to noise ratio and a ratio greater than 4 is desirable. For the model, the ratio of 5.986 indicates an adequate signal. This model can be used to navigate the design space.

As shown in Table 3, X_3 (cosubstrate, glucose concentration) and X_2^2 (second order effect of substrate concentration) are the most important model parameters because the Prob> F value is 0.0049 for X_3 and 0.0013 for X_2^2. Model terms with a prob> F value greater than 0.1 are not significant. These results show that the second order effect of substrate concentration and glucose concentration have a direct effect on (*S*)-1-phenylethanol concentration. The response surface contour-plots (Figures 2-7) were drawn to predict the effects of the independent variables on the (*S*)-1-phenylethanol concentration. Each contour curve represents an infinite number of combinations of two test variables with the other two maintained at their respective zero level. (*S*)-1-phenylethanol concentration increases with increasing initial substrate concentration (Figures 2, 5 and 6) and biocatalyst concentration (Figures 4, 6 and 7). On the other hand, (*S*)-1-phenylethanol concentration decreases as the reaction time increases (Figures 2-4). This is thought to be due to the presence of a lot of a number of enzymes in the structure of the plant cells and the product, (*S*) -1-phenylethanol, to be converted into unknown products during the reaction. Increasing glucose concentration can cause cosubstrate inhibition and because of this inhibition, (*S*)-1-phenylethanol concentration decreases with increasing of the glucose concentration (Figures 3, 5 and 7). In order to maximize the concentration of (*S*)-1-phenylethanol, the optimum conditions were obtained by the response surface

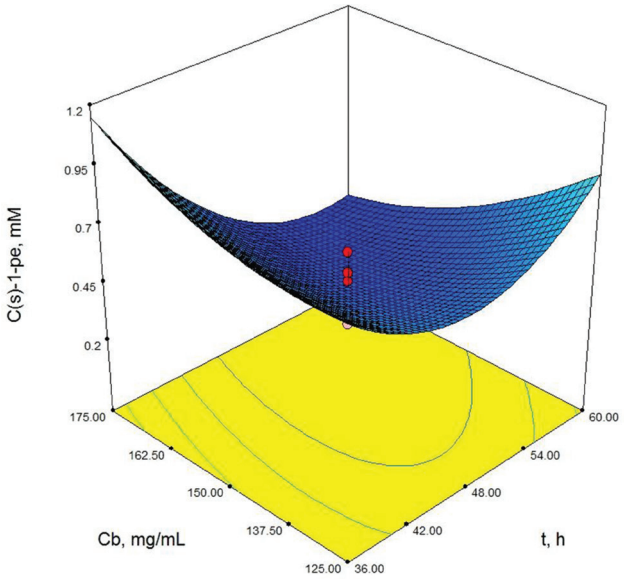

Figure 4: Contour-plot of (*S*)-1-phenylethanol concentration: The effect of time and biocatalyst concentration. Other variable is held at zero level. C_{so}=10 mM, C_g=30 mM.

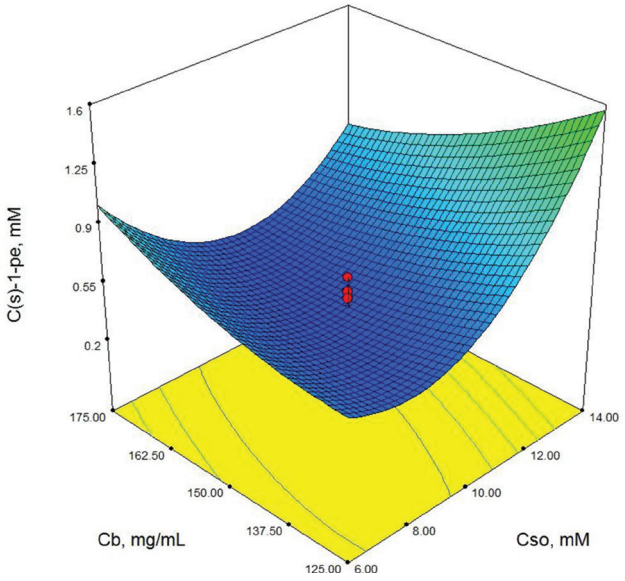

Figure 6: Contour-plot of (*S*)-1-phenylethanol concentration: The effect of initial substrate concentration and biocatalyst concentration. Other variable is held at zero level. t=48 h, C_g=30 mM.

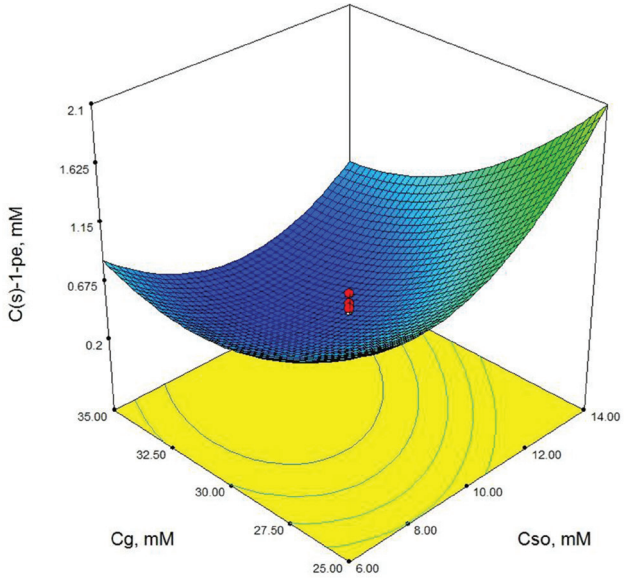

Figure 5: Contour-plot of (*S*)-1-phenylethanol concentration: The effect of initial substrate concentration and glucose concentration. Other variable is held at zero level. t=48 h, C_b=150 mg/mL.

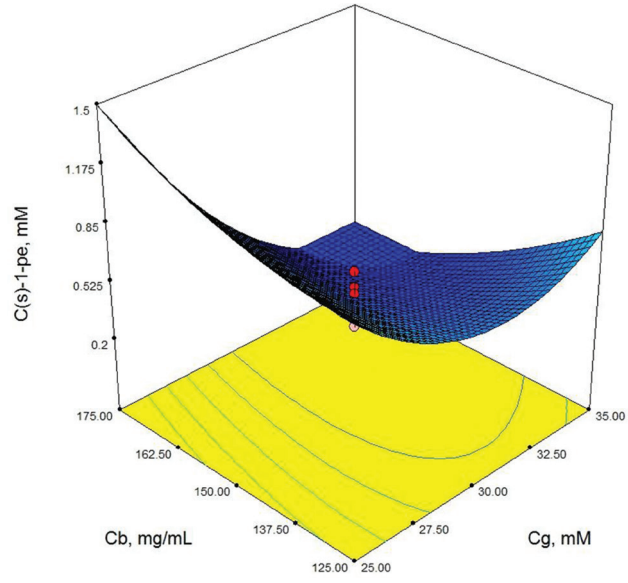

Figure 7: Contour-plot of (*S*)-1-phenylethanol concentration: The effect of glucose concentration and biocatalyst concentration. Other variable is held at zero level. t=48 h, C_{so}=10 Mm.

methodology with the Design expert (6.01) program are given in Table 4.

With these optimum values, (*S*)-1-phenylethanol concentration was obtained at 2.58 mM. Enantiomeric excess and conversion values were >99% and 40%, respectively. In order to verify these results, an experimental study was carried out it these conditions and (*S*)-1-phenylethanol concentration was obtained at

Table 4: Optimum values of independent variables obtained with RSM.

Independent variables	Optimum value
t, h	36
C_{So}, mM	6
C_g, mM	25.15
C_b, mg/mL	175

2.40 mM. The relative deviation between the computer solution and the experimental results was calculated as 6.9%.

In the literature, for (*S*)-1-pheylethanol production, Chang et al. [16] reported 96% enantiomeric excess value with carrot as biocatalyst, Xie et al. [9] reported 98% enantiomeric excess value with acetone powder of adzuki bean as biocatalyst, Yang et al. [13] reported 96.4%, 75.8%, 73.8% and 72.8% enantiomeric excess values with carrot, cucumber, onion and radish as biocatalyst, respectively. In our previous study, enantiomeric excess, conversion and (*S*)-1-pheylethanol concentration were obtained as >99%, 58% and 0.6 mM, respectively using carrot as biocatalyst [23].

When compared with the literature, our results which show that the enantiomeric excess value is above 99% are encouraging.

4 Discussion

In this study, asymmetric reduction of acetophenone to (*S*)-1-phenylethanol resulted in >99% enantiomeric excess and 40% conversion, using Ispir bean which was pretreated with acetone powder methodology. Response surface methodology was used to optimize the production of (*S*)-1-phenylethanol. It was found that the most effective parameters were glucose concentration and second order effect of acetophenone concentration. Our results showed that, Ispir bean is a good biocatalyst for asymmetric reduction of acetophenone with its enantioselective reaction capability. In commercial products, it is desirable that the enantiomeric excess is >99%. As a result, the main goal of this study was to reach high enantiomeric excess and this was achieved. In order to increase the conversion of asymmetric reduction of acetophenone, our studies are going on.

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