REGULAR ARTICLE

Factors screening to statistical experimental design of racemic atenolol kinetic resolution via transesterification reaction in organic solvent using free *Pseudomonas fluorescens* lipase

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Abstract

As the (*R*)-enantiomer of racemic atenolol has no β-blocking activity and no lack of side effects, switching from the racemate to the (*S*)-atenolol is more favorable. Transesterification of racemic atenolol using free enzymes investigated as a resource to resolve the racemate via this method is limited. Screenings of enzyme, medium, and acetyl donor were conducted first to give *Pseudomonas fluorescens* lipase, tetrahydrofuran, and vinyl acetate. A statistical design of the experiment was then developed using Central Composite Design on some operational factors, which resulted in the conversions of 11.70–61.91% and substrate enantiomeric excess (*ee*) of 7.31–100%. The quadratic models are acceptable with R^2 of 95.13% (conversion) and 89.63% (*ee*). The predicted values match the observed values reasonably well. Temperature, agitation speed, and substrate molar ratio factor have low effects on conversion and *ee*, but enzyme loading affects the responses highly. The interaction of temperature–agitation speed and temperature–substrate molar ratio show significant effects on conversion, while temperature–agitation speed, temperature–substrate molar ratio, and agitation speed–substrate molar ratio affect *ee* highly. Optimum conditions for the use of *Pseudomonas fluorescens* lipase, tetrahydrofuran, and vinyl acetate were found at 45°C, 175 rpm, 2000 U, and 1:3.6 substrate molar ratio.

KEYWORDS

central composite design, enzymatic transesterification, kinetic resolution, *Pseudomonas fluorescens* lipase, racemic atenolol

1 | INTRODUCTION

Atenolol is a β-adrenergic receptor antagonist drug (β-blocker) recognized as one of the best-selling drugs in the world,[1,2] used to treat high blood pressure (hypertension), angina pectoris (chest pain), and arrhythmia.[3,4] The drug is mainly found in the form of a racemic mixture. Since its β-blocking activity is related to the (*S*)-enantiomer,[5] which avoids the side effects produced by the racemic form,[6] and

the (*R*)-atenolol has no β-blocking activity and no lack of the side effects,[7,8] switching from the racemic form to the (*S*)-atenolol is more favorable.

The (*S*)-atenolol is produced via two general methods, i.e., the asymmetric syntheses and kinetic resolutions of the racemic atenolol. The asymmetric synthesis is conducted through reactions that convert racemic or achiral substrates asymmetrically using chiral metal catalysts[9-11] or chiral addenda.[12-14] The second method resolves the available

racemic atenolol via chromatographic resolutions, which employ many types of chiral selectors,[15,16] microbial fermentation of the racemate or its derivative,[17] and enantioselective esterification or hydrolysis of the racemate using immobilized enzymes.[18,19] Recently, formation of the enantiomer was offered through an enzymatic transesterification reaction of a racemic alcohol, precursor of the (S)-atenolol, in ionic liquids.[20] Since the racemic atenolol is available in markets, the kinetic resolution is the faster strategy to produce the single enantiomer than asymmetric syntheses.[21]

Of all the methods used in the kinetic resolutions, enzymatic transesterification is the most dominant method.[22] As the resources to develop the enzymatic transesterification of racemic atenolol using free enzymes are limited, a study of batch free enzyme catalysis is necessary to gain knowledge for its preparation. A statistical experimental design of the racemic atenolol enzymatic transesterification by *Pseudomonas fluorescens* lipase (Amano) using vinyl acetate in an organic medium batch was conducted. Since one-factor-at-a-time tests the factors one at a time, varying the experimental factors simultaneously through a factorial experimental design was considered as the correct way to deal with the experimental factors.[23]

2 | MATERIALS AND METHODS

2.1 | Materials

(R,S)-atenolol (99% USP) was purchased from Nanjing Chemlin Chemical Industry (China). (S)-atenolol (97%, Tocris Bioscience (UK)) and (R)-atenolol (99%, Sigma-Aldrich (Malaysia)) were used to prepare the standard solutions. Chemicals were of analytical grade except for analysis (of high-performance liquid chromatography (HPLC) grade) and were supplies by EOS Scientific (Malaysia), Fisher Scientific (Malaysia), and Merck (Malaysia). *Pseudomonas fluorescens* lipase (Amano 534730, 20 U/mg) was bought from Modern-Lab Chemicals (Malaysia). All materials were used without pretreatment.

2.2 | Enzyme and medium screening

Flow of the experiments is shown in Figure 1. The work began by conducting the screening processes on enzyme(s), medium, and acetyl donor and finished with the statistical design of the experiment. The enzyme and medium screening procedure are described below.

The 20–24 mL racemic atenolol solution was mixed with vinyl acetate at a ratio of 1:1.5 (mole/mole) in 100-mL flasks. The mixtures were shaken in orbital shakers (Max Q4000 Barnstead Lab-Line Infors HT Ecotron or Incu-Shaker Mini Benchmark) at 200 rpm and 40°C for 30 min. The enzyme in a certain unit of activity was added. The enzymatic
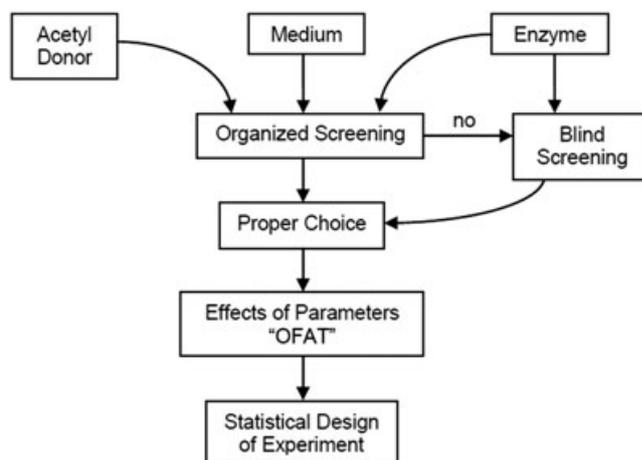

FIGURE 1 Flow chart of the study

transesterification was conducted at 155 or 200 rpm and 40°C. After initial samples were collected for a certain interval of time, 1 mL of aliquot was taken and placed in a glass vial, and then kept in a refrigerator prior to analyses using ultrafast liquid chromatography (UFLC). For mixed organic solvents, the reactions were conducted as follows: 50 mg of racemic atenolol was dissolved in 10 mL dimethylsulfoxide (DMSO) or dimethylformamide (DMF). After a clear solution was formed, 25 mL toluene was added. Vinyl acetate was pipetted in at the same ratio. After a certain amount of enzyme activity was placed into the solution, the reaction was conducted at 40°C and 200 rpm for 6 h. Samples were collected at the initial and end time of the incubation period.

The enzymatic transesterification in buffer solutions was prepared as follows: the racemate was dissolved in Sorensen buffers (50–100 mM, pH 6.6–7.8) to form the 35.6 mM solution. Then the 24 mL solution was placed in flasks. Vinyl acetate was added at the ratio of 1:1 (mole/mole). The flasks were shaken at 40°C and 200 rpm for 30 min. Lipase was finally put in (CALB: 665 μL, CRL: 7 mg). The reactions were conducted at the same operating conditions as the previous section for 10 h.

2.3 | Acetyl agent screening

The 24 mL solution of racemic atenolol dissolved in tetrahydrofuran (THF) (18.8 M) was prepared in 100-mL flasks. Each acetylating agent was mixed at the substrate to agent ratio of 1:2.4 (mole/mole). After shaking for 30 min, to every flask was added with 1000 U enzyme. After the initial samples were taken, the flasks were incubated at 40°C and 155 rpm. Samples (1 mL) were collected at certain intervals of time and kept in a refrigerator prior to analyses using UFLC.

2.4 | Design of experiment (DOE)

The transesterification process was studied using a central composite design (CCD). The design was prepared and analyzed using Design-Expert software (v. 6.0.6, Stat Ease, Minneapolis, MN). Four reaction factors at five factorial levels were studied as described in Table 1 (all factors and levels were selected from one-factor-at-a-time experiments, which were conducted previously). Two responses were set, i.e., racemic atenolol conversion (X) and enantiomeric excess of substrate (*ee*).

2.5 | CCD experimental procedure

The 25 mL of racemic atenolol dissolved in tetrahydrofuran was prepared in 100-mL flasks. Vinyl acetate was then put in as required. After shaking for 30 min, to every flask was added a certain amount of enzyme. The mixtures were incubated in orbital shakers (Max Q4000 Barnstead Lab-Line and Infors HT Ecotron). Samples were taken out at a certain interval time and directly analyzed using UFLC. All experiments used operating conditions provided by DOE.

2.6 | Analysis of reaction product and calculation of results

The atenolol enantiomers were analyzed with a Shimadzu (Japan) UFLC LC-20A Prominence using a Chiralcel OD column (250 mm x 4.6 mm). The mobile phase consisted of hexane-ethanol-diethyl amine (75–25–0.1% *v*/v) and flow at 0.5 mL/min. U*V*/Vis detector was set at 275 nm wavelength. The UFLC was operated at 35°C. Two μL samples were injected at a time. For chromatograms of the atenolol enantiomers, please refer to the previous article (Agustian et al.).[24]

Conversions (X) and enantiomeric excess of substrate (*ee*) of the reaction were calculated using the equations:

$$X\ (\%) = \frac{C_0 - C_t}{C_0} \times 100 \tag{1}$$

$$X_S\ (\%) = \frac{C_{S0} - C_{St}}{C_{S0}} \times 100 \tag{2}$$

$$X_R\ (\%) = \frac{C_{R0} - C_{Rt}}{C_{R0}} \times 100 \tag{3}$$

$$ee_S(\%) = \frac{(C_{Rt} - C_{St})}{(C_{Rt} + C_{St})} \times 100 \tag{4}$$

where C_0 is the initial amount of racemic atenolol (mM), C_t is the amount of racemic atenolol (mM) at reaction time t, C s_t is the amount of (*S*)-atenolol at reaction time t, and C_{Rt} is the amount of (*R*)-atenolol at reaction time t.

3 | RESULTS AND DISCUSSION

3.1 | Screening of enzyme

Many lipases were employed to resolve the racemic atenolol via the transesterification reaction as given in Table 2. At the beginning of the experiments, an organized screening process was prepared by focusing the enzymatic reactions on the free enzymes from *Candida antarctica* lipase of fraction B (CALB), *Candida rugosa* lipase (CRL), and lipase PS (PSL) since they are frequently used to resolve the racemic compounds.[18,25-34] However, these enzymes cannot be used, as low results are produced, which are not acceptable. Hence, the racemic atenolol resolution was finally conducted through a blind screening process until a suitable enzyme was discovered.

In general, the conversions of 19–100% were found on the (*S*)-atenolol, but 0–77% of the (*R*)-atenolol was also changed (Table 2). Most of the employed enzymes were found converting both the atenolol enantiomers. Although a previous study found that the immobilized CALB is a prospective enzyme,[20] the enzyme changed both atenolol enantiomers as most other biocatalysts. Previously, lipase PS immobilized on diatomite earth (lipase PS-D) had

TABLE 1 The experimental factors and their levels

Experimental factor	Symbol	Unit	Levels				
			(− α)	(− 1)	(0)	(+1)	(+α)
Temperature	x_1	°C	39	42	45	48	51
Agitation speed	x_2	rpm	125	150	175	200	225
Enzyme activity	x_3	U	1000	1500	2000	2500	3000
Substrate molarrRatio	x_4	mol/mol	1:20	1:2.4	1:3.6	1:4.8	1:60

TABLE 2 Comparison of enzymes and reaction media

Enzyme	Load (mg)	Conversion (%)									
		DMSO		DMF		THF		DMSO + Toluene		DMF + Toluene	
		X_R	X_S	X_R	X_S	X_R	X_S	X_R	X_S	X_R	X_S
CRL	10	21.88	32.87	3.86	4.67	0	0	2.92	8.35	6.73	10.28
PCL	10	14.83	26.25	3.35	4.20	6.72	11.40	0	0	4.61	4.62
CCL	50	13.54	23.76	12.77	14.74	4.88	5.37	0	0	6.25	9.64
LP 62336	10	12.60	22.92	2.18	2.60	0	100.00	0	0	39.70	54.79
LP 62335	1.5	50.75	19.45	0	0.53	0	0	0	0	1.28	2.12
LAPS	30	13.63	24.19	2.27	3.21	0	0	0	0	0	0
MML	10	12.05	21.15	0	0.83	0	0	0	0	0	0
RAL	30	17.88	29.62	3.37	3.88	8.99	9.58	0	0	0	0
RNL	30	10.59	20.72	0	0.17	2.10	2.99	0	0	76.99	100
HPL	30	0	0	22.09	31.67	0	0	1.86	1.81	0	0
PFL 28602	8–20	16.13	23.23	3.20	3.63	6.94	70.42	0	0	0	0
MJL	35	26.26	35.21	4.25	5.14	27.16	19.75	0	0	12.15	17.75
CALB	2400	29.82	42.82	18.17	19.57	47.27	52.72	0	0.77	9.33	13.55
AN	30	27.80	38.60	6.56	6.48	17.25	18.26	0	0	0	0
AOL	20	27.19	39.46	8.64	9.02	0	0	0	0	0	0
ROL	30	23.87	33.62	10.62	11.52	7.63	7.66	2.32	3.37	0	0

CRL: *Candida rugosa*; PCL: *Pseudomonas cepacia*; CCL: *Candida cylindracea*; LP: Lipoprotein; LAPS: Amano Lipase from *Burkholderia cepacia*; MML: *Mucor miehei*; RAL: *Rhizopus arrhizus*; RNL: *Rhizopus niveus*; HPL: Hog Pancreatic; PFL: *Pseudomonas fluorescence*; MJL: *Mucor javanicus*; CALB: *Candida antarctica* fraction B; AN: *Aspergilus niger*; AOL: *Aspergilus oryzae*; ROL: *Rhizopus oryzae*; DMSO: dimethylsulfoxide; DMF: dimethyl formamide; THF: tetrahydrofuran; Operational conditions: 40°C, 200 rpm (except LP 62336, PFL, CALB: 155 rpm); $X_R = \{(C_{R0}-C_{Rt})/C_{R0}\} \times 100$; $X_S = \{(C_{S0}-C_{St})/C_{S0}\} \times 100$; C_0 is the initial concentration of racemic atenolol (mM), C_t is the concentration of racemic atenolol (mM) at reaction time t.

successfully catalyzed the kinetic resolution of the racemic atenolol,[18] but the free-form lipase Amano PS from *Burkholderia cepacia* used in the experiments was not able to resolve the compound. A similar case was observed on *Candida cylindracea* lipase, which is a powerful enzyme to resolve the racemic propranolol,[35] where it had low capability to resolve the racemic atenolol. Hence, from all the experimented enzymes, several enzymes could be used in the kinetic resolution of the racemic atenolol. Other enzymes need further exploration in order to make them more specific on a certain enantiomer of this racemic compound.

A further comparison of lipoprotein and PFL (Figure 2) indicated that the lipoprotein produced 100% conversion of (*S*)-atenolol in a short time, while the PFL-Fluka and PFL-Sigma reached conversions of 70.73% and 80.80%, respectively, of the same enantiomer after 24 h. The lipoprotein was found to have no activity on the (*R*)-atenolol, but high conversions on this enantiomer were developed by the PFL-Fluka (i.e., 7.20–13.74%) and PFL-Sigma (i.e., 25.17–32.34%). In term of enantiomeric ratio (*E*), the lipoprotein and PFL-Fluka gave high *E*, but the PFL-Sigma produced low results. Experiments with PFL-Amano gave the conversion of 46.36% for the (*S*)-atenolol and 0%

conversion for the (*R*)-atenolol after 24 h observation. The PFL Amano enzyme was considered comparable to the PFL-Fluka, as it produced a good enantiomeric ratio (~15) at this screening process. The PFL-Amano was
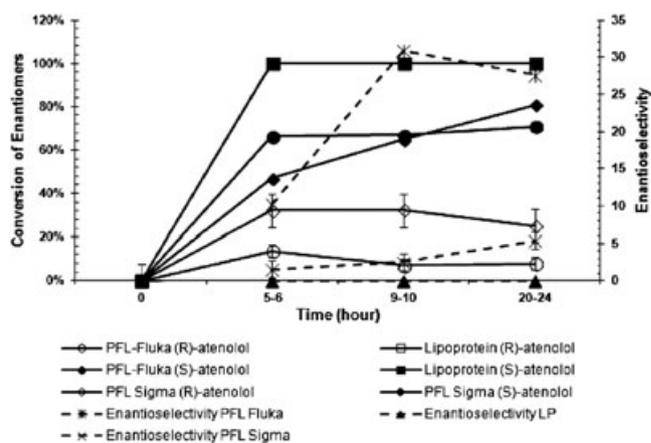

FIGURE 2 Comparison of enzymes performance [155 rpm, 40°C, 18.8 mM racemic atenolol in THF (lipoprotein, PFL-Sigma) or 30.04 mM racemic atenolol in THF (PFL-Fluka); 2.4 molar ratio, lipoprotein: 3200 U, PFL-Fluka: 800 U, PFL-Sigma: 3200 U]

decided to be used in the next experimental steps of the enzymatic transesterification reaction because the enzyme prefers the (S)-enantiomer ((S)-atenolol enantiopreference) and it is not expensive.

3.2 | Screening of reaction medium

Only organic water-miscible solvents and buffer solutions were used to dilute the racemic atenolol, as it is a hydrophilic compound. As described in Table 2, most lipases were active in DMSO and DMF where both atenolol enantiomers were converted by the enzymes at low conversion values. Although many lipases in THF showed poor or no activity, it was suitable for the racemic atenolol kinetic resolution, as high conversions were developed by lipoprotein 62336 and PFL 28602 on the (S)-atenolol in this solvent. A previous enzymatic process of the racemic atenolol used THF as the reaction medium to give 40–42% yields (overall) and 94% *ee* of product.[18] Since enzyme activity is high in nonpolar and water immiscible solvents,[36] combined media such as DMSO + Toluene and DMF + Toluene were also studied. Poor results were observed with DMSO + Toluene. The reaction in DMF + Toluene produced high conversions for both atenolol enantiomers when lipoprotein 62336 and *Rhizopus niveus* lipase were used, where the (S)-atenolol was changed faster than the (R)-atenolol.

Since the racemic atenolol has higher solubility in water (~27 mg/mL) than in DMSO and DMF (<20 mg/mL at room temperature) or THF (<10 mg/mL at room temperature), and by considering the production capacity of the (S)-atenolol, the enzymatic transesterification reaction in phosphate buffer solutions were conducted on free CALB, LAPS, CCL, and CRL. It failed to observe the CCL-based reaction using 67 mM phosphate buffer pH 7.4, as its samples created high pressures in the UFLC column, hence the samples were not analyzed. The LAPS changed both the atenolol enantiomers, where conversions were below 15%.
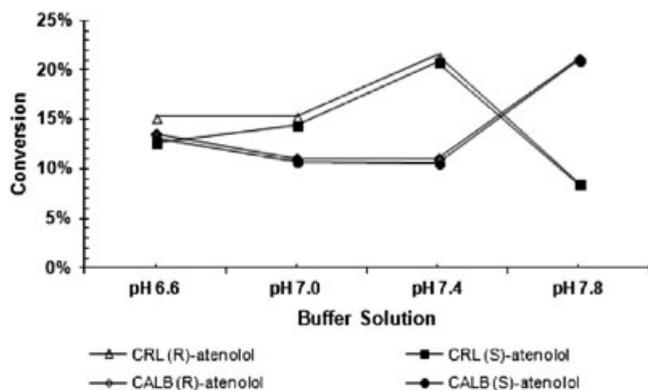
The free CALB and CRL results are shown in Figure 3. Conversions were low (8.62–22.25%), and both lipases reacted on both enantiomers. Lower results than in the organic solvents were obtained, although the reaction used higher racemate concentrations. Efforts to use a biphasic system similar to an earlier work carried out by Barbosa et al.[19] were also developed. The racemic atenolol dissolved in some mixtures of hexane and 67 mM phosphate buffers pH 6.6–7.8 were incubated with 75 mg free CALB at 200 rpm and 40°C. These biphasic reactions using the free CALB were also not successful.

From these descriptions, THF was chosen for the reaction solvents, as several enzymes showed their preference on the (S)-atenolol.

3.3 | Screening of acetyl donor

Vinyl acetate, isoprophenyl acetate, and ethyl acetate were compared to find the proper acetyl donor (Table 3). Vinyl acetate converted 15.07–49.02% of the (S)-atenolol with no reaction on the (R)-atenolol. On the contrary, isoprophenyl acetate and ethyl acetate changed only as high as 6% of both the atenolol enantiomers, which indicated that PFL-Amano did not catalyze the transesterification of these enantiomers. Previous experiments described that the isoprophenyl acetate and ethyl acetate were associated with the kinetic resolutions of (R)- or (S)-enantiomers conducted in hydrophobic solvents, while the vinyl acetate was effectively used in either the hydrophobic or hydrophilic solvents. Furthermore, the use of the vinyl acetate as the acetyl donor offers an effective solution to overcome the reaction equilibrium because enol coproduct is immediately transformed irreversibly into acetaldehyde or acetone.[37-39] Hence, only vinyl acetate can be used to produce better conversion and the enzymatic transesterification reaction work stereoselectively.

3.4 | Statistical DOE

Statistical DOE was developed by taking benefits from the PFL Amano, tetrahydrofuran, and vinyl acetate. It was designed by combining four factors and five factorial levels using the CCD that gave 30 experimental runs (16 factorial points, 8 axial points, and 6 center points) as described in Table 4. The conversion and *ee* were in the range of 11–70-61.91% and 7.31–100%, respectively. The highest conversion and *ee* were produced at a temperature of 42°C, 200 rpm agitation speed, 2500U enzyme activity, and substrate molar ratio of 1:2.4.

The statistical quadratic models for the racemic atenolol conversion (X) and substrate enantiomeric excess (*ee*) on the operational factors are defined as follows:

FIGURE 3 Enzymatic transesterification in phosphate buffers

TABLE 3 Comparison of the acetylating agent

	X_S (%)				X_R (%)			
Compound	0	12	20	36	0	12	20	36
Vinyl Acetate	0	15.07	22.40	49.02	0	0	0	0
Isoprophenyl Acetate	0	6.01	0	0	0	5.66	0	0
Ethyl Acetate	0	5.63	0	0	0	5.49	0	0

18.80 mM racemic atenolol in THF, 2.4 molar ratio, PFL-Amano: 1000 U, 40°C; 155 rpm.

TABLE 4 Central composite design matrix

	Independent variables				Response	
Run	x_1: Temperature (°C)	x_2: Agitation speed (rpm)	x_3: Enzyme activity (U)	x_4: Molar ratio	X (%)	ee (%)
1	48	200	2500	1: 2.4	51.80	100
2	48	200	2500	1: 4.8	51.65	100
3	48	200	1500	1: 2.4	39.44	59.30
4	42	200	2500	1: 2.4	61.91	100
5	42	200	1500	1: 2.4	30.09	36.17
6	45	175	1000	1: 3.6	11.70	7.31
7	48	150	1500	1: 4.8	47.06	82.75
8	42	200	2500	1: 4.8	59.75	100
9	45	175	2000	1: 1.2	47.44	85.10
10	45	125	2000	1: 3.6	51.87	100
11	45	175	2000	1: 3.6	47.16	83.33
12	48	200	1500	1: 4.8	43.98	73.38
13	45	175	2000	1: 6.0	51.03	100
14	45	175	3000	1: 3.6	51.21	100
15	45	175	2000	1: 3.6	44.80	74.63
16	45	175	2000	1: 3.6	53.59	100
17	42	150	2500	1: 2.4	60.11	100
18	45	175	2000	1: 3.6	55.95	100
19	45	175	2000	1: 3.6	54.08	100
20	42	200	1500	1: 4.8	36.02	48.28
21	42	150	1500	1: 2.4	36.90	55.41
22	48	150	2500	1: 4.8	51.58	100
23	39	175	2000	1: 3.6	51.57	100
24	48	150	1500	1: 2.4	37.06	53.96
25	42	150	2500	1: 4.8	58.70	100
26	42	150	1500	1: 4.8	32.83	44.55
27	45	175	2000	1: 3.6	55.26	100
28	45	225	2000	1: 3.6	51.61	100
29	51	175	2000	1: 3.6	56.34	72.57
30	48	150	2500	1: 2.4	51.55	100

$$X = 51.81 + 0.31x_1 - 0.07x_2 + 9.28x_3 - 0.83x_4 + 0.59x_1{}^2$$
$$+ 0.034x_2{}^2 - 5.04x_3{}^2 - 0.59x_4{}^2$$
$$+ 0.024x_1x_2 - 4.10x_1x_3 + 1.01x_1x_4 + 0.47x_2x_3$$
$$+ 0.23x_2x_4 - 1.26x_3x_4$$

$$(5)$$

$$ee = 92.99 + 1.25x_1 - 0.81x_2 + 22.15x_3 + 3.08x_4 - 2.47x_1{}^2$$
$$+ 0.96x_2{}^2 - 10.63x_3{}^2 - 0.90x_4{}^2 + 0.72x_1x_2 - 5.31x_1x_3$$
$$+ 2.60x_1x_4 + 1.22x_2x_3 + 0.52x_2x_4 - 2.76x_3x_4$$

$$(6)$$

The proposed models fit well and are highly satisfactory (Table 5). The coefficient of determination (R^2) for conversion is more than 95%, which is acceptable. The R^2 for *ee* model is only 89.63%. As a comparison, Soyer et al.[40] obtained the R^2 for *ee* of 80.05% during enzymatic racemic 1-phenyl 1-propanol resolution, while Cunha et al.[41] described that the R^2 of 91% was adequate for accuracy and applicability of the polynomial model used to describe the optimized conditions in the kinetic resolution of racemic 1,2-*O*-isopropylidene-3,6-di-*O*-benzyl-myo-inositol. The predicted values match the experimental data reasonably well, with the R^2 of 95.13% (conversion) and 90.95% (substrate *ee*). Hence, the models are applicable and reliable and can be used to simulate the reaction. Effects of the individual operational factors (x_1-x_4) are summarized in Table 6.

3.5 | Mutual effects of factors on conversion

As shown in Table 6, the temperature (x_1) and substrate molar ratio (x_4) had low effects on the conversion. Although both factors increased the conversions, there was only a small conversion difference between the highest ($\pm51.5\%$) and lowest conversion ($\pm50\%$). A similar condition was considered on

TABLE 5 Summary of the factorial effects

Factor	Effects			
	Conversion		ee	
x_1	Low	Synergist	Low	Synergist
x_2	Low	Antagonist	Low	Antagonist
x_3	High	Synergist	High	Synergist
x_4	Low	Synergist	Low	Synergist
x_1x_2	High	Significant	High	Significant
x_1x_3	Low	Not significant	Low	Not significant
x_2x_3	Low	Not significant	Low	Not significant
x_3x_4	Low	Not significant	Low	Not significant
x_2x_4	Low	Not significant	High	Significant
x_1x_4	High	Significant	High	Significant

the agitation speed (x_2). The slight conversion differences found on each factor could be caused by the fact that the optimum temperatures are found in the range of 40–60°C,[42] the substrate molar ratio of 1:1–1:5 was frequently used in the transesterification-based enzymatic process in order to prevent depletion of the acetyl donor in the reaction mixtures,[43-48] and the agitation speeds of 200–300 rpm were generally employed in the transesterification processes of the racemic compounds. However, the enzyme activity showed a sharp increase on the conversion (Figure 4). Zhang et al.[49] concluded that one of the factors that highly influenced the conversions was the enzyme activity.

Six interactions between factors were found. Temperature-agitation speed and temperature-substrate molar ratio interaction produced high and significant effects on conversion. High temperatures and agitation speed were required to give high conversions found at high operating temperature (~ 47°C). High temperatures and substrate molar ratios also gave high conversions. The interactions of temperature-enzyme activity, agitation speed-enzyme activity, agitation speed-substrate molar ratio, and enzyme activity-substrate molar ratio were considered not significant and had low effects, as the difference between the highest and lowest conversion found during the experiments was less than 1%.

3.6 | Mutual effects of factors on *ee*

Individually, low effects on *ee* were developed by the temperature and substrate molar ratio. Both factors indeed increased the *ee*, but a slight difference between the highest and lowest *ee* was observed (<5%). High effect on the *ee* was shown by enzyme activity, where a sharp increase from 59% *ee* (1500 U) to 100% *ee* (2500 U) was obtained. However, the agitation speed decreased the *ee* slightly.

The six factorial interactions also affected *ee*. High and significant interactions were shown by the temperature-agitation speed, temperature-substrate molar ratio, and agitation speed-substrate molar ratio. The highest *ee* was found at temperatures of 43–47°C and agitation speed of 150–160 rpm, but increasing the temperature produced similar *ee*. High *ee*'s were achieved only at the high temperature-substrate molar ratio interaction. Similarly, high *ee*'s were obtained at high agitation speeds and substrate molar ratio. Other interactions had low effects and were considered not significant.

From the statistical DOE, the constraints used to obtain the optimum values for the enzymatic transesterification process were temperature (level: 45°C; range: 42–48°C), agitation speed (level: 175 rpm; range: 150–200 rpm), enzyme loading (level: 2000 U; range: 1500–2500 U), molar ratio (level: 1:3.6; range: 1:2.4–1:4.8), conversion (range: 11.70–61.91%), and *ee* (range: 7.31–100%).

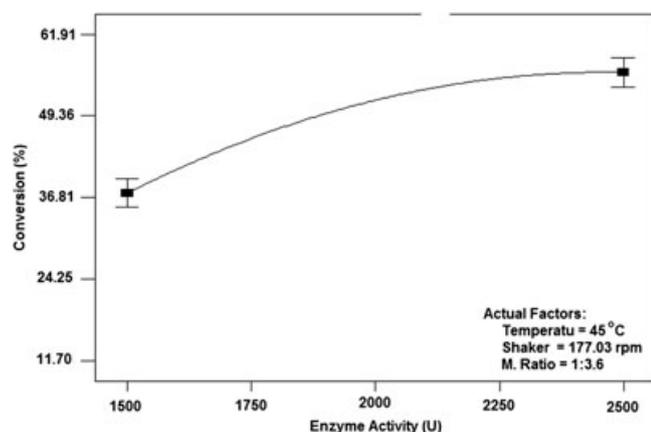**TABLE 6** Predicted and experimental results at the optimum operating conditions

Solution	T (°C)	Agitation speed	Enzyme loading	Molar ratio	Predicted		Experimental	
					X (%)	ee_S (%)	X (%)	ee (%)
1	42	199	1555	3.90	36.06	55.89	32.79	23.70
2	47	184	2496	2.71	53.17	98.47	58.75	100
3	47	168	2126	3.78	53.78	96.78	50.34	100
4	42	165	2237	2.59	57.30	100.00	52.75	100
5	43	152	2219	4.34	55.60	100.00	50.68	100

3.7 | Attaining optimum conditions and model verification

The above constraints were used to verify the models. Five of 10 solutions provided by the software for the optimum condition estimation were investigated (Table 6). Most of the experimental conversions were reasonably close to the values predicted using the RSM models. The difference between the predicted and experimental conversions was less than 5%, but solution-2 gave higher experimental conversion than its predicted value, showing the maximum operating condition as temperature and enzyme activity were at their highest conditions. The ee values exceeded the predicted data except for solution-1, which obtained a low conversion. These descriptions confirmed the validity and adequacy of the predicted models. During the solution-1 experiment, it was found that not only the (S)-atenolol was changed, but the (R)-atenolol was also converted (~15% conversion). This could be caused by high agitation speed, which increases movement of the substrate molecules in the reaction solvent, hence the enzyme has difficulties to adsorb the (S)-atenolol.

4 | CONCLUSION

Kinetic resolutions of racemic atenolol were investigated using free enzymes in batch via the transesterification reaction. The screening process of the free enzymes, media, and acetyl donors gave the PFL Amano, THF, and vinyl acetate, respectively. The statistical design of the experiment using Central Composite Design Response Surface Method produced the conversion of 11.70–61.91% and ee of 7.31–100%. The proposed quadratic equations are acceptable where R^2 are 95.13% (X) and 89.63% (ee). Predicted values matched the observed values reasonably well. Individually, the factors of the temperature, agitation speed, and substrate molar ratio factor had little effect to cause a high increase or decrease on the conversion and ee, but enzyme loading affected these responses highly. From six factorial interactions available, only temperature-agitation speed and temperature-substrate molar ratio interaction gave significant effects on the conversion, while other interactions had low/insignificant effects. The temperature-agitation speed, temperature-substrate molar ratio, and agitation speed-substrate molar ratio interaction affected the ee highly/significantly. Optimum conditions found from the design constraints were 45°C, 175 rpm, 2000 U, and 1:3.6 molar ratio.

FIGURE 4 Effect of enzyme activity on conversion

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