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Study on Synthesis of 1-Phenylethyl Acetate by Enzymatic Kinetic Resolution

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Abstract

In this study, porcine pancreatic lipase (PPL), Candida antarctica lipase (CAL-B, free state) and Novozyme 435 lipase were used as catalysts to establish an efficient, highly selective and environmentally friendly biocatalysis system for the synthesis of 1-phenylethyl acetate (PEA). Lipase species, acyl donor, reaction solvent, amount of lipase, lipase repeatability, reaction time, substrate concentration and reaction temperature were used as variables to study the characteristics of lipase catalytic kinetics for the resolution of 1-phenylethanol (PE). The results showed that Novozym 435 lipase was the best enzyme in the n-hexane solvent system. The optimal reaction temperature was 60˚C, the optimal lipase addition amount was 40 mg/ml, the concentration of PE was 100 mmol/ml, and the concentration of vinyl acetate was 500 mmol/L. After 24 hours of reaction, The yield of PEA was 61.49%. When Novozym 435 lipase was recovered and applied for 4 times or more, the reaction time was reduced to 1 h, the reaction temperature was reduced to 30˚C, the addition of lipase was reduced to 20 mg/ml, and the yield of PEA remained stable above 40%. The $K_m/V_{\max}$ was 0.8943 h, $V_{\max}$ was 0.118, and K_m was 0.105 mol/L. Obtain the Michaelis-Menten model: $V_0 = \frac{0.118 \cdot [S]}{0.105 + [S]}$. This study not only enriched the basic theoretical knowledge of non-aqueous enzymology, but also provided a reference for the application of lipase in the industrial production of spices.

Subject Areas

Chemical Engineering & Technology

Keywords

Lipase, Kinetic Resolution, 1-Phenylethyl Acetate, Dynamics

1. Introduction

Among many fragrances, 1-phenylethyl acetate (PEA) was a non-essential class of chiral fragrances. In particular, among food flavors, esters were the most versatile and the most used category, and they could be reasonably formulated to make various fragrances to give special aromas to pastries, non-alcoholic beverages, candies, liquors, and other foods; Meanwhile, PEA could also be used to configure various cosmetics, perfumes, and other flavors [1] [2]. However, the existing chemical synthesis obtained from PEA had low optical purity and required the participation of heavy metal reagents, which was not conducive to the promotion of green chemistry concept, such as Kirilin [3] *et al.* The (R)-1-phenylethyl acetate obtained by using $PbZn/Al_2O_3$ as catalyst was prepared at 300˚C with only 32%.

Lipase, also known as glycerol ester hydrolase, was a biocatalyst commonly found in a variety of microorganisms. Its main application was in organic synthetic chemistry to solve the problem of obtaining highly enantioselective products from racemic alcohols [4]. With the development of non-aqueous enzymology, it had been found that lipases could be used in organic media to catalyze splitting reactions such as esterification, ester exchange and transesterification reactions. Due to the high specificity and selectivity of enzymatic reactions catalyzed by lipases, lipases had been widely used in medicine and health, food and nutrition, chemical industry, and energy development [5] [6] [7].

Meanwhile, enzymatic kinetic resolution (KR) deserved to be investigated thoroughly [8] [9] [10] because of its high efficiency, mild reaction conditions, environmental friendliness and avoidance of precious metal catalysts. For example, Spelmezan *et al.* [11] prepared a novel biocatalyzer by covalently binding Candida Antarctica lipase onto magnetic nanoparticles coated with chitosan activated by sebacic acid, and experimentally proved that the catalyst could be used to catalyze the transesterification of partially racemized aromatic alcohols and vinyl acetate. Under the optimum conditions (vinyl acetate as acyl donor, n-hexane as solvent, reaction temperature 45˚C), the biocatalyst maintained considerable activity after 10 cycles of application. Response surface methodology (RSM) and four-factor-five-level Centre Composite Rotatable Design (CCRD) were employed to optimization of kinetic resolution of (±)-2-octanol [12].

In this study, the enzymatic properties of porcine pancreatic lipase, Candida Antarctica enzyme B (free state) and Novozyme 435 lipase were investigated in relation to the application of kinetic resolution for the preparation of PEA, making a contribution to the application of lipase in the industrial production of fragrances (**Figure 1**).

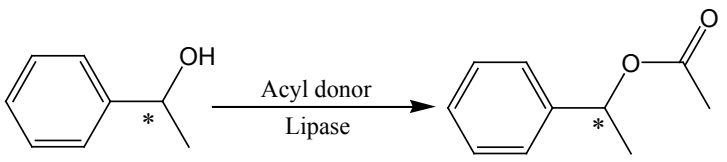

Figure 1. Synthesis of PEA by enzymatic kinetic resolution.

The research will provide important reference for the mass production of PEA.

2. Materials and Methods

2.1. Materials

Porcine pancreatic lipase (PPL) was purchased from Tisci Chemical Industry Co., LTD.; Candida Antarctica enzyme B (CAL-B, free state) was purchased from Beijing Grusen Technology Co., LTD.; Novozyme 435 Lipase was purchased from Beijing Novozyme; 1-phenylethanol and PEA were purchased from Sahn Chemical Technology (Shanghai) Co., LTD. All other reagents used in this work were of analytical grade and purchased from different commercial suppliers.

2.2. Analysis Conditions

Gas chromatographic analysis conditions: Fuli 9790 gas chromatographic column type KB-5 30 m × 0.32 mm × 0.25 μm; The flow rate of hydrogen was 30 mL/min, the flow rate of air was 300 mL/min, the gauge pressure of nitrogen was 0.1 mpa, the column temperature was 230˚C, the detector temperature was 230˚C, the injector temperature was 140˚C, and the injection volume was 0.5 μL. The internal standard was benzyl alcohol. Under the above gas chromatographic conditions, the retention time of benzyl alcohol, PE and PEA were 2.13 min, 2.22 min and 2.99 min respectively.

Yield was calculated by applying the following equation:

$$\text{Yield} = \frac{C_B}{C_{A0}} \times 100\%$$

C_{A0}: The initial concentration of PE;

C_B: The concentration of PEA;

$$V_0 = \frac{C_B}{t}$$

V_0: the increase in sulforaphane acetate per unit time at the beginning of the reaction, t: The reaction time.

2.3. Procedure

2.3.1. Experimental Operation

In this study, microcentrifuge tubes (EP tubes) were used as reaction vessels. First, PE was heated and melted, subsequently the required PE, solvent, acyl donor and lipase were added sequentially to the EP tubes. The EP tubes were placed in a shaker and the reaction was carried out at 200 r/min, controlled temperature. Samples were taken at regular intervals between reactions. The sample was left to stand, the supernatant was taken (separated using a centrifuge if necessary) and the internal standard benzyl alcohol was added and analyzed by gas chromatography (GC). The final determination of the optimum reaction conditions was based on the GC analysis.

At the end of the reaction, if Novozyme 435 lipase was to be recovered, the reaction solution (including solid Novozyme 435 lipase) could be filtered and washed with the reaction solvent for three times. Finally, the initially treated lipase was dried to a constant weight in an oven at 40˚C.

2.3.2. Kinetics of Enzymatic Reactions

The initial reaction velocity was calculated from the concentration of PEA measured at 0.5 h, and K_m and $V_{\max}$ were obtained according to Hans-Woolf curve. This reaction referred to the Michaelis-Menten model and used the following enzyme catalytic concepts: (**Figure 2**).

Where the rate constants K_1, K_2 and K_3 describe the reaction rates associated with each step of the catalytic process. The enzyme (E) combines with its substrate (S) to form the enzyme-substrate complex (ES), which can be re-dissociated to form $E + S$ or can continue the chemical reaction to form P and E. It is assumed that the reverse reaction of the enzyme with the product ($E + P$) forms an ES complex at an insignificant rate. Observations of the properties of many enzymes show that at low substrate concentrations (S), the lower initial rate (V_0) is directly proportional to [S], while at high substrate concentrations [S], the rate tends to the maximum, and the reaction rate is independent of [S]. The maximum rate is $V_{\max}$. Meanwhile, based on the above assumptions and empirical observations, the Michaelis-Menten model can be deduced as follows [13] [14]:

$$V_0 = \frac{V_{\max} \cdot [S]}{K_m + [S]}$$

3. Results and Analysis

3.1. Lipase Species Screening

Firstly, the kinetic resolution of three species of lipases was investigated in this study to select lipases with high reactivity and selectivity. The specific results were shown in **Figure 3**.

The experimental data were shown in **Figure 3**, under the condition of no catalyst, there was almost no transesterification reaction between PE and ethyl acetate, and the yield was only 0.23% without catalyst. However, the kinetic resolution reaction of tetraethoxylanol has been improved to varying degrees with the addition of different lipases. At the same time, the catalytic activities of porcine pancreatic lipase, Candida Antarcticus enzyme B (free state) and Novozym 435 increased sequentially, especially Novozym 435 showed higher reactivity compared with other lipases, with the yield increased to 38.06%. At the same time, considering that Novozym 435 had the best reaction activity, Candida Antarctica enzyme B was selected as the lipase for subsequent experiments.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E+P$$

Figure 2. Organic phase enzymatic reaction process.

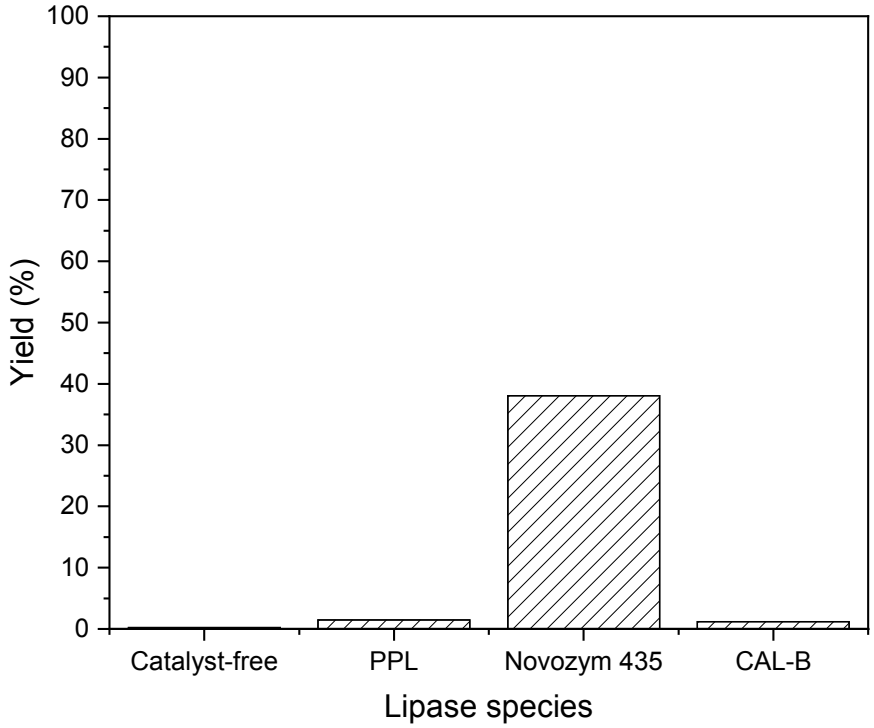

Figure 3. Effects of lipase species on the kinetic resolution of PE.

3.2. Screening of Acyl Donors

As substrates in the reaction, acyl donor played an important role in the kinetic resolution of PE acetate reaction. In the selection of acyl donor, the conversion rate of reaction, the activity of lipase and the stereoselectivity of the enzyme should be fully considered. In this study, four different types of acyl donors were investigated for kinetic resolution of PE, so as to select acyl donors with higher reactivity. The specific results were shown in **Table 1**.

The experimental data were shown in **Table 1**, ethyl acetate and vinyl acetate, which were widely studied acyl donors, have achieved good yields, while methyl acetate and ethyl formate had low yields. At the same time, considering that enol ester as acyl donor was an irreversible transesterification reaction with the highest yield of 45.80%, this study chose vinyl acetate as the acyl donor for subsequent experiments.

3.3. Screening of the Optimal Solvent

As the reaction medium, the solvent played an important role in the kinetic resolution of PE acetate reaction. This was not only because the solvent determined the diffusion rate and solubility of reactants in the reaction, but also affected the catalytic activity of lipase to a certain extent. In this study, five different types of reaction solvents and solvent-free systems were investigated for kinetic resolution of PE, so as to select the reaction solvent with high reaction activity. The specific results were shown in **Table 2**.

As could be seen from **Table 2**, when tetrahydrofuran and acetonitrile were

Table 1. Effect of acyl donor on the kinetic separation of PE.

Entry	Acyl donor	Yield (%)
1	ethyl acetate	38.06
2	acetic acid vinyl ester	45.80
3	methyl acetate	31.51
4	ethyl formate	26.98

Table 2. Effect of solvent on the kinetic separation of PE.

Entry	Solvent	Yield (%)
1	tetrahydrofuran	13.89
2	toluene	48.20
3	n-hexane	58.45
4	n-heptane	57.19
5	acetonitrile	44.21
6	solvent-free	45.80

used as solvent, their catalytic activity was lower than that of the solvent-free group. Especially in tetrahydrofuran solution, the reaction yield was only 13.89%. The catalytic activity of Novozym 435 in this solvent was much lower than that of other groups. It was speculated that tetrahydrofuran as a solvent can not well dissolve tetrahydrofuran alcohol or vinyl acetate; however, toluene, n-hexane and n-heptane as reaction solvents showed better catalytic activity, and considering that n-hexane as solvent had the best reaction yield of 58.45%, n-hexane was selected as the reaction solvent for subsequent experiments.

3.4. Screening of the Optimal Amount of Lipase

The addition of lipase as a catalyst in the kinetic resolution of PEA played an important role in this study. Although lipase was theoretically not consumed as a catalyst, it had the disadvantage of being expensive compared to common chemical reagents, and the amount of lipase added to the reaction was an issue that must be considered in order to improve the economics of the study. In this study, the kinetic splitting of sulphoxide was investigated at 5 mg/ml, 10 mg/ml, 20 mg/ml, 40 mg/ml and 50 mg/ml of lipase, in order to select a more economically viable amount of lipase with higher reactivity, as shown in **Figure 4**.

As could be seen from **Figure 4**, when the amount of lipase added was less than 20 mg/ml, the yield decreased significantly with the decrease of the amount of lipase added, and the yield was only 45.81% when the amount of lipase was 5 mg/ml. When the lipase content was between 20 mg/ml and 40 mg/ml, the yield changed steadily and increased slightly. When the lipase content was 40 mg/ml, the yield reached the highest point and then began to decline slowly. When the lipase content was 40 mg/ml, the yield was 61.49%. Therefore, considering the catalytic activity and economic feasibility of lipase comprehensively, 20 mg/mL

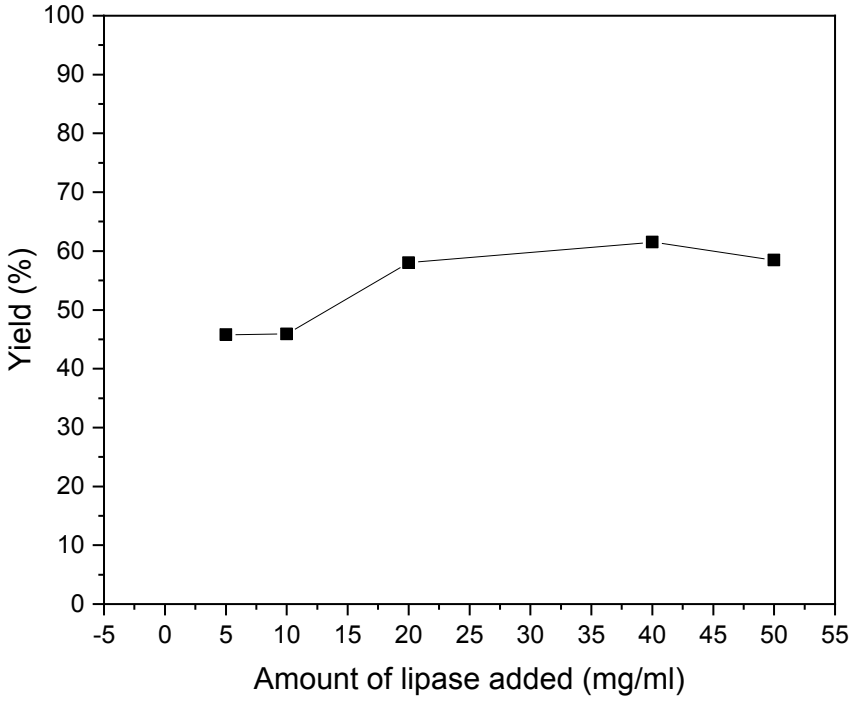

Figure 4. Effects of different lipase addition amounts on the kinetic resolution of PE reaction.

lipase addition was selected as the reaction condition for subsequent experiments.

3.5. Performance Test of Lipase Reuse

As an immobilized enzyme, Novozym 435 had important and long-term significance in industrial applications, and its reuse performance deserved special attention. In this study, lipase was recovered by recycling operations in Section 1.3.1, and the treated lipase was applied repeatedly to obtain the experimental results of the influence of the number of lipase uses on the catalytic activity of lipase. The specific results were shown in **Figure 5**.

By repeated cycle, to paraphrase the **Figure 5** showed that Novozym 435 lipase catalytic activity showed a trend of slow decline, but the catalytic activity of lipase can remain at more than 45%, this may be due to between the lipase and immobilized carrier form relatively stable covalent bond, this limited the lipase molecules idea of radical changes. Therefore, from the perspective of economic feasibility, Novozym 435 lipase would be used as catalyst in subsequent experiments.

3.6. Screening of the Optimum Reaction Time and Substrate Concentration

The concentration of the substrate played an important role in the kinetic resolution of the reaction of PE and vinyl acetate. Studies had pointed out that substrate inhibition was easy to occur in enzymatic reactions. That was, when substrate

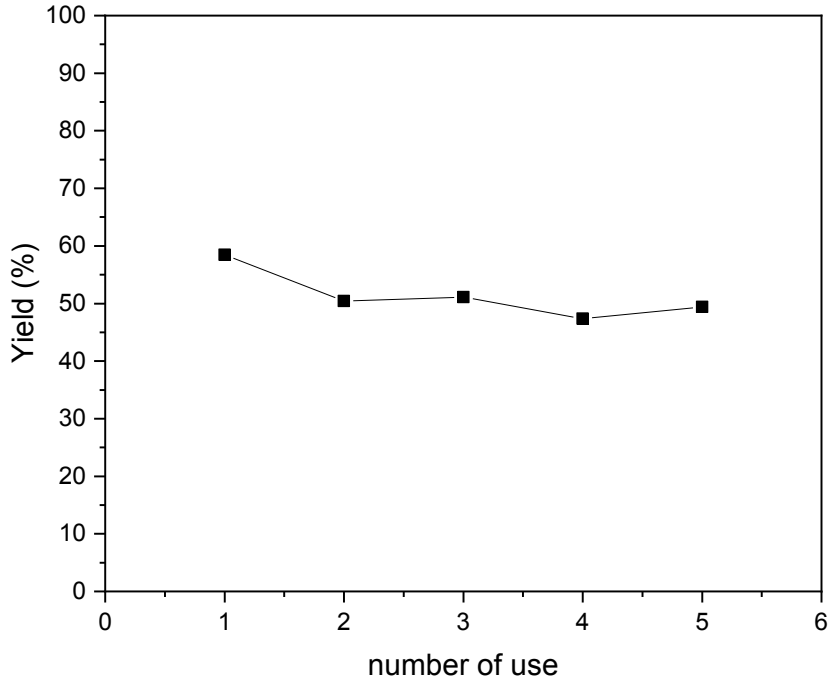

Figure 5. Effect of lipase application times on kinetic resolution of PE reaction.

concentration increased to a certain threshold, the initial reaction rate would decrease with the increase of substrate concentration. In addition, appropriate enzymatic reaction time had great reference value for future industrial application. In this study, the kinetic resolution reaction was investigated with the concentration of 50 mmol/L, 100 mmol/L, 200 mmol/L and 500 mmol/L, respectively, and the sampling analysis was conducted at intervals of 0 h, 0.5 h, 1 h, 3 h, 5 h and 7 h to select the appropriate reaction conditions. The specific results were shown in **Figure 6**.

It could be seen from **Figure 6** that in the first 1h of the reaction, the yield of the reaction decreased significantly with the increase of substrate concentration, which also indirectly indicated that the initial reaction rate of higher substrate concentration was lower. For example, for 500 mmol/L substrate concentration, the yield was only 17.20% for 0.5 h. After about 2 hours of reaction time, the yield of different substrate concentration reached the peak (50 mmol/L, 100 mmol/L substrate concentration, only needed about 1 hour to reach the peak). Meanwhile, from the experimental data of 3 h, 5 h and 7 h, it could be seen that the reaction time and substrate concentration had no significant influence on the final reaction yield.

3.7. Kinetic Study of the Enzymatic Reaction

In this study, the kinetic parameters of the enzymatic reaction were explored based on the Hanes-Woolf curve (**Figure 7**), from which the kinetic equations for the enzymatic reaction could be derived by substituting the Michaelis-Menten model equation. Besides, whether an enzymatic reaction had potential industrial application was largely determined by the catalytic efficiency (K_m / V_{max}).

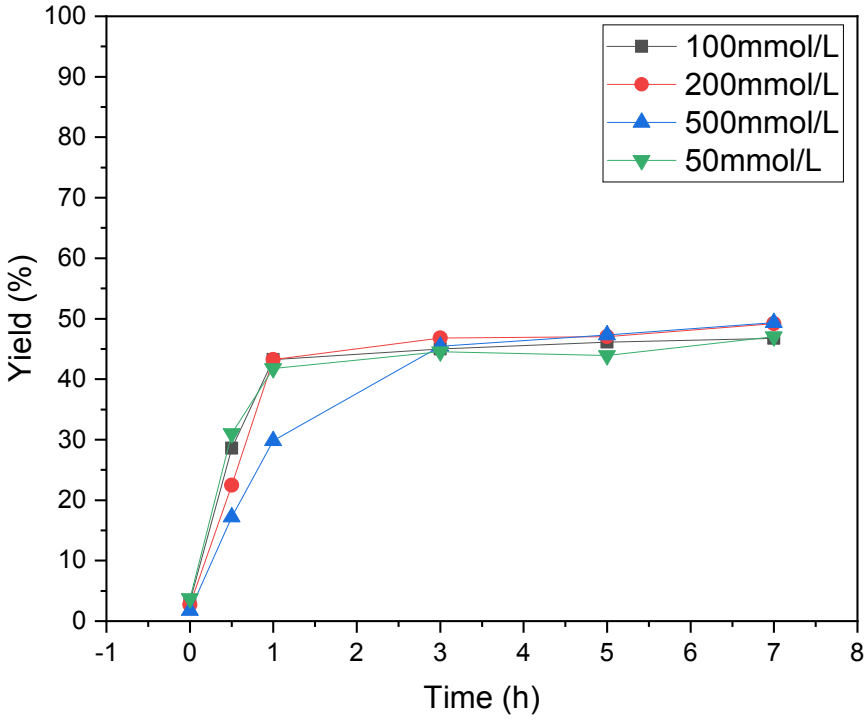

Figure 6. Effects of different substrate concentration and reaction time on kinetic resolution of PE.

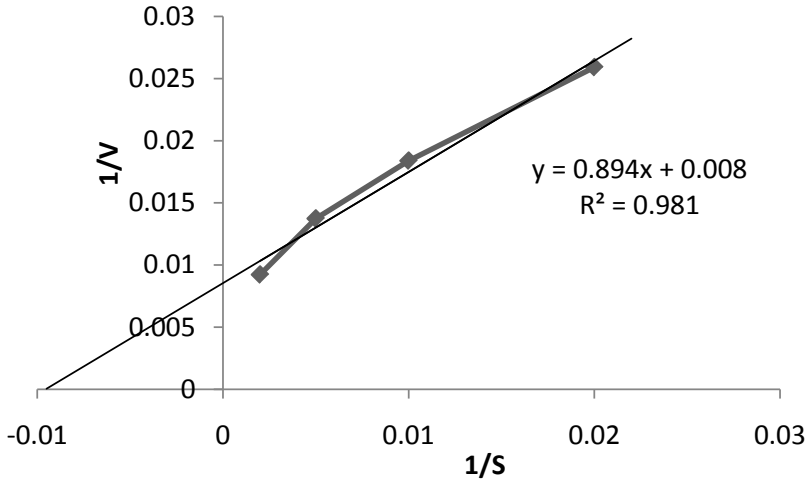

Figure 7. Double inverse plot ($1/v$ plotted against $1/S$).

The K_m/V_{max} measured for this reaction was 0.8943 h, V_{max} was 117.65 mmol·L^{-1}·h^{-1} approximately 0.118 mmol·L^{-1}·h^{-1} and K_m was 105.21 mmol/L *i.e.* 0.105 mol/L. The resulting Michaelis-Menten model was:

$$V_0 = \frac{0.118 \cdot [S]}{0.105 + [S]}$$

3.8. Screening of the Optimum Reaction Temperature

For ordinary chemical reactions, as the temperature rose, the molecules involved

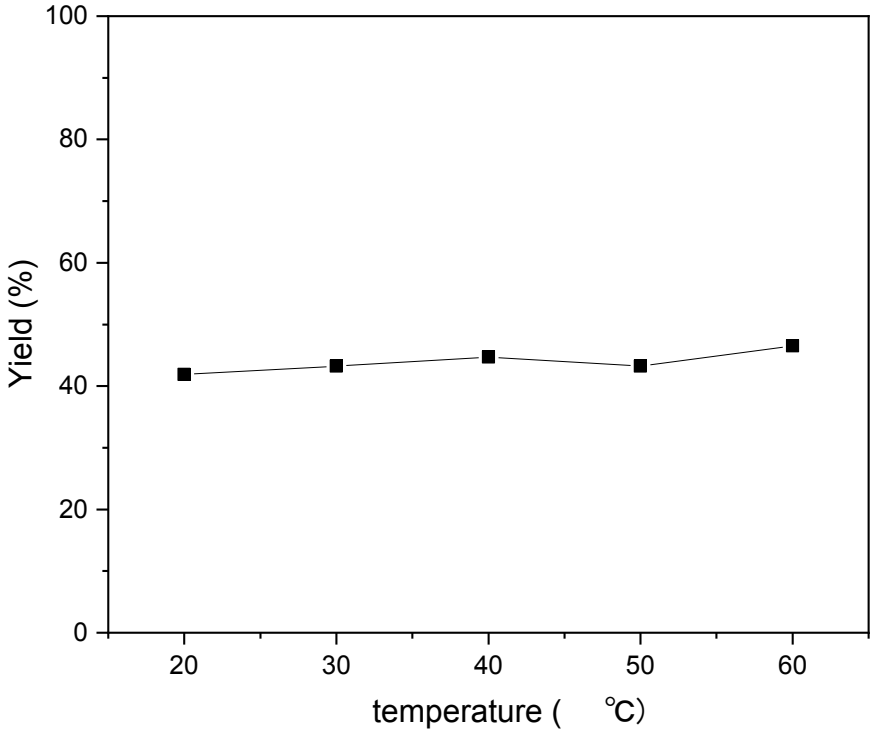

Figure 8. Effect of different reaction temperatures on kinetic resolution of PE。

gain more energy, the chance of collisions increases, and the reaction rate increased. But for enzymatic reactions, when the temperature rose to a certain threshold, the enzyme protein molecules denature and lose catalytic activity. In this study, the reaction temperature of 20˚C, 30˚C, 40˚C, 50˚C and 60˚C was investigated for kinetic resolution of PE, so as to select the reaction temperature with high reaction activity. The specific results were shown in **Figure 8**.

Figure 8 showed that with the increase of reaction temperature, the reaction yield increased slightly. Generally speaking, the improvement of reaction temperature on the reaction yield was not significant. By the way, the Novozym 435 lipase used in this experiment was a 4-times recycled lipase, so the catalytic efficiency was lower compared to the yields of the previous experiments.

4. Conclusion

In this study, PE was used as substrate and lipase was used as catalyst to conduct enzymatic kinetic resolution of aromatic alcohol compounds, and factors such as Lipase species, acyl donor, reaction solvent, lipase addition amount, lipase reuse performance, reaction time, substrate concentration and reaction temperature were investigated. Meanwhile, on the basis of the above experiments, the kinetic parameters of enzymatic reaction were explored in this study according to Hanes-Woolf curve. The results showed that Lipase species, acyl donor and solvent played an important role in influencing the catalytic activity of the enzymatic reaction, because most of the enzymes were highly specific to the substrate and the catalyzed reaction. The reaction temperature and the amount of lipase

had no significant effect on the catalytic activity of the enzymatic reaction. Further kinetic studies also revealed the degree of influence of substrate concentration on the catalytic activity of enzymatic reaction. This study provided a new way for enzymatic kinetic resolution of aromatic compounds, and also provided a reference for the application of lipase in the industrial production of spices.

Conflicts of Interest

The authors declare no conflicts of interest.

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