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Kinetic Resolution of (*R*,*S*)-2-Butanol Using Enzymatic Synthesis of Esters

Maria Dolores Romero · Jose Maria Gomez · Beatriz Diaz-Suelto · Alicia Garcia-Sanz · Nicola Baster

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Abstract Kinetic resolution of (*R*,*S*)-2-butanol using enzymatic synthesis of esters has been studied. (*R*,*S*)-2-Butanol is commonly found as a racemic mixture, and the products of its esterification are racemic mixtures too. This work is of great significance in the field of the enzymatic kinetic resolution due to the little information found in literature about the resolution of (*R*,*S*)-2-butanol as pure compound. So, this article is a contribution about the enzymatic resolution of (*R*,*S*)-2-butanol. The reaction here studied is the esterification/transesterification of (*R*,*S*)-2-butanol in organic media (*n*-hexane) using as biocatalyst the lipase Novozym 435®. The main target of this study is to analyze the influence of certain variables in this reaction. Some of these variables are acyl donor (acids and esters), concentration of substrates, enzyme/substrate ratio, and temperature. The main conclusions of this study are the positive effect of higher substrates concentration (1.5 M) and larger amount of enzyme (13.8 $g mol^{-1}$ substrate) on kinetic resolution rate but not a very noticeable effect on enantiomeric excesses. The longer the carboxylic acid chain is, the better results are obtained. Besides to achieve a satisfactory kinetic resolution, it is recommendable to select reaction times (180 min) at which the highest substrate enantiomeric excess is reached (~60%). The temperature has not an appreciable influence on the resolution in the range studied (40–60 °C). When an ester (vinyl acetate) is used as acyl donor, the resolution shows better results than when using a carboxylic acid as acyl donor (ee_s~90% at 90 min). Moreover, Michaelis–Menten parameters, v_{max} and K_M, were determined, 0.04 mol l^{-1} min^{-1} and 0.41 mol l^{-1}, respectively.

Keywords Esterification · Kinetic resolution · Enzymatic · (*R*,*S*)-2-Butanol

Introduction

Racemic Mixture Kinetic Resolution

Racemic mixture resolution is a very important fact for pharmaceutical, food, and cosmetic industries because most of their products are racemic mixtures and in some cases only one isomer is useful for their purposes. For example, *S*-limonene and *R*-limonene are optical isomers from limonene, both act as flavoring. Anyhow *S*-limonene is extracted from orange peels, and

M. D. Romero (✉) · J. M. Gomez · B. Diaz-Suelto · A. Garcia-Sanz · N. Baster
Chemical Engineering Department, Universidad Complutense de Madrid, 28040 Madrid, Spain
e-mail: mdolores@quim.ucm.es

R-limonene is extracted from lemon peels. Not only racemic mixtures are present in food industry but also in the pharmaceutical one. For instance, *S*-ibuprofen, a well-known anti-inflammatory drug, has therapeutic activity [1] while *R*-ibuprofen does not show therapeutic activity. On the contrary, *S*-thalidomide has teratogen activity while *R*-thalidomide has sedative effect. Its use as a racemic mixture can lead to birth defects when pregnant women take it.

Not only the study of racemic mixtures and their resolution has importance for human health but also it has an environmental importance too [2]. Degradation of chiral drugs during wastewater treatment and in the environment can be stereoselective and can lead to chiral products of varied toxicity. The distribution of different enantiomers of the same chiral drug in the aquatic environment and biota can also be stereoselective and its toxicity too. As a result, the same drug might reveal different activity and toxicity that will depend on its origin and exposure to several factors governing its fate in the environment. The most important environmentally relevant groups of chiral drugs are nonsteroidal anti-inflammatory drugs, central nervous system drugs, cardiovascular, respiratory, gastrointestinal, antimicrobials, and chemotherapy drugs.

Different methods are considered suitable for racemic mixture resolution [3]; nonetheless, the current preference for the so-called natural products has brought about an increasing interest toward biotechnology in order to obtain these pure isomers. The first method developed was the spontaneous resolution [4]. It consisted of the precipitation of the isomer of interest from a supersaturated solution of the racemic mixture in a spontaneous way or by previous seeding of crystals of the interesting isomer (resolution by entrainment). This method can be used if the racemic substance crystallizes as a conglomerate composed of observably different particles of the two enantiomers, which can be physically sorted; anyhow, seldom is applicable. Zaugg proposed it as a method to obtain the much more active *l*-methadone, an analgesic drug [5].

Another way of producing pure enantiomers is the use of chiral resolving agents. It is based upon the conversion of the mixture of enantiomers into a mixture of diastereoisomers (optical isomers that are not mirror images of one another), which differ in physical properties and therefore can be separated by physical processes. This transformation requires the use of a previously obtained optically active substance. The two salts present in the mixture, therefore, have different solubility and so are separable. Synthesis of duloxetine, an antidepressant drug, can be set as an example [6].

Nowadays, processes which involve biotechnology are likely to succeed, so kinetic resolution using enzymes seems to be the best one. It consists on using a highly selective catalyst (enzymes or other) which reacts preferably with one of the isomers of the mixture. It is an asymmetric synthesis which leads to obtain one free isomer and the other one as a different product.

Direct esterification is an equilibrium reaction where one of the products is water, so in order to avoid hydrolysis, it must be carried out in non-aqueous solvents, like organic solvents [7, 8], supercritical fluids (SCFs) [9–11], and ionic liquids (ILs) [12]. Recently, some authors [13, 14] have studied the use of lipases in esterification reactions on byphasic systems (phase-separable catalysis) based on different solvents such as ILs, SC-CO_2, fluorous solvents, and liquid polymers. These systems bring together the benefits from the SCFs, reactants, and/or products reside largely in the SC-CO_2 and those from the ILs, which are helpful to stabilize the activity of lipases. Despite these apparent benefits of these new solvents, there are some cases in which the best results are obtained using organic solvents (as *n*-hexane). To set an example, the transesterification of 2-butanol using vinyl acetate as acyl donor and Novozym 435 as biocatalyst shows higher esterification extents at shorter times in *n*-hexane media than in SC-CO2 media (results not published yet).

In this paper, the biocatalyst used has been a hydrolase enzyme, Novozym 435®, which is being successfully used in esterification reactions [15]. A wide number of racemic

mixtures have been resolved using Novozym 435® as biocatalyst, for example, alcohol and silane mixtures. Kinetic resolution of 1-phenylethanol was developed using vinyl acetate as acyl donor and an ionic liquid as solvent [16]. Vinyl acetate was also used as acyl donor in kinetic resolution of α-hydroxysilanes in organic media (pentane), providing enantiomeric excesses near 99% for 1-hydroxyallyltrimethylsilane resolution [17]. Kinetic resolution of 1-(3,4-methylenedioxyphenyl)propan-1-ol, better known as marginatumol, led to determine the absolute configuration of natural marginatumol, by comparison of the optical properties of both compounds, the synthetic and the natural one [18]. Novozym 435 can also be used in the production of compounds which are of importance for pharmaceutical industry [19].

Therefore, the aim of this work is the kinetic resolution of (*R*,*S*)-2-butanol by esterification or transesterification of (*R*,*S*)-2-butanol with an acyl donor, using as catalyst the immobilized lipase Novozym 435® from *Candida antarctica*. The reaction media used has been *n*-hexane. The effect of some variables as substrates concentration, enzyme/substrate ratio, temperature, carboxylic acid chain length, and type of acyl donor was studied. Additionally kinetic data were experimentally determined (not found in bibliography) and fitted to the Michaelis–Menten equation.

Michaelis–Menten Model

The Michaelis–Menten model is one of the simplest and best-known approaches to enzyme kinetics. It takes the form of an equation relating reaction rate to substrate concentration for a system where a substrate S binds reversibly to an enzyme E to form an enzyme–substrate complex ES, which then reacts irreversibly to generate a product P and to regenerate the free enzyme E. This system can be represented schematically as follows:

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

To arrive at the familiar form of the Michaelis–Menten equation, some assumptions [20] and substitutions are needed. Michaelis–Menten equation for this system can be written as (Eq. 1),

$$v = \frac{v_{\max} \cdot [\mathrm{S}]}{K_{\mathrm{M}} + [\mathrm{S}]} \tag{1}$$

Here, $v_{\max}$ represents the maximum rate achieved by the system, at maximum (saturating) substrate concentration. [*S*] is the concentration of the substrate S. K_{M} (Michaelis constant) is the substrate concentration at which the reaction rate is 50% of the $v_{\max}$, and it is defined in terms of the rate constants as follows (Eq. 2):

$$K_{\mathrm{M}} = \frac{k_2 + k_3}{k_1} \tag{2}$$

Materials and Methods

Reagents and Materials

All chemicals—acetic acid (Panreac), *n*-decane and butyric acid (Aldrich), (*R*,*S*)-2-butanol, hexanoic and octanoic acids, acetic anhydride, vinyl acetate, and *n*-hexane (Fluka)—were of analytical grade. The enzyme was Novozym 435® from *C. antarctica* hydrolase B,

immobilized on a macro porous acrylic resin with a water content of 1–2% (*w*/*w*) and kindly provided by Novo Nordisk, Denmark.

Method for (*R*,*S*)-2-Butanol Kinetic Resolution

Reactions in *n*-hexane were carried out in a shaker, equipped with a temperature controller. In all the synthesis remained constant not only the agitation rate (200 rpm) but also the atmospheric pressure. All the experiments were carried out at equimolar concentrations of reactants, and the volume of the reaction mixture was 20 ml. *n*-Decane was used as internal standard (1%, *w*/*w*). After different reaction times, 200 μl of sample was taken in order to follow the reaction. Samples were analyzed by GC.

With the aim of choosing the best conditions to get a proper (*R*,*S*)-2-butanol kinetic resolution, the influence of different variables in the reaction rate and enantiomeric excesses was studied. Substrate concentrations varied from 0.3 to 1.5 M. Two different enzyme/substrate ratios (E/S), 6.3 and 13.8 $g\,mol^{-1}$, were used and temperatures of 30 °C, 40 °C, and 60 °C were studied. The carboxylic acids used were acetic, butanoic, hexanoic, and octanoic. Different types of acyl donors as carboxylic acids, esters, and anhydrides were used.

Substrate and product enantiomeric excesses (ee_S and ee_P) were determined by the following equations (Eqs. 3 and 4):

$$ee_S = \frac{C_B - C_A}{C_B + C_A} \cdot 100 \tag{3}$$

$$ee_P = \frac{C_{A_0} \cdot X_A - C_{B_0} \cdot X_B}{C_{A_0} \cdot X_A + C_{B_0} \cdot X_B} \cdot 100 \tag{4}$$

where the subscripts A and B represent the fast and the slow (*R*,*S*)-2-butanol isomer, respectively.

Taking into account the studies of molecular recognition of sec-alcohol enantiomers by *C. antarctica* lipase B [21], the isomer which reacts faster is *R*-2-butanol while *S*-2-butanol reacts in a slower way.

Method for Kinetic Data Measurement

Experimental setup for the kinetic modeling experiments consisted of a glass reactor equipped with three openings submerged into a silicon bath. Temperature was controlled using a heating plate equipped with a contact thermometer. The reaction mixture was agitated using a magnetic stirrer (200 rpm). A water cooled condenser was used in order to avoid solvent evaporation.

Reaction mixture (50 ml) consisted of the organic solvent (*n*-hexane), substrates at equimolar concentrations ((*R*,*S*)-2-butanol and vinyl acetate), internal standard (*n*-decane), and 6.9 $g\,l^{-1}$ of enzyme. Substrate concentrations varied from 0.1 to 1 M while enzyme concentration remained constant (6.9 $g\,l^{-1}$). Samples were withdrawn at short reaction times. Samples were analyzed by GC.

Relationship between amount of product and reaction time is lineal only during the first reaction minutes, and the slope represents the initial reaction rate (v_0). This value changes with the amount of enzyme and substrate concentration.

Once initial reaction rates (v_0) at each substrate concentration were calculated, and these were fitted to the Lineweaver–Burk equation. Lineweaver–Burk equation is the result of the Michaelis–Menten equation linearization (Eq. 5).

$$\frac{1}{v_0} = \frac{K_M}{v_{max}} \cdot \frac{1}{[S]} + \frac{1}{v_{max}} \tag{5}$$

Analytical Methods

Substrates and products concentrations were determined by gas chromatography with a Varian gas chromatograph equipped with a hydrogen flame ionization detector and a CHIRALDEXTM B-PM column (30 m length×0.25 mm i.d.). Helium was used as carrier gas at a flow rate of 1 ml min^{-1}. In all cases, the initial temperature of the column oven was 40 °C with a heating rate of 2 °C min^{-1} to 54 °C, and after 1 minute of stabilization, the temperature was increased up to 150 °C (at 15 °C min^{-1}). Injector temperature was 200 °C and detector temperature was 250 °C. All the samples were analyzed three times to determine the experimental error, which was less than 4% in all cases.

Retention times for (*R*,*S*)-2-butanol isomers were 5.861 and 6.145 min, respectively. Taking into account that the w_h (peak width at half height) for both peaks is 0.11 min, the resolution (R_S) was 2.65 which let us to be sure that the analytical method used is optimal. In the same way, the resolution for the (*R*,*S*)-2-butyl acetate was determined, giving a higher value of 23.14.

Results and Discussion

Effect of Substrates Concentration

In order to study the influence of substrates concentration, kinetic resolution was performed using substrates concentration from 0.3 to 1.5 M. The enzyme/substrate ratio used was 6.3 gmol $alcohol^{-1}$, the acyl donor was acetic acid, and temperature was 40 °C.

Figure 1 depicts the evolution of substrate enantiomeric excess with the conversion of (*R*,*S*)-2-butanol. As can be seen, the higher the substrates concentration is (1.5 M), the higher substrate enantiomeric excesses are obtained at the same (*R*,*S*)-2-butanol conversions.

Figure 2 depicts the evolution of product enantiomeric excess with (*R*,*S*)-2-butanol conversion. At the beginning of the reaction, product enantiomeric excess is close to 100% because only the fastest isomer (*R*-2-butanol) reacts so the product only contains *R*-2-butyl acetate. As the reaction continues, product enantiomeric excess decreases because *S*-2-butyl acetate is formed. At low substrate concentrations (up to 0.5 M), product enantiomeric excess remains close to 100% till (*R*,*S*)-2-butanol conversions of 10%, while at higher substrate concentrations (from 0.8 to 1.5 M), product enantiomeric excess decreases fast and reaches values of 85% at (*R*,*S*)-2-butanol conversions of just 3%. Consequently, it is recommendable to work with lower substrate concentrations to get an adequately pure product (eep~100%) and enough amount of it. It would be proper to work with higher substrate concentrations if the target of the resolution is the substrate, whereas if the target is the product, it would be better to use lower substrate concentrations.

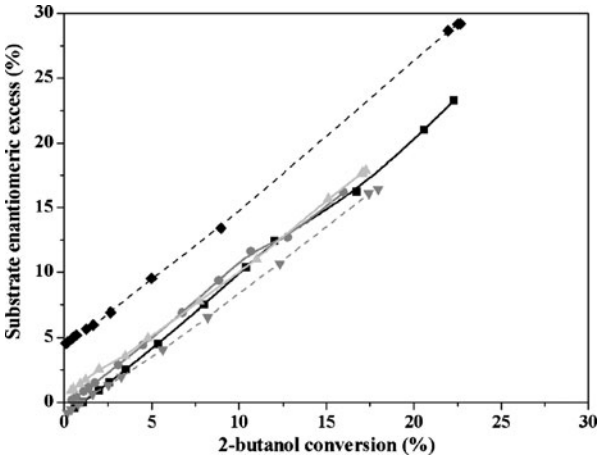

Fig. 1 Substrate enantiomeric excess with (*R*,*S*)-2-butanol conversion at different substrate concentration (*square* 0.3 M, *circle* 0.5 M, *triangle* 0.8 M, *inverted triangle* 1 M, *diamond* 1.5 M). E/S (enzyme/substrate ratio) 6.3 gmol alcohol^{-1}, acyl donor: acetic acid, *T* 40 °C

Effect of Enzyme/Substrate Ratio

In order to study the influence of enzyme/substrate ratios, kinetic resolution was performed using two different ratios, 6.3 and 13.8 gmol alcohol^{-1}. These values were selected from previous studies [22]. The substrates concentration used was 0.5 M, the acyl donor was acetic acid, and temperature was 40 °C.

Figure 3 depicts the evolution of the two isomers obtained with the reaction time. In both cases, the yield of *R*-2-butyl acetate is higher than the yield of *S*-2-butyl acetate. When an enzyme/substrate ratio of 13.8 gmol alcohol^{-1} is used, the yield of the fastest isomer (*R*-2-butyl acetate) is higher than when a lower ratio is used. As it was expected, a larger amount of catalyst contributes to a higher reaction rate (Fig. 4).

Evolution of substrate and product enantiomeric excess was studied. Enzyme/substrate ratio has not a noticeable influence on substrate enantiomeric excess, whereas on product enantiomeric excess, it presents a more remarkable effect, although similar values are obtained at the end.

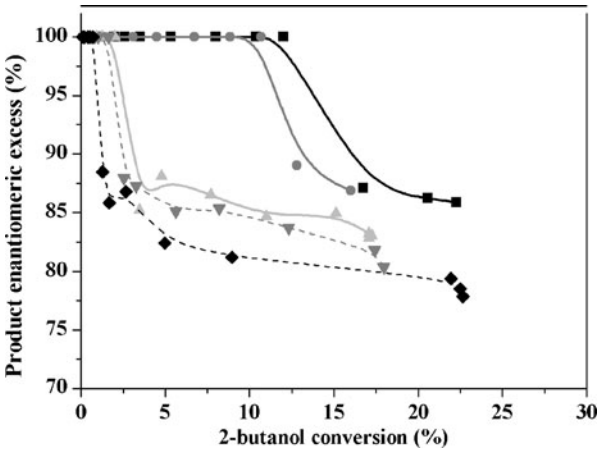

Fig. 2 Product enantiomeric excess with (*R*,*S*)-2-butanol conversion at different substrate concentration (*square* 0.3 M, *circle* 0.5 M, *triangle* 0.8 M, *inverted triangle* 1 M, *diamond* 1.5 M). E/S 6.3 gmol alcohol^{-1}, acyl donor: acetic acid, *T* 40 °C

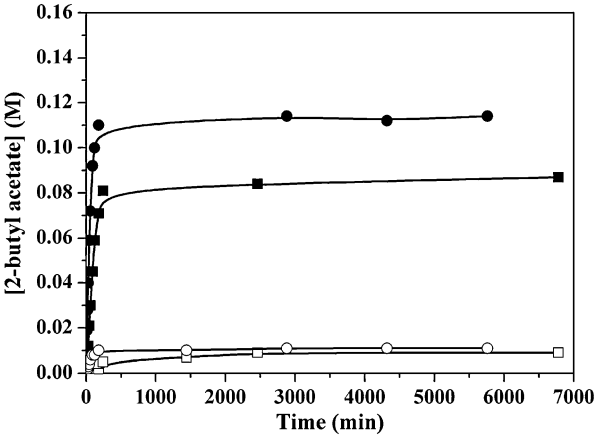

Fig. 3 *R*/*S*-2-butyl acetate (*filled R*, *empty S*) concentration with time at different E/S (*square* 6.3 gmol alcohol^{-1}, *circle* 13.8 g mol alcohol^{-1}). Substrate concentration 0.5 M, acyl donor: acetic acid, *T* 40 °C

As a result, lower enzyme/substrate ratios provide a purified product in *R*-2-butyl acetate (eep~100%) but low yields ((*R*,*S*)-2-butanol conversion~10%). However, higher enzyme/substrate ratios provide a not so purified product (eep~85%) but faster and also provide large amount.

Effect of Acid Chain Length

In order to study the influence of acid chain length, kinetic resolution was performed using acetic, butanoic, hexanoic, and octanoic acid as acyl donors. The substrates concentration was 0.5 M, the enzyme/substrate ratio was 13.8 gmol alcohol^{-1}, and temperature was 40 °C.

Figure 5 depicts the evolution of *R*-2-butyl esters produced when different acids are used as acyl donor. These are *R*-2-butyl acetate, butyrate, hexanoate, and octanoate. It can be seen that the use of acetic acid as acyl donor provides lower yields (~30%) than when longer chain acids are used. In such a case, the yield of esters produced is close to 92% and seems to be independent of acid chain length.

Further studies [23] have proposed that the better enantioselectivity got, when longer acyl chains are used, is due to the big differences of the thermodynamic components (enthalpy and entropy) of the enantioselectivity between the longer acyl chains and the

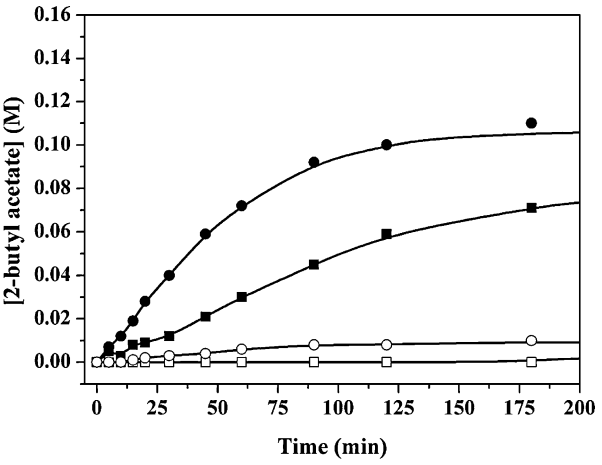

Fig. 4 *R*/*S*-2-butyl acetate (*filled R*, *empty S*) concentration with time (t<200 min) at different E/S (*square* 6.3 gmol alcohol^{-1}, *circle* 13.8 gmol alcohol^{-1}). Substrate concentration 0.5 M, acyl donor: acetic acid, *T* 40 °C

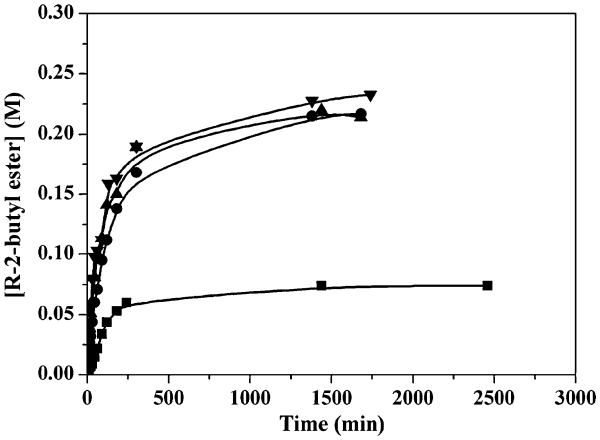

Fig. 5 *R*-2-butyl ester concentration with time using different carboxylic acids (*square* C_2, *circle* C_4, *triangle* C_6, *inverted triangle* C_8) as acyl donor. Substrate concentration 0.5 M, E/S 13.8 gmol alcohol^{-1}, *T* 40 °C

shorter ones. Figures 6 and 7 depict substrate and product enantiomeric excesses, respectively. Acetic acid has an effect on these variables which significantly differs from other acids. On one hand, substrate enantiomeric excess reaches a maximum near 60%, when acyl donors are butyric, hexanoic, and octanoic, and this behavior is characteristic of reversible reactions. On the other hand, when acetic acid is the acyl donor, substrate enantiomeric excess does not reach any maximum, which may be due to the previous commented inhibitory effect of acetic acid on the enzyme or to the lower thermodynamic components of the enantioselectivity for the shorter acids.

Product enantiomeric excess decrease is more markedly when the acid chain is longer. When acetic acid is used as acyl donor, product enantiomeric excess decrease is less pronounced due to the low esters yields obtained.

Effect of Temperature

In order to study the influence of temperature, kinetic resolution was performed at 30 °C, 40 °C, and 60 °C. The substrates concentration used was 0.5 M, the enzyme/substrate ratio was 13.8 gmol alcohol^{-1}, and the acyl donor was hexanoic acid.

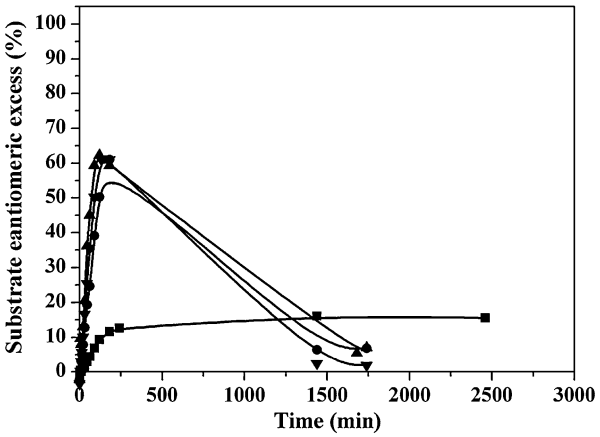

Fig. 6 Substrate enantiomeric excess with time using different carboxylic acids (*square* C_2, *circle* C_4, *triangle* C_6, *inverted triangle* C_8) as acyl donor. Substrate concentration 0.5 M, E/S 13.8 gmol alcohol^{-1}, *T* 40 °C

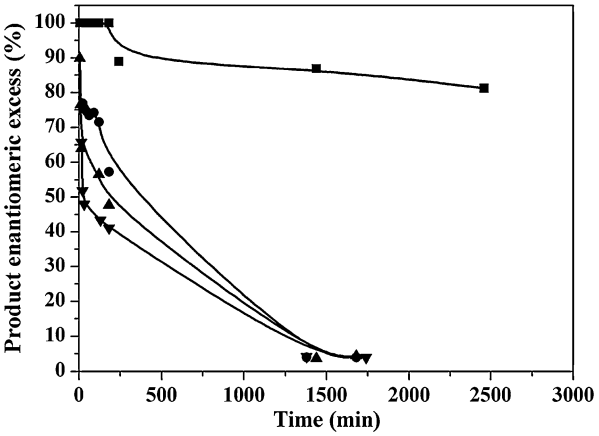

Fig. 7 Product enantiomeric excess with time using different carboxylic acids (*square* C_2, *circle* C_4, *triangle* C_6, *inverted triangle* C_8) as acyl donor. Substrate concentration 0.5 M, E/S 13.8 gmol alcohol^{-1}, *T* 40 °C

Temperature seems not to have a significant effect on the production of the ester. Temperature has a positive effect on kinetics, but it can have also a negative effect on enzyme activity. Both effects can be counteracted each other and that can be the reason why none noticeable influence is observed. Anyhow, it would be interesting to make some extra experiments in a wider temperature range in order to find out if temperature has an effect on (*R*,*S*)-2-butanol esterification kinetics.

Variation of substrate enantiomeric excess was also studied, and the trend observed is characteristic of reversible reactions in all the cases and temperature also seems not to have an effect on the kinetic resolution of (*R*,*S*)-2-butanol. The same conclusion can be drawn when the variation of product enantiomeric excess is analyzed.

Effect of Acyl Donor

In order to study the influence of acyl donor, kinetic resolution was performed using acetic anhydride and vinyl acetate as acyl donor. The substrates concentration used was 0.5 M, the enzyme/substrate ratio was 13.8 gmol alcohol^{-1}, and temperature was 40 °C.

Two different kinds of reactions were performed: on one hand esterification using acetic anhydride as acyl donor and on the other hand transesterification using vinyl acetate as acyl donor. Reaction's diagrams are depicted in Fig. 8.

Figure 9 depicts the evolution of *R*-2-butyl acetate (the fastest produced). As it can be observed, not only the yield is higher when using an ester as acyl donor (vinyl acetate) but also the reaction rate than when using the acetic anhydride. With vinyl acetate, the

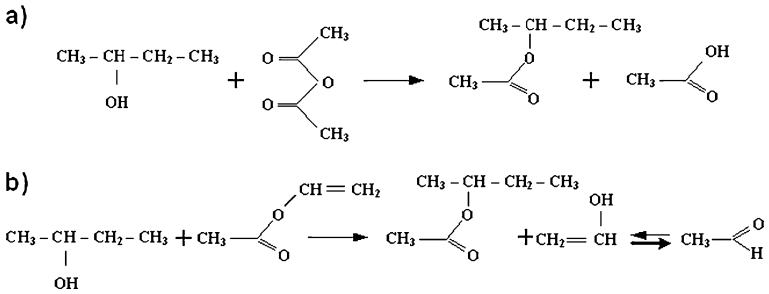

Fig. 8 Reaction's diagrams. **a** Acyl donor: acetic anhydride. **b** Acyl donor: vinyl acetate

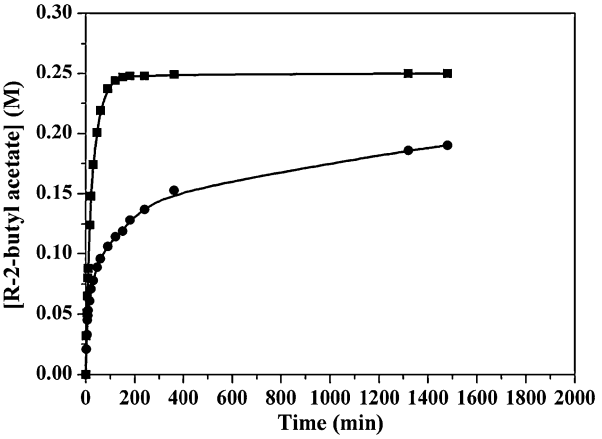

Fig. 9 *R*-2-butyl acetate concentration with time using different types of acyl donor (*square* vinyl acetate, *circle* acetic anhydride). Substrate concentration 0.5 M, E/S 13.8 gmol alcohol^{-1}, *T* 40 °C

maximum amount of *R*-butyl acetate (0.25 M) is reached at 3 h (~180 min), whereas with acetic anhydride, this is reached at times longer than 1 day (~1,500 min).

Substrate and product enantiomeric excesses were studied. The trend of substrate enantiomeric excess is alike for both acyl donor and is expected for irreversible reactions, in which substrate enantiomeric excess grows up with the reactants conversion during the whole range. When using vinyl acetate as acyl donor, the residual substrate purity ((*R*,*S*)-2-butanol conversion~90%) is close to 100%, which means that a high pure substrate can be obtained but with scarce amount of it. The same happens when the acyl donor is acetic anhydride.

Product enantiomeric excess presents the same trend for both acyl donors. A mixture of both isomers is obtained at the end of the reaction ((*R*,*S*)-2-butanol conversion~95%). The use of an ester as acyl donor improves kinetic resolution of (*R*,*S*)-2-butanol by means of an enzymatic synthesis, not only because of the high yields obtained but also because of the high reaction rate.

Michaelis–Menten Equation

In order to study (*R*,*S*)-2-butanol esterification kinetics, experiments were developed at different equimolar substrate concentrations (from 0.1 to 1 M). Reactions were performed using 6.9 gl^{-1} of enzyme at 40 °C and vinyl acetate as acyl donor.

Figure 10 depicts the evolution of (*R*,*S*)-2-butyl acetate (isomers mixture) concentration up to 3 h of reaction, at different substrate concentrations. Initial reaction rate is determined by means of the slope at short times.

Table 1 depicts the values obtained for the initial reaction rates and their inverses for each substrate concentration. As can be seen in Table 1, the higher the substrate concentration is, the higher the initial reaction rate. Therefore, no substrate inhibition is observed at least up to substrate concentrations of 1 M.

The linear fitting of the experimental data to the Lineweaver–Burk equation (Eq. 5) allows the determination of the Michaelis–Menten parameters, v_{max} and K_M.

$$\frac{1}{v_{max}} = 24.94\text{l min mol}^{-1} \rightarrow v_{max} = 0.04 \text{ mol l}^{-1} \text{ min}^{-1}$$
$$\frac{K_M}{v_{max}} = 10.19\text{min} \rightarrow K_M = 0.41 \text{ mol l}^{-1}$$

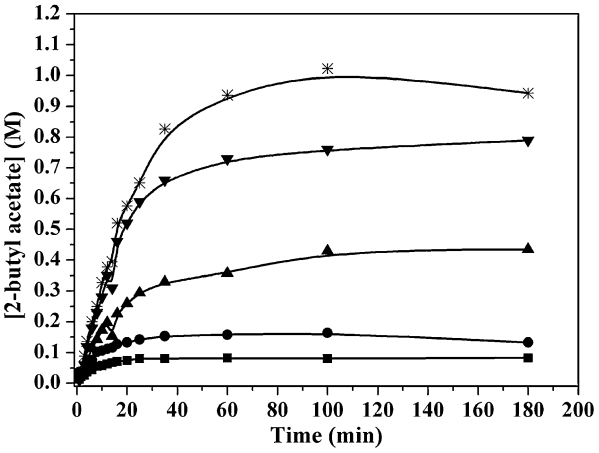

Fig. 10 Evolution of (*R*,*S*)-2-butyl acetate concentration with time at different substrate concentration (*square* 0.1 M, *circle* 0.16 M, *triangle* 0.5 M, *inverted triangle* 0.8 M, *asterisk* 1 M). E 6.9 gl^{-1}, acyl donor: vinyl acetate, *T* 40 °C

Equation 6 is the Michaelis–Menten equation for the (*R*,*S*)-2-butanol esterification with vinyl acetate using Novozym 435® as biocatalyst.

$$v = \frac{0.04 \cdot [S]}{0.41 + [S]} \tag{6}$$

Conclusions

Substrate concentration has a positive effect on reaction rate. The optimal concentration depends on the target of kinetic resolution. In order to get a pure substrate, it is better to work with high concentrations (1.5 M of substrates) and conversions; nonetheless, lower concentrations (0.3 M of substrates) and conversions (less than 10%) provide a purer product. Enzyme/substrate ratio has a more remarkable effect on reaction rate than on kinetic resolution. The advantage of using higher enzyme/substrate ratios (13.8 g mol $alcohol^{-1}$) is the shorter time needed to get an appropriate purity. Acids with more than four carbon atoms lead to a better kinetic resolution. Besides in order to get a substrate and a product of enough purity, it is advisable to finish the reaction at *R*-2-butanol equilibrium conversion (~55%). Temperature has not a noticeable effect on kinetic resolution in the studied range (30–60 °C). Using an ester as acyl donor (vinyl acetate) provides isomers of high purity in short times (180 min), so it is the best way of the presented study to develop (*R*,*S*)-2-butanol kinetic resolution. Michaelis–Menten equation for (*R*,*S*)-2-butanol esterification using Novozym 435® as biocatalyst and vinyl acetate as acyl donor has been determined.

Table 1 Values of v_0 and $1/v_0$ for each substrate concentration

[*S*] (mol l^{-1})	0.1	0.16	0.5	0.8	1
v_0 (mol l^{-1} min^{-1})	7.5×10^{-3}	1.3×10^{-2}	1.7×10^{-2}	2.9×10^{-2}	3.1×10^{-2}
$1/v_0$ (l min mol^{-1})	133.3	75.2	57.8	34.8	32.4
1/[*S*] (l mol^{-1})	10	6.25	2	1.25	1

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