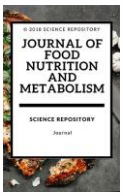

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Research Article

The mechanism of lipase-catalyzed synthesis of food flavoring ethyl butyrate in a solvent-free system

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ABSTRACT

Immobilized Lipase-B from *Candida antarctica* catalyzed the esterification of butyric acid and ethanol under anhydrous solvent-free reaction conditions toward the synthesis of ethyl butyrate, a compound significant as food and perfume flavoring, as well as biofuel. The proton inventory technique was efficiently applied in mixtures of the anhydrous polar solvents ethanol and deuterated ethanol (CH_3CH_2OD). Subsequently, and by suitable analysis of the experimental data, the aforementioned synthetic procedure seems likely to follow the kinetic mechanism ordered bi-bi involving single substrate dead-end inhibition by ethanol, whereas were estimated values of important parameters. So far is the first experimental evidence that the synthesis of ethyl butyrate, which is catalyzed by immobilized lipase, it follows an entirely different mechanism when it is performed in anhydrous solvent-free system *vs.* that in anhydrous *n*-hexane; then it may be constructive for the industrial production of fixed quality of ethyl butyrate.

Introduction

The production of esters as food flavorings, by means of lipase-catalyzed synthetic processes is industrially important due to the favored substrate specificity; this latter leads to the synthesis of exceptional pure esters by utilizing raw materials of low molecular weight. If the raw materials (i.e. fatty acids and alcohols) originate from natural sources, then the produced esters can be considered as natural when their enzymatic synthesis take place in solvent-free systems where no traces of organic solvents exist. Besides, solvent-free procedures are environmentally friendly and less costly by the reason of avoiding volatile organics and further purifying processes [1]. Among the most common synthetic food-flavorings, the ethyl butyrate has been found useful as resembling various flavors as oranges, pineapple, grapefruit, apples and apricots; additionally, ethyl butyrate has been used as solvent in perfumery products, as cellulose plasticizer, as well as biofuel [1, 2].

Many published studies have reported on the lipase-catalyzed esterification of fatty acids and alcohols in solvent-free systems, using short chain reactants, and they put forward very few information regarding their detailed kinetic investigation; additionally, there are not reports on the elucidation of the corresponding mechanisms [3]. Enzymatic mechanisms and detailed kinetic studies constitute essential tools in managing the industrial production of high quality standardized biotechnological compounds [4].

The catalytic potential of lipases in reactions of ester synthesis is based on the structure of their active site, as well as on the microenvironment which is established after the binding of substrates. In that case it is more likely that the substrate binding promotes the stabilization of the electric dipole moments in the transition states rather than in the ground states, and it allows lipases to function through dissimilar catalytic mechanisms in solvents of substantially different polarities (electric field catalysis) [5]. Therefore, and due to the formation of anionic intermediates in the transition states, and the stabilization of the carbonyl oxygen of scissile ester bonds through hydrogen bonding onto the oxyanion holes, the reaction of esterification is favored in non-polar solvents [6].

In this work is reported a thorough and novel kinetic study of the synthesis of ester ethyl butyrate, catalyzed by immobilized *Candida antarctica* Lipase-B (CALB) under anhydrous solvent-free conditions, by means of a systematic application of the proton inventory technique, as well as by using appropriate equations, curve fitting procedures and suitable statistical analysis [7-9]. It was found that the aforementioned

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synthesis of ethyl butyrate in a solvent-free system follows the ordered bi-bi mechanism involving single dead-end substrate inhibition by ethanol [10].

Materials and Methods

I Materials

The compounds absolute ethanol, anhydrous deuterated ethanol (≥ 99.5 atom % D) and butyric acid, which were dehydrated and stored over activated (at 250 °C) molecular sieves, as well as ethyl butyrate standard for Gas chromatography (GC) analyses, were of analytical grade and purchased from Sigma-Aldrich (St. Louis, USA). Pervaporation membranes, immobilized Lipase-B from *Candida antarctica* (CALB) and activated molecular sieves (3Å), were purchased as previously [10].

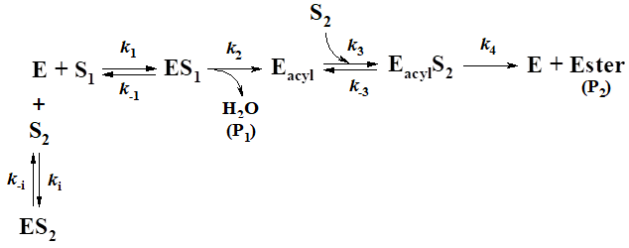
II Esterification reactions, activity and kinetic measurements

All esterification reactions of CALB-catalyzed synthesis of ethyl butyrate were carried out in identical solvent-free systems, in a jacketed filter reactor, as it has been described previously [10]. In all cases, the method of initial velocities was followed, whereas the concentration of substrate butyric acid was ranged from 0.10 M up to 2.75 M. The use of butyric acid concentrations higher than 2.75 M would exceed the 20% of the whole esterification reaction mixtures applied in this work. A typical kinetic run comprised the addition of the reactants in the reactor, in that order: 28.5 ml anhydrous ethanol or anhydrous deuterated ethanol, the appropriate quantity of anhydrous butyric acid (e.g. 1.40 mL, i.e. 0.50 M), and 20 mg of CALB ($[E]_0$ = 4.04 M), which initiated the reaction. Subsequently, the reactor was thermostated at 40°C and stirred continuously (100 rpm), whereas the formed water was maintained stable (0.01 $^{v}/_{v}$) by pervaporation under vacuum of 10 mbar [10]. The esterification reactions were followed up to the first 25 min, when the % of esterification was less than 5%; at that time the concentration of ethyl butyrate, as well as its yield were monitored. For this reason, aliquots were withdrawn at time intervals, diluted in anhydrous ethanol and analyzed by CG - FID chromatography, as previously [1, 10]. The kinetic runs were performed in triplicates and their mean values were considered; the standard deviations of triplicates were found less than ± 5%, in all cases. At the end of the esterification procedures the contained water was estimated by the coulometric method of Karl Fischer [1, 10]. The quantitative determinations concerning the % yields and the concentrations of the synthesized ethyl butyrate were performed by means of DANI MASTER GC Fast Flame ionization detector system whose the GC-profiles were analyzed and quantified as previously [1, 10].

III The potential esterification mechanisms and their kinetic expressions

Furthermore, and since the concentration of substrate butyric acid should be considered very low as compared to that of experimentally constant solvent-ethanol, we suggest that the herein esterification reactions may follow either the kinetic mechanism ping-pong bi-bi or the ordered bi-bi, by including single-substrate dead-end inhibition. The aforementioned mechanisms are described by equation (1) and scheme 1, as well as by equation (2) and scheme 2, respectively. Both equations (1) and (2) were developed as previously, and they can be rearranged to equations (1a) and (2a) respectively, as $K_{ms1} + [S_2] \neq \mathbf{0}$ is valid in all cases.

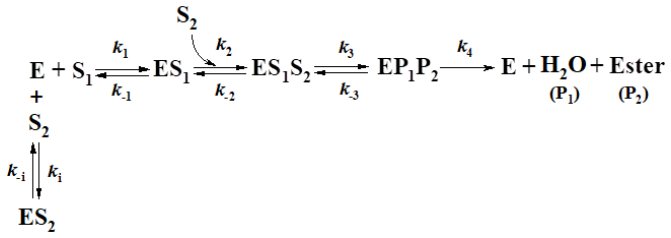
Scheme 1: Ping-pong bi-bi reaction scheme (substrate inhibition by ethanol)



$$v = \frac{[E]_0 k_{cat}[S_1][S_2]}{K_{mS1}[S_2]\left(1+\frac{[S_2]}{K_{iS2}}\right) + K_{mS2}[S_1] + [S_1][S_2]} \quad (1)$$

$$v = \frac{[E]_0 \frac{k_{cat}[S_2]}{K_{mS2}+[S_2]}[S_1]}{\frac{K_{mS1}[S_2]\left(1+\frac{[S_2]}{K_{iS2}}\right)}{K_{mS2}+[S_2]} + [S_1]} \quad (1a)$$

Scheme 2: Ordered bi-bi reaction scheme (substrate inhibition by ethanol)



$$v = \frac{[E]_0 k_{cat}[S_1][S_2]}{K_{ii}K_{mS2} + K_{mS1}[S_2]\left(1+\frac{[S_2]}{K_{iS2}}\right) + K_{mS2}[S_1] + [S_1][S_2]} \quad (2)$$

$$v = \frac{[E]_0 \frac{k_{cat}[S_2]}{K_{mS2}+[S_2]}[S_1]}{\frac{K_{ii}K_{mS2} + K_{mS1}[S_2]\left(1+\frac{[S_2]}{K_{iS2}}\right)}{K_{mS2}+[S_2]} + [S_1]} \quad (2a)$$

$$v = \frac{[E]_0 k_{cat}^{app}[S_1]}{K_m^{app} + [S_1]} \quad (3)$$

Therefore, both equations (1a) and (2a) can be simplified to the Michaelis-Menten like equation (3). Although the parameter $k_{cat}^{app} = \frac{k_{cat}[S_2]}{K_{mS2}+[S_2]}$

is common among equations (1a) and (2a), however the parameter k_m^{app} is expressed differently, i.e. as $K_m^{app} = \frac{K_{mS1}[S_2]\left(1+\frac{[S_2]}{K_{iS2}}\right)}{K_{mS2}+[S_2]}$ and $K_m^{app} = \frac{K_{ii}K_{mS2}+K_{mS1}[S_2]\left(1+\frac{[S_2]}{K_{iS2}}\right)}{K_{mS2}+[S_2]}$ respectively. Furthermore and in both cases of the aforementioned mechanisms the parameters $\left(\frac{k_{cat}}{K_m}\right)^{app}$ are easily generated, and are expressed as

$\left(\frac{k_{cat}}{K_m}\right)^{app} = \frac{k_{cat}}{K_{mS1}\left(1+\frac{[S_2]}{K_{iS2}}\right)}$ and $\left(\frac{k_{cat}}{K_m}\right)^{app} = \frac{k_{cat}}{\frac{K_{ii}K_{mS2}}{[S_2]}+K_{mS1}\left(1+\frac{[S_2]}{K_{iS2}}\right)}$

for the mechanisms of scheme 1 and scheme 2, respectively.

By taking into account reasonable approximations (see Appendices), the parameter k_{cat}^{app} degenerates to k_{cat}, under all circumstances. In the same way, the relation $K_m^{app} = \frac{K_{mS1}[S_2]\left(1+\frac{[S_2]}{K_{iS2}}\right)}{K_{mS2}+[S_2]}$ (according to scheme 1) degenerates to: $K_m^{app} = \frac{K_{mS1}}{K_{iS2}}[S_2]$ whereas the relation $K_m^{app} = \frac{K_{ii}K_{mS2}+K_{mS1}[S_2]\left(1+\frac{[S_2]}{K_{iS2}}\right)}{K_{mS2}+[S_2]}$ (according to scheme 2) degenerates to: $K_m^{app} = \frac{k_{cat}}{k_1K_{ii}}[S_2]$ Consequently, the expressions for the $\left(\frac{k_{cat}}{K_m}\right)^{app}$ parameter, in the cases of both mechanisms of scheme 1 and scheme 2, were considered to approximate as $\left(\frac{k_{cat}}{K_m}\right)^{app} = k_{cat}\frac{K_{iS2}}{K_{mS1}}\frac{1}{[S_2]}$ (according to scheme 1), and $\left(\frac{k_{cat}}{K_m}\right)^{app} = k_1K_{ii}\frac{1}{[S_2]}$ (according to scheme 2), respectively.

These series of experimental data were fitted by the equation (3). In (Table 1) are depicted the parameters of equations (1), and (2) as they derived from the rate constants of mechanisms, which appeared in schemes 1 and 2, respectively.

Table 1: The dependencies of parameters of equations (1) and (2), on the rate constants of ping-pong bi-bi and ordered bi-bi mechanisms, involving single substrate dead-end inhibition, are depicted according to schemes 1 and 2, respectively.

ping-pong bi-bi	ordered bi-bi
$k_{cat} = \frac{k_2k_4}{k_2+k_4}$	$k_{cat} = \frac{k_3k_4}{k_3+k_{-3}+k_4}$
$K_{mS1} = \frac{k_4(k_{-1}+k_2)}{k_1(k_2+k_4)}$	$K_{mS1} = \frac{k_2k_3k_{-i}k_4+k_{-1}k_3k_ik_4+k_{-1}k_{-2}k_ik_{-3}+k_{-1}k_{-2}k_ik_4}{k_1k_2k_{-i}(k_3+k_{-3}+k_4)}$
$K_{mS2} = \frac{k_2(k_{-3}+k_4)}{k_3(k_2+k_4)}$	$K_{mS2} = \frac{k_{-2}k_3+k_{-2}k_4+k_3k_4}{k_2(k_3+k_{-3}+k_4)}$
$K_{iS2} = \frac{k_{-i}}{k_i}$	$K_{iS2} = \frac{k_2k_3k_{-i}k_4+k_{-1}k_3k_ik_4+k_{-1}k_{-2}k_ik_{-3}+k_{-1}k_{-2}k_ik_4}{k_2k_3k_4k_i}$
$\frac{k_{cat}}{K_{mS1}} = \frac{k_1k_2}{k_{-1}+k_2}$	$\frac{k_{cat}}{K_{mS1}} = \frac{k_1k_2k_3k_4k_{-i}}{k_2k_3k_{-i}k_4+k_{-1}k_3k_ik_4+k_{-1}k_{-2}k_ik_{-3}+k_{-1}k_{-2}k_ik_4}$
$\frac{k_{cat}}{K_{mS2}} = \frac{k_3k_4}{k_{-3}+k_4}$	$\frac{k_{cat}}{K_{mS2}} = \frac{k_2k_3k_4}{k_{-2}k_3+k_{-2}k_4+k_3k_4}$
	$K_{ii} = \frac{k_{-i}}{k_i}$

K_{mS1}, K_{mS2}, K_{iS2}, and K_{ii} are: the corresponding Michaelis-Menten parameters, and the analogous parameter due to the substrate inhibition by the ethanol(s), as appeared in Schemes 1 and 2.

IV Proton inventories and solvent isotope effects (S.I.E.)

The proton inventory measurements were performed similarly to the aforementioned kinetic runs, although by using a fixed and low concentration of butyric acid (0.30 M, i.e. $[S_1]_0 << K_m^{app}$, in order to study the variation of the parameter $\left(\frac{k_{cat}}{K_m}\right)^{app}$. These latter measurements were carried out in mixtures of anhydrous CH_3CH_2OH and CH_3CH_2OD, where the deuterium atom fraction $n = \frac{CH_3CH_2OH}{CH_3CH_2OD}$ was well defined in all cases [11 and therein references]. A total of eight different mixtures were prepared with n = {0.00, 0.10, 0.25, 0.40, 0.50, 0.60, 0.75, 0.99}. Similar measurements concerning the study of parameter k_{cat}^{app} under the given reaction conditions, were considered as unachievable and impractical requiring [butyric acid] > 5x k_m^{app} where insolubility drawbacks were involved. The experimental data from proton inventories were fitted by the equations (4), (5) and (6), whose the parameters have been described previously [4, 12]. These equations are referred to medium effects (Z) and to transition states comprising a physical step (C_{Ph}), which precedes the chemical step (C_{Ch}) and where v protons are transferred; then the relation $C_{Ph} + C_{Ch} = 1$ holds true. In cases where the value of C_{Ph} approaches zero, equation (4) degenerates to equation (5) and/or to (6) when v=1. In these equations the ratio $\frac{k_n}{k_0}$ stands for the quotient $\frac{\left(\frac{k_{cat}}{K_m}\right)_n^{app}}{\left(\frac{k_{cat}}{K_m}\right)_0^{app}}$

$$\frac{k_n}{k_0} = \frac{Z^n}{C_{Ph} + \frac{1 - C_{Ph}}{(1 - n + n\phi^T)^v}} \quad (4) \qquad \frac{k_n}{k_0} = Z^n(1 - n + n\phi^T)^v \quad (5)$$

$$\frac{k_n}{k_0} = Z^n(1 - n + n\phi^T) \quad (6)$$

The value of an additional index-parameter was estimated ($\Box$C) in order to conclude on the non-linear character of proton inventories; values of $\Box C > 0$, or $\Box C < 0$, and/or $\Box C = 0$, denote dome-shaped, or bowl-shaped, and/or linear character, respectively of proton inventories of the constant under consideration [12]. It should be mentioned that the rule of the geometric mean holds true in mixtures of CH_3CH_2OH/CH_3CH_2OD due to that there is only one hydrogenic site on the ethanol molecule [13, 14]. Therefore, the general equation Gross-Butler-Kresge, and its simplified forms, as they are equations (4), (5) and (6) are valid too. [6, 10].

V Analysis and fitting of the experimental data

The fitting of experimental data by the employed appropriate equations (Eqs:3, 4, 5 and 6) were curried out (17) through the OriginPro 2018 trial version. Various weighted least-squares tests as convergence criteria, as well as goodness of the fit criteria e.g. Akaike's Information Criterion (AIC), Akaike Weights (AICW) etc, were taken into account according to the case, as previously [10]. Subsequently, the equation which best fitted the experimental data was selected, and it contributed in the elucidation of the mechanism of the CALB-catalyzed synthesis of ethyl butyrate in a solvent-free system, as it is described by schemes 1 and 2, respectively.

As far as it concerns the ping-pong bi-bi mechanism, water is firstly released and promptly removed in following the binding of substrate butyric acid onto CALB; the release of water precedes the binding of alcohol-substrate. The rate-constant k_4 (scheme 1) rate-limits the ester formation and it is the only one which may be influenced by the occurrence of isotopic exchanges (H/D) on the alcoholic hydroxyl. Dissimilarly in the ordered bi-bi mechanism, the sequential binding of both acid and alcohol substrates onto CALB is followed by the simultaneous formation of ester and the removed water. This latter irreversible step (scheme 2) is partially rate-limited by the rate-constant k_4, it depends obviously on the previous equilibria, though the ratio k_3/k_{-3} and it may be much more influenced by the occurrence of isotopic exchanges (H/D) on the alcoholic hydroxyl [10, 15].

Results and discussion

I Non-linear fitting of experimental data from Michaelis-Menten-like kinetics

Equation (3) best fitted the experimental data obtained from the CALB-catalyzed synthesis of ethyl butyrate in a solvent-free system, under continuous removal of the formed water from the reaction mixtures. The results are appeared in (Figure 1); the values of the important parameters were estimated as $\boldsymbol{k_{cat}^{app}} = \boldsymbol{k_{cat}} = 10.26 \pm 0.71\ s^{-1}$, $\boldsymbol{K_m^{app}} = 4.28 \pm 0.35$ M, and $\left(\frac{k_{cat}}{K_m}\right)^{app} = 2.40\ M^{-1} \times s^{-1}$; the dual character of parameter $\left(\frac{k_{cat}}{K_m}\right)^{app}$ (section Materials and Methods III), has been taken into account, and it will be commented below (section Results and discussion III). The active concentration of employed enzyme ($[E]_0$) was estimated previously and equals to 4.04×10^{-6} M [10].

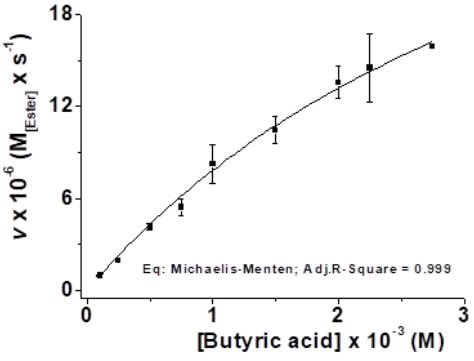


Figure 1: Fitting of experimental data of the CALB-catalyzed solvent-free synthesis of ethyl butyrate; the data were best fitted by equation $v = \frac{4.04 \times 10^{-6} \times 10.26 \times [\text{Butiric acid}]}{4.28 \times 10^{-3} + [\text{Butiric acid}]}\ (M \times s^{-1})$

II Non-linear fitting of experimental kinetic data from proton inventories

The experimental data of $\left(\frac{k_{cat}}{K_m}\right)^{app}$ proton inventories were fitted successively by equations (4), (5), and (6); nevertheless, equation (6)

best fitted these data as it is shown in Table 2, according to the estimated values of both the criteria AIC and AICW [9, 16]. Subsequently, it was found that equation (6) best fitted the proton inventories about 11624 times *vs*. equation (4) and about 20 times *vs*. equation (5), while equation (5) best fitted the proton inventories about 586 times *vs*. equation (4). In (Figure 2) is shown the best fit of experimental data from proton inventories of $\left(\frac{k_{cat}}{K_m}\right)^{app}$ parameter by equation (6), whereas a value of □C > 10 was estimated validating that these proton inventories are dome-shaped. The estimated normal S.I.E., which was generated from protonic bridges in the transition state, seems to be counterbalanced to some extent by an inverse medium effect (*Z*). Dome-shaped proton inventories may originate from various mechanisms such as the ping-pong bi-bi and the ordered bi-bi ones, involving single substrate dead-end inhibition [9, 17]. Consequently, the calculation of one fractionation factor in the transition state (□$^T \approx 0.23$), along with the estimation of a medium effect ($Z \approx 1.43 > 1$), are compatible to the transfer of one proton, as well as to the existence of successive interactions of hydrogen bonds in the transition state. These hydrogen bonds are being strengthened during the enzyme-substrate (butyric acid) binding in the reaction course from the ground state to the transition state [18].

Table 2: The results of fitting, of proton inventories related to the $\left(\frac{k_{cat}}{K_m}\right)^{app}$ parameter, in the CALB-catalyzed esterification reactions of butyric acid by ethanol, in solvent-free systems (anhydrous mixtures CH_3CH_2OH/CH_3CH_2OD).

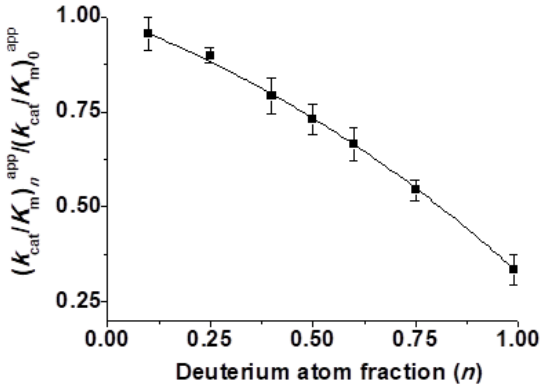
	Equations			
Parameters	(4)	(5)	(6)	Statistical comparisons
Adj.R-Square	0.999	0.999	0.999	
Z	2.230	1.999	1.427	
C_ph	0.014	-	-	
f^T	0.342	0.312	0.228	
v	1.798	1.556	1	
S.I.E.	3.046	3.055	3.078	
A.I.C.	-55.044	-67.774	-73.766	AIC(4) > AIC(5) > AIC(6)
AICW	0.819E-4	0.048	0.952	AICW(5)/AICW(4) ≈ 586 AICW(6)/AICW(5) ≈ 20 AICW(6)/AICW(4) ≈ 11624

Herein, the proton inventories are referred to the parameter $\left(\frac{k_{cat}}{K_m}\right)^{app}$ which takes on different meanings between the two mechanisms, by based on reasonable assumptions (see Appendices), i.e.

$$\left(\frac{k_{cat}}{K_m}\right)^{app} = k_{cat}\frac{K_{iS_2}}{K_{mS_1}}\frac{1}{[S_2]} \quad \text{or} \quad \left(\frac{k_{cat}}{K_m}\right)^{app} = k_1 K_{ii}\frac{1}{[S_2]}$$

for the mechanisms ping-pong bi-bi or ordered bi-bi, respectively. Obviously, in the case of ping-pong bi-bi mechanism, the value of $\left(\frac{k_{cat}}{K_m}\right)^{app}$ depends proportionally on the enzyme-butyric acid binding, the formation of first acyl enzyme, as well as the formation of ester; the opposite is valid concerning the enzyme-ethanol binding, the substrate inhibition and the formation of the ES_2 complex. Conversely, in the case of the ordered bi-bi mechanism, the value of $\left(\frac{k_{cat}}{K_m}\right)^{app}$ depends straightforward on the simultaneous and competitive binding of both substrates (butyric acid and ethanol) on the enzyme, and the formation of both complexes ES_1 and ES_2.

Furthermore, the estimated value of ϕ^T argues that a proton in transfer is connected, more likely, to low-barrier hydrogen bonding in the catalytic triad of CALB [4, 6, 19]. Likewise, the estimated value of *Z* could reveal that during the enzyme-substrate (butyric acid) binding many strongly bonded protons contribute in decreasing the flexibility of the enzyme molecule [4, 6].



Proton inventories of CALB-catalyzed solvent-free synthesis of ethyl butyrate in mixtures of anhydrous CH_3CH_2OH/CH_3CH_2OD. Equation $\frac{\left(\frac{k_{cat}}{K_m}\right)_n^{app}}{\left(\frac{k_{cat}}{K_m}\right)_0^{app}} = 1.427^n(1-n+0.228n)$ best fitted the experimental data.

These results were relatively expected for the reasons that in this work the CALB-catalyzed esterification reactions were carried out in mixtures of anhydrous CH_3CH_2OH and CH_3CH_2OD, as well as that an

immobilized enzyme was used. In this respect, the estimated value of the physical step (C_{ph}), which approaches zero in equation (4) argues that a charge-relay system was not activated; prerequisite for this activation is the development of interactions of substrate (s) by the remote subsites of enzyme. Besides, the anhydrous organic solvents hinder the enzyme-substrate(s) interactions and thus obstruct the activation of a charge-relay system. Moreover, organic solvents lower the values of the fractionation factors due to solvation of any formed charged species, and thus they affect positively the catalytic power of the active-site serine [6, 20-22]. Moreover, a relatively high normal S.I.E.≈3 was estimated from the best fitting of $\left(\frac{k_{cat}}{K_m}\right)^{app}$ proton inventories by equation (6), arguing that the breakdown of the -O-D bond of CH_3CH_2OD should rate-limit, at least partially, the reaction course, which is governed by the parameter $\left(\frac{k_{cat}}{K_m}\right)^{app} = k_1 K_{ii} \frac{1}{[S_2]}$ [23,24]. Therefore, by taking into consideration the dependencies of parameter $\left(\frac{k_{cat}}{K_m}\right)^{app}$ in both cases of the investigated mechanisms (Table 1 and Appendices), we should deduce that the CALB-catalyzed synthesis of ethyl butyrate, under the herein described reaction conditions, should follow the ordered bi-bi mechanism which involves single substrate (ethanol) dead-end inhibition. This result is novel, and it is reported for first time in this work.

Conclusions

Results and Discussion suggest that the CALB-catalyzed synthesis of ethyl butyrate in a solvent-free system follows, more likely, the ordered bi-bi mechanism involving single substrate (ethanol) dead-end inhibition. This major finding is mainly deduced through the application of the proton inventory technique in mixtures of the polar organic solvents CH_3CH_2OH and CH_3CH_2OD, as well as by the consequent estimated values of important parameters whose, the informative character was statistically explained in details. This novel and firstly reported finding is validated by the confirmed differentiation of the dependencies and meanings of $\left(\frac{k_{cat}}{K_m}\right)^{app}$ parameter between the two studied, herein, mechanisms. Additionally, only one proton is transferred in the transition state ($\Phi^{T} \approx 0.23$) during the rate-limiting step where the parameter $\left(\frac{k_{cat}}{K_m}\right)^{app} = k_1 K_{ii} \frac{1}{[S_2]}$ dominates; this latter, along with the estimated medium effect ($Z \approx 1.43$), support the existence of an acid-base mechanism, which corresponds to that appeared in scheme 2 [18, 21, 23]. For comparison purposes it should be pointed out that the synthesis of the same compound in anhydrous *n*-hexane (non-polar organic solvent), and under similar reaction conditions, follows the ping-pong bi-bi mechanism involving double dead-end substrate inhibition [10]. Finally, the findings of this work could be exceptionally helpful for a well organized production of ethyl butyrate, and other food-flavoring additives, in fixed quality at appropriate industrial reactors

Abbreviations

CALB: immobilized lipase-B from Candida antarctica
S.I.E: solvent isotope effect
AIC: Akaike's Information Criterion
AICW: Akaike weights

Appendices

(1) Since $[S_2]$ > 13 M, as it concluded from (section Materials and Methods III), then it seems more likely that the relation $\mathbf{[S_2] >> K_{mS2}}$ is valid. Therefore, the parameter k_{cat}^{app} equals to $\boldsymbol{k_{cat}}$, according to $k_{cat}^{app} = \frac{k_{cat}[S_2]}{K_{mS2}+[S_2]} \approx \frac{k_{cat}[S_2]}{[S_2]} = k_{cat}$ this latter is applicable in both cases of ping-pong bi-bi and ordered bi-bi mechanisms.

(2) Similar approximations may be considered for the parameters K_m^{app} and $\left(\frac{k_{cat}}{K_m}\right)^{app}$ as follows:

(a) In the case of ping-pong bi-bi mechanism it was considered (section Materials and Methods III) the relation which may be $K_m^{app} = \frac{K_{mS1}[S_2]\left(1+\frac{[S_2]}{K_{iS2}}\right)}{K_{mS2}+[S_2]}$ approximated according to the aforementioned conditions and assumptions ($k_{ms1} << \frac{K_{mS1}}{K_{iS2}}[S_2]$); as

$$K_m^{app} = \frac{K_{mS1}[S_2]\left(1+\frac{[S_2]}{K_{iS2}}\right)}{K_{mS2}+[S_2]} \approx \frac{K_{mS1}[S_2]\left(1+\frac{[S_2]}{K_{iS2}}\right)}{[S_2]} = K_{mS1}+\frac{K_{mS1}}{K_{iS2}}[S_2] \approx \frac{K_{mS1}}{K_{iS2}}[S_2]$$

Thus, the relation $K_m^{app} = \frac{K_{mS1}}{K_{iS2}}[S_2]$ is valid

(b) In the case of ordered bi-bi mechanism it was considered (section Materials and Methods III) the relation which may $K_m^{app} = \frac{K_{ii}K_{mS2}+K_{mS1}[S_2]\left(1+\frac{[S_2]}{K_{iS2}}\right)}{K_{mS2}+[S_2]}$ be approximated according to the aforementioned conditions and assumptions ($\boldsymbol{K_{ms1}} << \frac{K_{mS1}}{K_{iS2}}[S_2]$ and $\frac{K_{ii}K_{mS2}}{[S_2]} << \frac{K_{mS1}}{K_{iS2}}[S_2]$); as

$$K_m^{app} = \frac{K_{ii}K_{mS2}+K_{mS1}[S_2]\left(1+\frac{[S_2]}{K_{iS2}}\right)}{K_{mS2}+[S_2]} \approx \frac{K_{ii}K_{mS2}}{[S_2]}+K_{mS1}+\frac{K_{mS1}}{K_{iS2}}[S_2] \approx \frac{K_{mS1}}{K_{iS2}}[S_2] =$$

$$\frac{\frac{k_2k_3k_{-i}k_4+k_{-1}k_3k_ik_4+k_{-1}k_{-2}k_ik_{-3}+k_{-1}k_{-2}k_ik_4}{k_1k_2k_{-i}(k_3+k_{-3}+k_4)}}{\frac{k_2k_3k_{-i}k_4+k_{-1}k_3k_ik_4+k_{-1}k_{-2}k_ik_{-3}+k_{-1}k_{-2}k_ik_4}{k_2k_3k_4k_i}}[S_2] = \frac{k_3k_4k_i}{k_1k_{-i}(k_3+k_{-3}+k_4)}[S_2] = \frac{k_{cat}}{k_1K_{ii}}[S_2]$$

Thus, the relation $K_m^{app} = \frac{k_{cat}}{k_1 K_{ii}}[S_2]$ is valid.

c) In the case of ping-pong bi-bi mechanism the relation for the parameter $\left(\frac{k_{cat}}{K_m}\right)^{app}$ results as the ratio of the above (1) and (2a), and it may be approximated accordingly as:

$$\left(\frac{k_{cat}}{K_m}\right)^{app} = \frac{k_{cat}}{\frac{K_{mS1}}{K_{iS2}}[S_2]} = k_{cat}\frac{K_{iS2}}{K_{mS1}}\frac{1}{[S_2]}$$

Thus, the relation $\left(\frac{k_{cat}}{K_m}\right)^{app} = k_{cat}\frac{K_{iS2}}{K_{mS1}}\frac{1}{[S_2]}$ is valid.

(d) In the case of ordered bi-bi mechanism the relation for the parameter $\left(\frac{k_{cat}}{K_m}\right)^{app}$ results as the ratio of the above (1) and (2b), and it may be approximated accordingly as:

$$\left(\frac{k_{cat}}{K_m}\right)^{app} = \frac{k_{cat}}{\frac{k_{cat}}{k_1 K_{ii}}[S_2]} = k_1 K_{ii}\frac{1}{[S_2]}$$

Thus, the relation $\left(\frac{k_{cat}}{K_m}\right)^{app} = k_1 K_{ii}\frac{1}{[S_2]}$ is valid

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