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Original scientific paper

## Enzymatic synthesis of a vitamin B<sub>6</sub> precursor

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**Abstract:** 3-Cyano-4-(ethoxymethyl)-6-methyl-2-pyridone, an important precursor in the synthesis of vitamin B<sub>6</sub>, is obtained in the addition reaction between 2-cyanoacetamide and 1-ethoxy-2,4-pentanedione catalyzed by lipase from *Candida rugosa* (triacylglycerol acylhydrolases, EC 3.1.1.3). This work shows new experimental data and mathematical modeling of the lipase-catalyzed synthesis of 3-cyano-4-(ethoxymethyl)-6-methyl-2-pyridone. Kinetic measurements were performed at 50 °C with an enzyme concentration of 1.2 % w/v. The experimental results were fitted with two kinetic models: the ordered bi-ter and ping-pong bi-ter model, and the initial rates of the reaction were found to correlate best with the ping-pong bi-ter mechanism with inhibition by 2-cyanoacetamide. The obtained specificity constants indicated that lipase from *C. rugosa* had a higher affinity towards 1-ethoxy-2,4-pentanedione compared to 2-cyanoacetamide.

**Keywords:** *Candida rugosa* lipase; ping-pong kinetics; pyridone; 1-ethoxy-2,4-pentanedione.

### INTRODUCTION

A large number of natural and synthetic compounds that possess interesting pharmacological activity contain a 2-pyridone ring in their structure. 3-Cyano-2-pyridones, especially their substituted analogs, were the subject of many studies in recent years.<sup>1</sup> They were found to be associated with a wide range of therapeutic activities, *i.e.*, antimicrobial,<sup>2,3</sup> and antiviral.<sup>1</sup> They can also possess anti-HIV,<sup>4</sup> anticancer,<sup>5,6</sup> and cardiotoxic activities,<sup>7,8</sup> and can serve as a basis for the synthesis of more complex systems<sup>9</sup> and precursors for the synthesis of biologically active compounds.<sup>10</sup>

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3-Cyano-4-(ethoxymethyl)-6-methyl-2-pyridone is the first in a series of intermediates in the synthesis of vitamin B<sub>6</sub> according to the Harris and Folkers method.<sup>10–12</sup> The reaction between 2-cyanoacetamide and 1-ethoxy-2,4-pentanedione gives this product *via* Michael addition. Very few reports are available on the chemical synthesis of this intermediate,<sup>13</sup> and they all include organic catalysts and polar solvents. In addition, a few investigations of enzyme-catalyzed carbon–carbon bond formation *via* Michael addition are reported,<sup>14–16</sup> and to the best of our knowledge, there are no reports on the synthesis of 3-cyano-4-(ethoxymethyl)-6-methyl-2-pyridone using an enzyme as catalyst.

Enzyme-mediated reactions are attractive alternatives to tedious and expensive chemical methods. Chemical methods have problems such as high reaction temperatures, toxic catalysts and solvents, larger amounts of raw materials due to the non-selectiveness of the process and high waste generation.<sup>17</sup> Enzymes, also known as “green” catalysts, can be used to overcome these shortcomings. The use of enzymes in water, instead of toxic catalysts in polar organic solvents, offers less extreme conditions of temperature and pressure and minimizes energy consumption. Moreover, the production of waste is lowered, because the high specificity of an enzyme leads to fewer unwanted side effects and by-products.<sup>18</sup> It is very important to mention that there is an increasing need for industries to nurse environmental protection and to find more environmentally friendly materials and conditions to perform syntheses.

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are ubiquitous enzymes that catalyze the hydrolysis of fats and oils.<sup>19,20</sup> Lipases are powerful catalysts and the most commonly used enzymes in synthetic organic chemistry. Due to their ability to utilize a variety of substrates, lipases are very important and increasingly employed enzymes in a large number of fields, such as in the food, textile, dairy, cosmetics, and pharmaceutical industries. They have been extensively used in the synthesis of many biologically active compounds and natural products.<sup>21–23</sup> Moreover, their high enantio- and regio-selectivity are extremely important in the production of key intermediates for organic and medicinal chemistry.<sup>24</sup>

It has been shown<sup>25–28</sup> that the cyclization reaction of 2-cyanoacetamide and 1,3-diketones can be catalyzed by the lipase from *Candida rugosa*. In a previous work,<sup>29</sup> the condensation of 2-cyanoacetamide and 2,4-pentanedione was optimized using the response surface methodology (RSM). In addition, a mechanism for the enzyme-catalyzed reaction was proposed.

In continuation of this work, herein, a study of the enzyme-catalyzed synthesis of 3-cyano-4-(ethoxymethyl)-6-methyl-2-pyridone is presented in which the kinetics of the lipase-catalyzed condensation of 2-cyanoacetamide (CAA) and 1-ethoxy-2,4-pentanedione (EPD), Fig. 1, was investigated. A kinetic mechanism

is proposed, and the inhibition effect of the substrates was investigated, since this phenomenon is quite often found in lipase-catalyzed reactions.

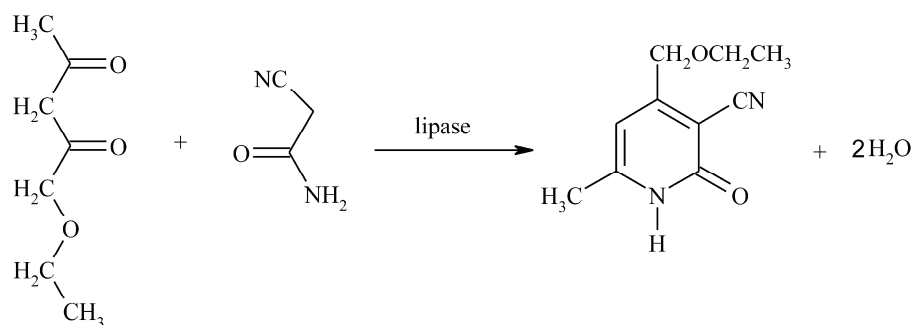


Fig. 1. Enzymatic synthesis of 3-cyano-4-(ethoxymethyl)-6-methyl-2-pyridone.

## EXPERIMENTAL

### Reagents

*Candida rugosa* lipase (3.1.1.3), Type VII, activity 1410 unit  $\text{mg}^{-1}$  of solid was purchased from Sigma (St. Louis, USA). 2-Cyanoacetamide was purchased from Fluka (Buchs, Switzerland) while 1,3-diketone (1-ethoxy-2,4-pentanedione) was prepared by the method described by Bruce *et al.*<sup>30</sup> All other chemicals were of analytical grade.

### Methods

The synthesis of 3-cyano-4-(ethoxymethyl)-6-methyl-2-pyridone was performed in a 50  $\text{cm}^3$  flask with a working volume of 5  $\text{cm}^3$  of deionized water containing appropriate amounts of 2-cyanoacetamide and 1-ethoxy-2,4-pentanedione. The enzyme (1.2 % w/v) was added to the freshly prepared reaction mixture and incubated on a shaker at 150 rpm at 50 °C. Aliquots of the reaction mixture were periodically withdrawn (0.5, 1, 2, 3 and 24 h) and analyzed using UV spectroscopy (UV Shimadzu 1700, Shimadzu Corporation, Kyoto, Japan) at 328 nm (maximum absorption of 3-cyano-4-(ethoxymethyl)-6-methyl-2-pyridone). Blanks were also run. All experiments were conducted in duplicate. All results present the difference between enzyme catalyzed and spontaneous chemical reaction. The initial reaction rates were determined as described previously.<sup>29</sup>

3-Cyano-4-(ethoxymethyl)-6-methyl-2-pyridone was isolated by filtration and purified using Akta Purifier HPLC equipped with a fraction collector. Chromatography was carried out on a semi-preparative reversed-phase C18 column (Hypersil Gold 5  $\mu\text{m}$  ODS, 10  $\text{mm} \times 250$  mm) using the mobile phases A (water + 0.1 % v/v formic acid) and B (acetonitrile + 0.1 % v/v, formic acid), at a flow rate of 6  $\text{cm}^3 \text{min}^{-1}$ . A linear concentration gradient, increasing from 20 to 100 % B, was applied. The length of the gradient was one column volume, and after that, the length of isocratic elution with 100 % B was one column volume. The detection wavelengths were 210 and 320 nm. Fractions containing the product were combined, evaporated and analyzed.

3-Cyano-4-(ethoxymethyl)-6-methyl-2-pyridone. m.p.: 208–209 °C (lit. m.p.: 209 °C<sup>13</sup>). IR (KBr,  $\text{cm}^{-1}$ ): 3290 (v, -NH), 3139, 3098 ( $\delta_s$ , -CH aromatic), 2983, 2974 ( $\delta_{\text{as}}$ , -CH<sub>3</sub>), 2902, 2897 ( $\delta_s$ , -CH<sub>3</sub>), 2217 (v, -CN), 1659 (amide band I), 1621 (amide band II), 1128 (v, -C-O). <sup>1</sup>H-NMR (200 MHz, DMSO-*d*<sub>6</sub>,  $\delta$  / ppm): 1.2 (3H, *t*, *J* = 7.0 Hz, -CH<sub>2</sub>OCH<sub>2</sub>CH<sub>3</sub>), 2.2 (3H, *s*,

pyridone  $-\text{CH}_3$ ), 3.5 (2H, *q*,  $J = 7.0$  Hz,  $-\text{CH}_2\text{OCH}_2\text{CH}_3$ ), 4.1 (2H, *s*,  $-\text{CH}_2\text{OCH}_2\text{CH}_3$ ), 6.3 (1H, *s*,  $-\text{CH}=\text{N}$ ), 12.5 (1H, *s*, NH).  $^{13}\text{C}$ -NMR (50 MHz,  $\text{DMSO}-d_6$ ,  $\delta$  / ppm): 15.1 ( $-\text{CH}_2\text{OCH}_2\text{CH}_3$ ), 19.4 (pyridone  $-\text{CH}_3$ ), 66.3 ( $-\text{CH}_2\text{OCH}_2\text{CH}_3$ ), 68.9 ( $-\text{CH}_2\text{OCH}_2\text{CH}_3$ ), 115.3 ( $-\text{CN}$ ), 96.6, 104.0, 152.9, 160.9, 161.2 (pyridone ring).

## RESULTS AND DISCUSSION

### Kinetic study

In order to perform the kinetic measurements under conditions that provide the maximum reaction rates, the influence of the initial enzyme concentration was tested. The effect of the initial enzyme concentration on the reaction rate and the yield of 3-cyano-4-(ethoxymethyl)-6-methyl-2-pyridone synthesis are shown in Fig. 2. The results indicate a linear increase in reaction rate with increasing enzyme concentration up to 1.2 % w/v, when the highest reaction rate of  $1.49 \cdot 10^{-3} \text{ mol dm}^{-3} \text{ h}^{-1}$  was achieved. With further increase in enzyme concentration to 1.8 %, w/v, the reaction rate, as well as the yield of pyridone, remained constant. Thus, an enzyme concentration of 1.2 % w/v was chosen for the determination of the kinetic parameters.

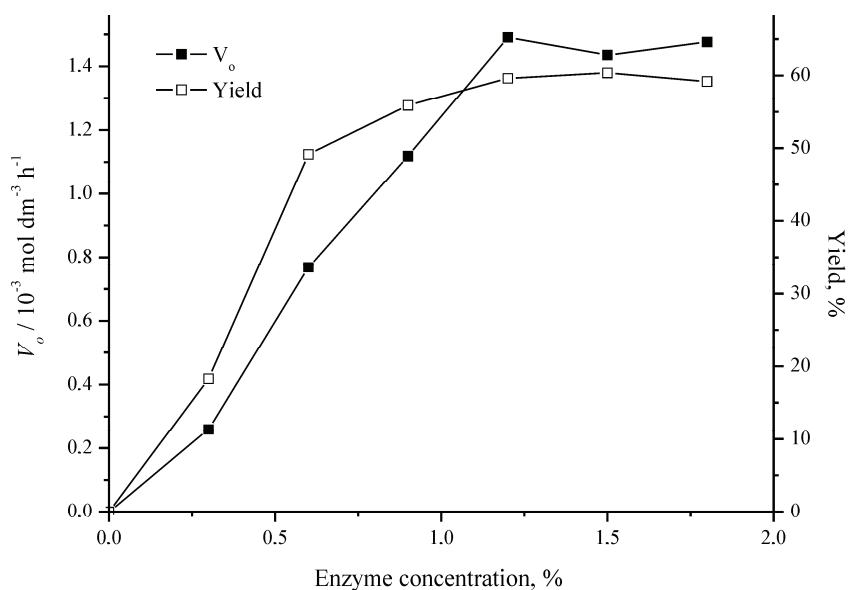


Fig. 2. Influence of the enzyme concentration on the initial reaction rate and the yield of pyridone. Concentrations of reactants were  $2.6 \times 10^{-2} \text{ mol dm}^{-3}$  and  $0.94 \text{ mol dm}^{-3}$  for EPD and CAA, respectively. Lipase concentration was varied between 0.3 and 1.8 %, w/v.

The aim of this work was to elucidate the mechanism of the condensation of 2-cyanoacetamide and 1-ethoxy-2,4-pentanedione mediated by lipase from *C. rugosa*. For this purpose, initial reaction rate analysis was employed as the most useful method. The kinetic parameters of this bi-substrate reaction were deter-

mined by measuring the initial reaction rates for different sets of substrate concentrations. The effect of both substrates was investigated systematically over a wide range of concentrations: the concentration for 1-ethoxy-2,4-pentanedione was varied from  $0.5 \times 10^{-2}$  to  $8 \times 10^{-2}$  mol dm<sup>-3</sup> and that for 2-cyanoacetamide from 0.2 to 4.8 mol dm<sup>-3</sup>. A large excess of 2-cyanoacetamide was applied since it was previously confirmed that an excess of this substrate significantly accelerates the lipase-catalyzed formation of pyridones.<sup>25–28</sup>

Graphical representations of obtained initial rates against 2-cyanoacetamide (or 1-ethoxy-2,4-pentanedione) concentrations at several fixed values of 1-ethoxy-2,4-pentanedione (or 2-cyanoacetamide) concentrations are shown in Figs. 3 and 4.

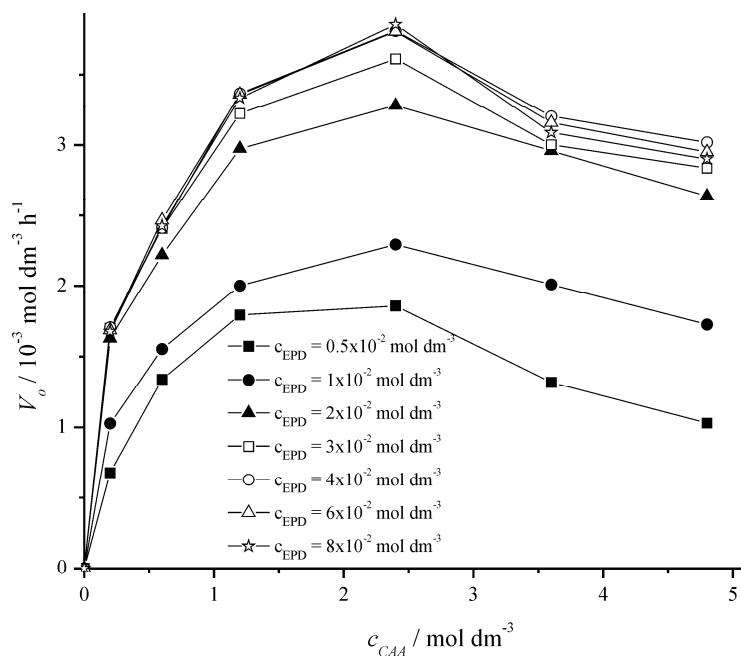


Fig. 3. The ping-pong model curves at a fixed concentration of 1-ethoxy-2,4-pentanedione.

It can be seen (Fig. 3) that with increasing concentration of 2-cyanoacetamide, the initial reaction rate increased up to an optimum value. The value of optimum 2-cyanoacetamide concentration slightly increased from 1.7 mol dm<sup>-3</sup> for lower 1-ethoxy-2,4-pentanedione concentrations to 2.4 mol dm<sup>-3</sup> for higher concentration. Nevertheless, at higher 2-cyanoacetamide concentrations a decrease of the initial velocity was observed, indicating that the excess 2-cyanoacetamide inhibited the catalytic activity of lipase. On the other hand, the effects of the 1-ethoxy-2,4-pentanedione concentration for various fixed concentrations of 2-cyanoacetamide (Fig. 4) resembled those typical Michaelis–Menten kinetics.

With increasing 1-ethoxy-2,4-pentanedione concentration, the reaction rates increased and slowly approached to a local maximum. The value of local maximum rate increased as the fixed concentration of 2-cyanoacetamide increased up to  $2.4 \text{ mol dm}^{-3}$ . Due to the inhibitory effect, with concentrations of 2-cyanoacetamide higher than  $2.4 \text{ mol dm}^{-3}$ , the local maximum rate were lower. Therefore, the maximum initial rate of  $3.85 \times 10^{-3} \text{ mol dm}^{-3} \text{ h}^{-1}$  was achieved with  $8 \times 10^{-2} \text{ mol dm}^{-3}$  of 1-ethoxy-2,4-pentanedione and  $2.4 \text{ mol dm}^{-3}$  of 2-cyanoacetamide.

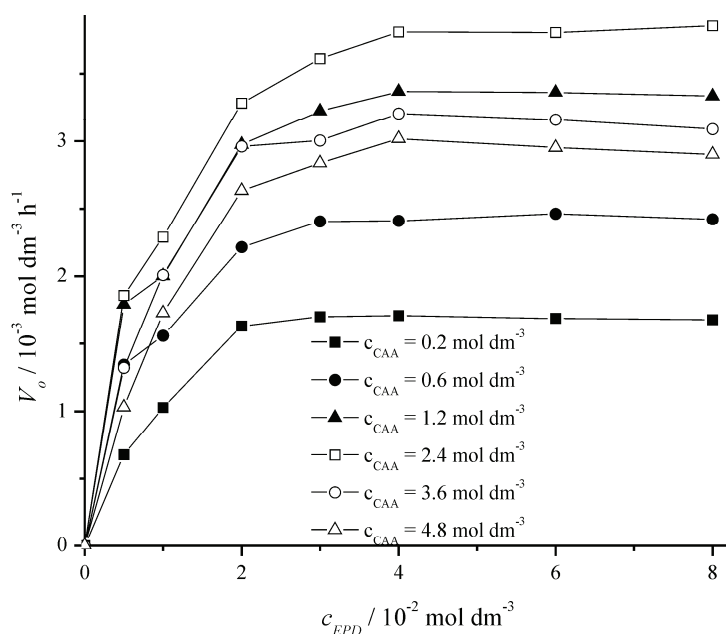


Fig. 4. The ping-pong model curves at a fixed concentration of 2-cyanoacetamide.

These results support a ping-pong mechanism with substrate inhibition, typical for lipase catalyzed reactions, and the same model was proposed in a previous study of the lipase-catalyzed synthesis of 4,6-dimethyl-3-cyano-2-pyridone.<sup>29</sup> A graphical illustration of the proposed mechanism is given in Fig. 5.

After statistical analysis, the ping-pong model with 2-cyanoacetamide inhibition was shown to be in good agreement with the experimental results, with a correlation coefficient of 0.94. According to this mechanism, the lipase was initially bound to 1-ethoxy-2,4-pentanedione forming a non-covalent complex, which was, with synchronal release of one water molecule, transformed to a 1-(ethoxymethyl)-3-oxo-but-1-enyl-enzyme complex. Subsequently, the modified enzyme reacted with the 2-cyanoacetamide and formed another binary complex which then released pyridone, water and the free enzyme. It is plausible that

inhibition by 2-cyanoacetamide occurs when a 2-cyanoacetamide molecule reacts with the enzyme directly to produce a dead-end complex (Fig. 6).

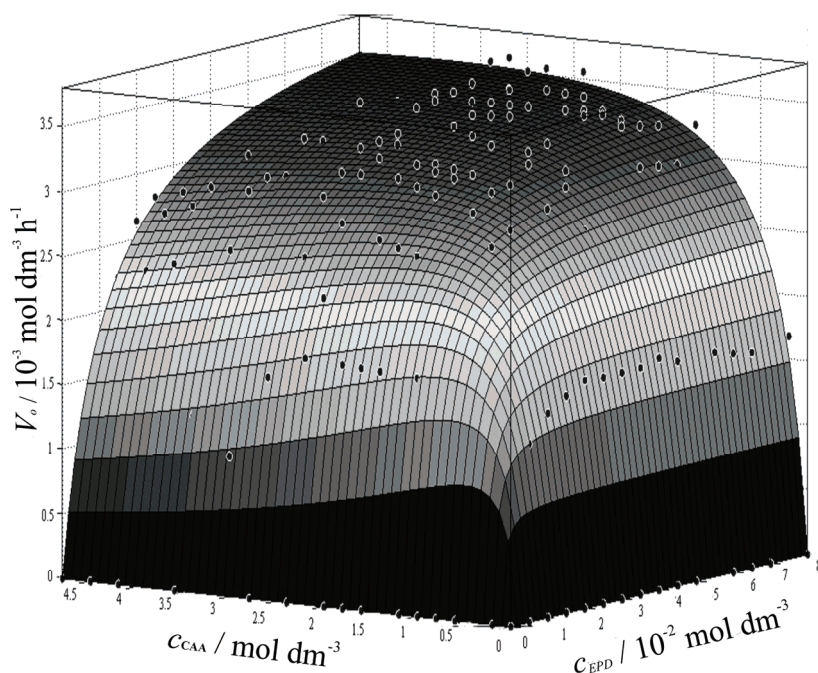


Fig. 5. Illustration of the proposed ping-pong bi-ter mechanism.

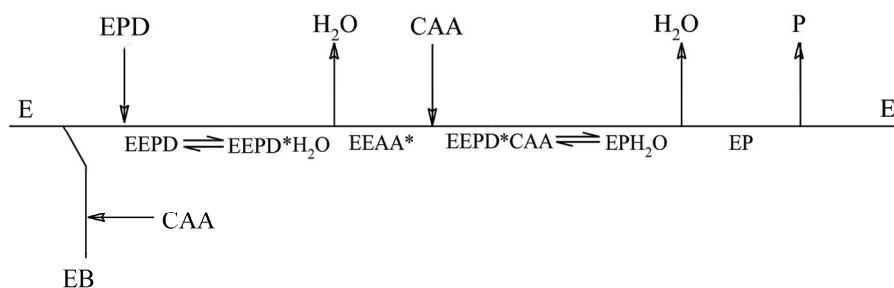


Fig. 6. Schematic representation of the ping-pong bi-ter mechanism: E: enzyme (lipase), CAA: 2-cyanoacetamide, EPD: 1-ethoxy-2,4-pentanedione, P: pyridone, EEPD and EP: complexes of lipase and EPD and P, respectively, EEPD\*: 1-(ethoxymethyl)-3-oxo-but-1-enyl-enzyme complex and EB: dead-end inhibition complex of the enzyme with 2-cyanoacetamide.

The kinetic constants, given in Table I, were obtained by multiple regression fitting of the experimental data:

$$v = \frac{V_{\max} [A][B]}{K_m^B [A] + K_m^A [B] \left( 1 + \frac{[B]}{K_{i,B}} \right) + [A][B]} \quad (1)$$

where  $v$  is the initial reaction rate,  $V_{\max}$  is the maximum reaction rate,  $[A]$  and  $K_{m,A}$  are the concentration and Michaelis constant of 1-ethoxy-2,4-pentanedione,  $[B]$  and  $K_{m,B}$  are the concentration and Michaelis constant of 2-cyanoacetamide, respectively, and  $K_{i,B}$  is the inhibitory constant of 2-cyanoacetamide.

TABLE I. Estimated values of the kinetic parameters for the synthesis of 3-cyano-4-(ethoxymethyl)-6-methyl-2-pyridone

Parameter	Value
$V_{\max}$	$4.34 \times 10^{-3} \text{ mol dm}^{-3} \text{ h}^{-1}$
$K_{m,A}$	$0.315 \times 10^{-2} \text{ mol dm}^{-3}$
$K_{m,B}$	$0.347 \text{ mol dm}^{-3}$
$K_{i,B}$	$1.603 \text{ mol dm}^{-3}$
$K_{s,A}$	$0.106 \text{ dm}^3 \text{ g}^{-1} \text{ h}^{-1}$
$K_{s,B}$	$0.963 \times 10^{-3} \text{ dm}^3 \text{ g}^{-1} \text{ h}^{-1}$

Specificity constants,  $K_s$ , for A and B can be defined as  $K_s = k_{\text{cat}}/K_m$ , where  $k_{\text{cat}}$  is the rate constant for the catalyzed reaction. The high value of  $K_{s,A}/K_{s,B}$  (109.6) indicates that the lipase from *C. rugosa* has a higher affinity towards the 1,3-diketone compared to 2-cyanoacetamide, which was also observed in a previous study.<sup>29</sup> The assumed reaction mechanism is schematically presented in Fig. 7.

By using specificity constant values, the preference of the enzyme for different 1,3-diketones (1-ethoxy-2,4-pentanedione and 2,4-pentanedione) can be compared. As the specificity constant of 2,4-pentanedione is about ninety fold higher than the specificity constant for 1-ethoxy-2,4-pentanedione, it is clear that lipase prefers 2,4-pentanedione as substrate. If the catalytic constants of these two reactions are compared, it is interesting to notice that the reaction was much faster ( $\approx 500$  fold) with 2,4-pentanedione, although the higher polarity of the ethoxymethyl group facilitates the attack on the carbonyl group of 1-ethoxy-2,4-pentanedione. It seems that the mechanism of the enzymatic synthesis of substituted 2-pyridones is different to that operative in their chemical synthesis that leads to different selectivities, which could be an additional motive for wider application of lipase-catalyzed reactions. The explanation could be in the specific tunnel shape of the active center of the lipase from *C. rugosa*.<sup>31</sup> The bulkier is the substrate, the more difficult is the approach to the catalytic triad and consequently the formation of the enzyme-substrate complex is slower.



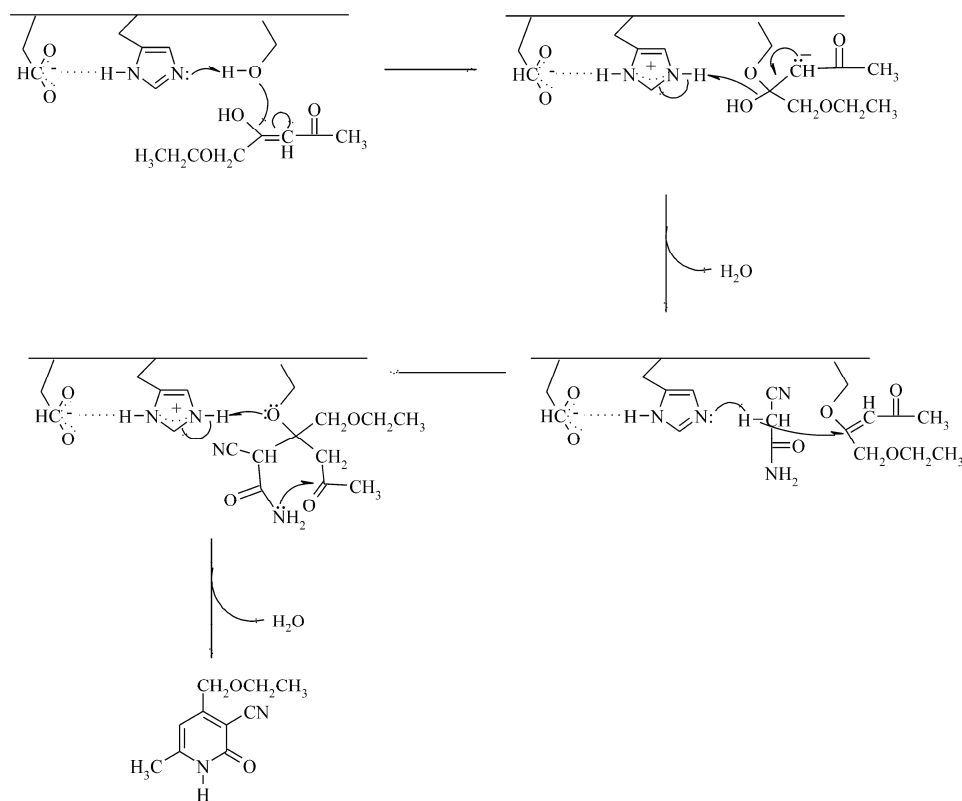


Fig. 7. Hypothetical mechanism of the enzyme catalyzed reaction of 1-ethoxy-2,4-pentanedione and 2-cyanoacetamide.

#### CONCLUSIONS

The enzymatic synthesis of 3-cyano-4-(ethoxymethyl)-6-methyl-2-pyridone, an important precursor for vitamin B<sub>6</sub>, starting from 1-ethoxy-2,4-pentanedione and 2-cyanoacetamide was described. In addition, a kinetic study was performed. The results indicate a linear increase in reaction rate with increasing enzyme concentration up to 1.2 %, w/v. Moreover, the mechanism of 2-cyanoacetamide and 1-ethoxy-2,4-pentanedione condensation mediated by lipase from *Candida rugosa* was elucidated using the initial reaction rate analysis. The best fit of the experimental data was achieved using the ping-pong bi-ter mechanism with inhibition by 2-cyanoacetamide. Values of the kinetic parameters demonstrated higher affinity of lipase from *C. rugosa* for 1-ethoxy-2,4-pentanedione rather than for 2-cyanoacetamide. Moreover, the values of specificity constants of 2,4-pentanedione and 1-ethoxy-2,4-pentanedione clearly demonstrated a greater affinity towards 2,4-pentanedione and less bulky substrates.

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## ИЗВОД

ЕНЗИМСКА СИНТЕЗА ПРЕКУРСОРА ВИТАМИНА Б<sub>6</sub>

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3-Цијано-4-(етоксиметил)-6-метил-2-пиридон је веома важан прекурсор у синтези витамина Б<sub>6</sub>. Добија се у реакцији између молекула 2-цијаноацетамида и 1-етокси-2,4-пентандиона катализованој липазом из *Candida rugosa* (триацилглицерол-ацил-хидролаза, ЕС 3.1.1.3). Резултати овог рада представљају сет нових експерименталних података брзине ензимске синтезе 3-цијано-4-(етоксиметил)-6-метил-2-пиридона. Математичким моделовањем ових података добијени су подаци о кинетици испитане реакције. Кинетичка мерења вршена су на температури од 50 °С при концентрацији ензима од 1,2 % m/v. Добијени резултати фитовани су са два различита математичка модела (пинг–понг модел са инхибицијом 2-цијаноацетамидом и секвенцијални модел са правилним редоследом везивања са инхибицијом 2-цијаноацетамидом). Вредности коефицијената линеарности показују да се почетне брзине реакције при различитим почетним концентрацијама супстрата најбоље могу описати пинг–понг би–тер моделом при чему постоји инхибиција 2-цијаноацетамидом. У раду је дат и графички приказ пинг–понг модела. Добијене константе специфичности указују на то да липаза из *C. rugosa* има већи афинитет према 1-етокси-2,4-пентандиону у поређењу са 2-цијаноацетамидом.

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