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Kinetics and thermodynamics of synthesis of propyl gallate by mycelium-bound tannase from *Aspergillus niger* in organic solvent

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Abstract

A kinetic and thermodynamic study was performed on the esterification of propyl gallate from gallic acid and 1-propanol by mycelium-bound tannase from *Aspergillus niger* in organic solvent. The activity of tannase increased with temperature up to a threshold at 47.5 °C, and successive fell beyond this value enlightened the occurrence of reversible biocatalyst inactivation. The experimental results confirmed that the deactivation process of mycelium-bound tannase follows first-order kinetics pattern, and the mycelium-bound enzyme showed improved stability in organic solvent. In consideration of both the activity and stability of tannase, the optimum reaction temperature for tannase-catalyzed esterification should be 40 °C. A kinetic model of esterification by mycelium-bound tannase was developed based on the Ping–Pong Bi–Bi kinetic mechanism, considering not only the effects by substrates and products, but also tannase denaturation. A reasonable quality of fit was observed by fitting experimental rate data to the kinetic mode with an average correlation coefficient of 0.977. Additionally, if neglecting the inactivation of tannase, the kinetic model fitted the corresponding experimental data poorly. These results indicated that it is important to study the enzyme stability to help simulate the enzymatic reaction process.

Keywords: Kinetics; Thermodynamics; Simulation; Tannase; Organic solvent

1. Introduction

The use of non-conventional media in industrial biocatalysis has grown considerably in recent years for its potential applications in fine-chemical, agrochemical, perfumery, flavor, pharmaceutical and drug industries [1]. The advantages of non-aqueous enzymology lie in dramatically higher substrate solubility, the ability to use enzymes synthetically rather than hydrolytically, and the capability to modify native selectivity by simply tailoring the reaction medium rather than the enzyme itself. Tannin acyl hydrolases (EC 3.1.1.20) are esterases able to hydrolyze the ester linkages of tannic acid in aqueous media and synthesize gallic acid esters in organic media. Propyl gallate is a very important gallic acid ester and widely used as antioxidant in foods, cosmetics, hair products, adhesives and lubricants industry; and also used as a prodrug of trimethoprim, a pharmaceutical antibacterial agent.

The study of activity and stability of enzymes is an important aspect to consider in biotechnological processes, as this can provide information on the structure of the enzymes and help optimize the economic profitability of enzymatic processes. Previous works have studied the characteristics of tannase in aqueous and organic media [2–7]. However, no kinetics and thermodynamics studies using mycelium-bound tannase as catalysts in organic solvents are performed up to now. In order to study the kinetics and thermodynamics of tannase-catalyzed esterification in organic solvent, we chose the synthesis of propyl gallate from gallic acid and 1-propanol in benzene as a model reaction. The initial part of this communication deals with deactivation kinetics of mycelium-bound tannase and estimation of thermodynamic parameters, which would help determination of the probable mechanism of deactivation to some extent; in the second part, a kinetic model for the mycelium-bound tannase synthesis of propyl gallate was deduced based on the Ping–Pong

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Nomenclature

Symbols in thermodynamics

ΔE_a	activation energy of reversible denaturation (kJ mol^{-1})
ΔE_d	activation energy of irreversible denaturation (kJ mol^{-1})
ΔG	free energy (kJ mol^{-1})
ΔH	enthalpy (kJ mol^{-1})
k_d	first-order deactivation rate constant (h^{-1})
ΔS	entropy (kJ mol^{-1} K^{-1})
$t_{1/2}$	half-life of the enzyme (h)
T	absolute temperature (K)
Ψ	activity coefficient

Subscripts

C_{E_t}	active enzyme concentration at time t
C_{E_0}	active enzyme concentration at the beginning of the thermal treatment

Symbols in kinetics

K_{m_i}	Michaelis constant for component i (mol L^{-1})
K_{i_j}	inhibition constant for component j (mol L^{-1})
K'_{iGA}	constant for K_{iGA}/K_{mAl} (mol L^{-1})
v	initial reaction rate (mol L^{-1} h^{-1} g^{-1})
V_{max}	maximum reaction rate (mol L^{-1} h^{-1} g^{-1})

Subscripts

Al	1-propanol
E.AC	enzyme-acyl complex
E.AC-Al	binary complex of enzyme-acyl and Al
E.AC-W	binary complex of enzyme-acyl and W
E-GA	binary enzyme–substrate GA complex
E-PG	binary enzyme–product PG complex
GA	gallic acid
GA-E-GA	ternary substrate GA–enzyme–substrate GA complex
PG	propyl gallate
W	water

Bi–Bi kinetic mechanism with enzyme deactivation and the validity of the model was tested experimentally.

2. Materials and methods

2.1. Microorganism, media and culture conditions

Aspergillus niger (China General Microbiological Culture Collection Center, No. 3.315) was grown in 500 mL Erlenmeyer flasks containing 100 mL of medium (tannic acid, 20 g L^{-1}; starch, 4 g L^{-1}; saccharose, 6 g L^{-1}; glucose, 2 g L^{-1}; soybean flour, 9 g L^{-1}; K_2HPO_4, 1 g L^{-1}; NH_4NO_3, 1 g L^{-1}; KC1, 0.5 g L^{-1}; $MgSO_4$, 0.5 g L^{-1}; $CaCl_2$, 0.2 g L^{-1}; $FeSO_4$, 0.01 g L^{-1}) at pH 6.0, 30 °C, 180 rpm. Suspensions of spores (1×10^7) were used as inoculums. After cultivated for 72 h, the mycelia were collected by centrifugation, washed thoroughly by 8.5 g L^{-1} NaCl solution and vacuum-dried to contain 71.4% of water, which were used in every experiment.

2.2. Enzymatic reactions

Unless stated otherwise, the typical enzymatic reactions were carried out by adding 0.35 g mycelia to the reaction mixture, which consisted of 5.56 mmol L^{-1} gallic acid, 7.3% (v/v) 1-propanol and 10 mL benzene in 25 mL glass flasks shaken at pH 3.6, 40 °C, 200 rpm. All solvents had been dried over 3 Å molecular sieves for 72 h. The molar conversion was defined as (molar of propyl gallate/molar of the initial fed gallic acid) × 100%. The initial reaction rate was defined as the amount of propyl gallate formed by 1 g dry mycelia h^{-1} under the assay conditions.

2.3. Analytical procedures

Analyses were performed on a Waters 2690 HPLC with a LiChrospherC18 column (LiChroCART 125 × 4, Merck KGaA, 64271 Darmstadt, Germany). Samples (200 μL) removed from the systems were vacuum evaporated, diluted with 0.15 mmol L^{-1} ethyl *p*-hydroxybenzoate in methanol and a volume of 20 μL was injected into the HPLC. The solvent system/mobile phase comprised of methanol:water in the ratio of 55:45 adjusted to pH 3 with phosphoric acid at a flow rate of 1 mL min^{-1} for 8 min. The ethyl *p*-hydroxybenzoate was the internal standard. The absorbance analysis was carried out at 275 nm. The reaction product was calculated from a calibration curve plotted ($Y = 0.03144X - 0.00194$, $R = 0.9999$) as propyl gallate concentration (X, μg mL^{-1}) versus the ratio (Y) between propyl gallate peak area and internal standard peak area. All the assays were done in duplicate and significant differences ($P < 0.05$) were measured.

2.4. Mycelium-bound tannase activity and stability studies

The effects of temperature on the activity of tannase were studied at various enzyme reaction temperature from 30 to 70 °C. Kinetics of irreversible denaturation was studied at temperature from 40 to 70 °C. *A. niger* (0.35 g) mixed with 10 mL of benzene was kept at each temperatures for variable times (0–96 h). The tannase residual activity was examined daily and compared to the cells not submitted to thermal treatment.

3. Results and discussion

3.1. Effect of temperature on mycelium-bound tannase activity

According to the Arrhenius model, it is well known that the enzyme activity increases with temperature up to a maximum value, which evidenced in this study. The thermal effect in a semilog plot versus the reciprocal temperature (Fig. 1) showed that the temperature threshold was 47.5 °C. Below that point the activity of tannase increased when the reaction temperature increased from 30 to 47.5 °C, while at temperature higher than

47.5 °C a negative effect was observed on the tannase activity. The thermal inactivation of mycelia might be the result of the solvent molecules interacting with the "membrane–enzyme" system, which reversibly produces a conformational change disturbing the active conformation of the enzyme [8]. The activation energy (ΔE_a) were calculated from the slope by plot of $\ln v$ versus $1/T$ below the T threshold, which was 23.297 kJ mol^{-1} ($R^2 = 0.9995$), and the energy of the reversible unfolding of the enzyme were estimated from the plot above the T threshold in Fig. 1, which was 62.856 kJ mol^{-1} ($R^2 = 0.9908$). The activation energy was quite small and suggested that there is not a high energy barrier for catalysis, and the higher reversible unfolding energy was evident that the unfolding of the enzyme requires much more energy, which were also observed for *Trichoderma reesei* xylanase [9], mycelium-bound carboxylesterase from *Aspergillus oryzae* [10] or immobilized *Candida antarctica* lipase B [11].

3.2. Effect of temperature on mycelium-bound tannase stability

The effect of temperature on the activity of tannase at temperature from 30 to 70 °C showed that the tannase activity was good at temperature from 40 to 50 °C (Fig. 1). In order to evaluate the effect of temperature on the stability of the enzyme the mycelium-bound tannase was deactivated under various temperatures from 40 to 70 °C. The extent of deactivation was measured by the apparent first-order rate constant of enzyme denaturation, k_d. The constant k_d was estimated at different temperatures from the slopes by plot $\ln \Psi$ versus time, defining the activity coefficient, Ψ, as the ratio of the total active enzyme concentration at time t to that at the beginning of the thermal treatment (Fig. 2). It was shown that k_d progressively increased from 2.8×10^{-3} to 2.84×10^{-2} h^{-1} with increasing temperature from 40 to 70 °C. The experimental results indicated that the deactivation process of mycelium-bound tannase follows first-order kinetics comparable to what has been found for immobilized *Candida rugosa* lipase [12]. As shown in Fig. 3, the half-life time of tannase decreased rapidly with increasing temperature. It was found that half-life time of tannase was 247 h at 40 °C, which was about

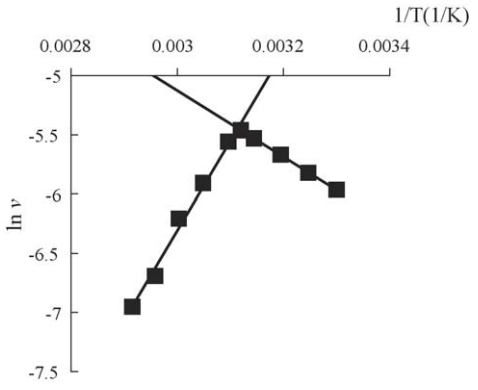


Fig. 1. Arrhenius plots for the estimation of the thermodynamic parameters. Reaction conditions: 5.56 mmol L^{-1} gallic acid, 7.3% (v/v) 1-propanol and 10 mL benzene, 0.35 g mycelia at pH 3.6, 200 rpm.

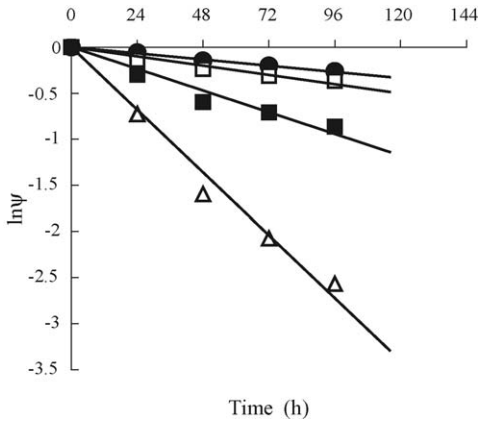


Fig. 2. Semilog plots of irreversible denaturation of mycelium-bound tannase. T: (●) 40 °C, (□) 50 °C, (■) 60 °C and (△) 70 °C. Reaction conditions: *A. niger* mixed with benzene was kept at each temperatures for variable times (0–96 h), then the tannase residual activity was investigated at 5.56 mmol L^{-1} gallic acid, 7.3% (v/v) 1-propanol and 10 mL benzene, 0.35 g mycelia at 40 °C, pH 3.6, 200 rpm.

10-fold longer than that at 70 °C. It only lost 1 and 3% of its original activity after 1 h incubation at 60 and 70 °C. In our previous study, the immobilized and free tannase of *A. niger* 3.315 just retained 87 and 30%, respectively, of its original activity in aqueous media after 1 h incubation at 60 °C [13]. Other reports showed that the tannase of *A. niger* van Tieghem retained 95% of its activity after 1 h incubation at 60 °C [14] and the tannase of *A. aculeatus* DBF 9 retained its original activity up to 50 °C for 1 h [15]. Farias et al. reported that *Cryophonectrica parasitica* tannase was stable at temperature below 30 °C while at 50 and 60 °C the activity was lost in 20 and 10 min [16]. These results suggested that the mycelium-bound tannase of *A. niger* 3.315 was rather heat-stable in organic solvent.

Based on the transition state theory as described by Eyring [17] the thermodynamic activation parameters of irreversible denaturation were estimated at temperature from 40 to 70 °C, which were 69.2 kJ mol^{-1} for ΔE_d; −0.1511 kJ mol^{-1} K^{-1} for ΔS; 66.48 kJ mol^{-1} for ΔH; 113.77–118.31 kJ mol^{-1} for ΔG (R^2 for plot of $\ln(k_d/T)$ versus $1/T$ is 0.951; R^2 for plot of $\ln k_d$ versus $1/T$ is 0.954). It was found that the entropy value of tan-

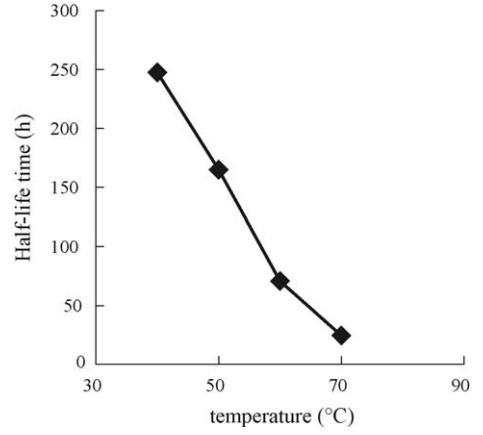


Fig. 3. Effect of temperature on half-life time of mycelium-bound tannase in benzene.

nase was negative, which is unique in biocatalytic systems. It has been predicted that not only enzyme itself but also the microenvironment around the catalyst could influence the entropy value significantly. In hydrophobic areas an ionic group or dipole is likely to be a much more effective catalyst than one shielded by water molecules in aqueous solution. On the other hand, mycelium, which could be seemed as the immobilization matrix for tannase might have effects on enzyme conformation [18]. Similar negative entropies were observed during stability studies for cytochrome *c* [19], pectolytic enzymes [20] and mycelium-bound carboxylesterase [10].

At reaction temperature of 47.5 °C the activity of tannase reached a climax (Fig. 1) while the enzyme stability study indicated that at this temperature tannase inactivated faster with time and at lower temperature of 40 °C tannase showed both good activity and stability. Therefore, 40 °C was selected as the optimum reaction temperature for the following kinetic studies.

3.3. Kinetic model development

Previous studies showed that the lipase-catalyzed esterification could be described by the Ping–Pong Bi–Bi kinetic model [21,22]. Similarly, since the model reaction in our research is a tannase-catalyzed esterification from acid and alcohol, we could assume that the tannase-catalyzed esterification follows the Ping–Pong Bi–Bi kinetic mechanism basically, considering the effects by both substrates and products at the same time. Before the kinetic model development, it is necessary to analyze the effects by substrates and products on the activity of tannase in detail.

The reaction catalyzed by tannase in organic solvent is:

$$\text{Gallic acid (GA)} + \text{1-propanol (Al)} \rightarrow \text{propyl gallate (PG)} + \text{water (W)} \quad (1)$$

It has been proven, that water activity has an effect on the kinetic parameters [23,24]. To cancel out any effect of water on the kinetic parameters, the experiments were carried out under the condition of a constant water activity of 1 measured by water activity meter (USA, AQUALAB PawKit), which exhibited the optimum activity of mycelium-bound tannase in our previous study.

The solvation of gallic acid is dependent on both the gallic acid and the 1-propanol concentration. In order to dissolve gallic acid sufficiently, the concentration of 1-propanol must be far beyond the concentration of gallic acid. Thus, in this research with the fixed concentration of 1-propanol the effect of 1-propanol on the tannase esterification could be neglected.

To examine the effect of gallic acid on the reaction rate, the initial concentrations of gallic acid were varied from 0.926 to 12.9 mmol L^{-1}. As shown in Fig. 4, the initial rate of esterification were observed to increase with increasing the concentration of gallic acid but reached a maximum at a critical concentration value (5.56 mmol L^{-1}), and subsequently increasing gallic acid concentration led to a decrease in the initial velocity. Therefore, gallic acid was assumed to act as a dead end inhibitor of the enzyme.

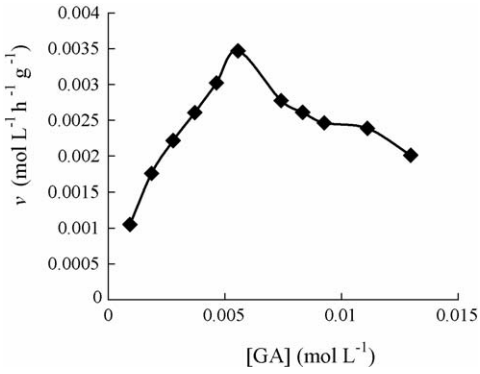


Fig. 4. Effect of the gallic acid concentration on the initial velocity of the reaction. Reaction conditions: 0.926–12.96 mmol L^{-1} gallic acid, 7.3% (v/v) 1-propanol and 10 mL benzene, 0.35 g mycelia at 40 °C, pH 3.6, 200 rpm.

Because of the equilibrium between ester and initial acid, it is also important to evaluate negative effect of propyl gallate on the reaction process by adding propyl gallate at the beginning of the reaction.

Based on the analysis of above effects of substrates and products and the tannase thermodynamics, we assume that tannase-catalyzed esterification follows the Ping–Pong Bi–Bi kinetic mechanism, not only with dead-end inhibition by gallic acid and negative effect by propyl gallate, but also with tannase denaturation. Since the tannase-catalyzed reaction in organic solvent favors esterification, to simplify the kinetic model, the model developed under the irreversible assumption. The mass transfer study (data not shown) indicated that at stirring speeds of 200 rpm the external mass transfer was negligible and the internal mass transfer was not the limiting factor with loading 0.35 g of mycelia in the reaction system.

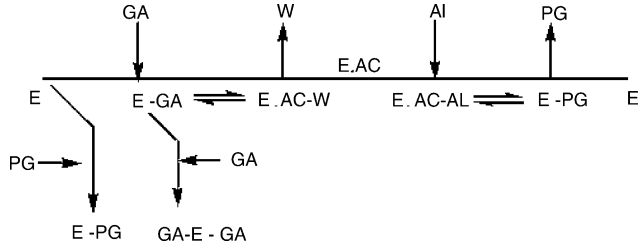
The kinetic model for tannase-catalyzed esterification is deduced as follows and the mechanism is shown in Scheme 1 [25].

When there is no inhibition, the initial rate of reaction at given concentrations of GA and Al is given by

$$v = \frac{V_{max}[GA][Al]}{K_{m_{GA}}[Al] + K_{m_{Al}}[GA] + [GA][Al]} \quad (2)$$

and if there is dead-end inhibition by GA and [Al] is varied, the equation is

$$v = \frac{V_{max}[GA]}{K_{m_{GA}} + [GA](1 + K_{m_{Al}}/[Al] + K_{m_{Al}}[GA]/K_{i_{GA}})} \quad (3)$$



Scheme 1. The scheme of Ping–Pong Bi–Bi kinetic model for tannase-catalyzed esterification, including negative effect by gallic acid and propyl gallate and tannase inactivation.

and when [Al] is fixed and in excess tends to infinity, Eq. (3) could be further reduced

$$v = \frac{V_{max}[GA]}{K_{m_{GA}} + [GA](1 + [GA]/K'_{i_{GA}})} \quad (4)$$

where $K'_{i_{GA}} = K_{i_{GA}}/K_{m_{Al}}$, and if there is negative effect by PG,

$$v = \frac{V_{max}[GA]}{K_{m_{GA}}(1 + [PG]/K_{i_{PG}}) + [GA](1 + [GA]/K_{i_{GA}})} \quad (5)$$

and under the reaction temperature of 40 °C the effect of the tannase denaturation (Eq. (7)) must be considered in the kinetic model (detailed in the thermodynamics section), and the final kinetic equation becomes:

$$v = \frac{V_{max}[GA] \times \exp(-k_d t)}{K_{m_{GA}}(1 + [PG]/K_{i_{PG}}) + [GA](1 + [GA]/K'_{i_{GA}})} \quad (6)$$

$$C_{E_t} = C_{E_0} \times \exp(-k_d t) \quad (7)$$

where [GA] is the concentration of gallic acid, [Al] the concentration of 1-propanol, [PG] the concentration of propyl gallate, v the initial reaction rate, V_{max} the maximum reaction rate, $K_{m_{GA}}$ and $K_{m_{Al}}$ the Michaelis constants for gallic acid and 1-propanol, $K_{i_{GA}}$ and $K_{i_{PG}}$ the inhibition constants for gallic acid and propyl gallate, C_{E_t} the total active enzyme concentration at time t, C_{E_0} the active enzyme concentration at the beginning of the thermal treatment, and k_d, the first-order deactivation rate constant is 0.0028 h^{-1} at 40 °C.

3.4. Apparent kinetic parameters estimation and model fitting

Calculation of apparent kinetic parameters is based on the initial rates, which lead to the resolution of the global kinetic model. For concision and clarity the data from Fig. 4 were drawn by Lineweaver–Burk double reciprocal plots at low gallic acid concentration (0.926–5.56 mmol L^{-1}) (Fig. 5) and at high gallic acid concentration (5.56–12.9 mmol L^{-1}) (Fig. 6), respectively. The parameters V_{max} and $K_{m_{GA}}$ of the Ping–Pong Bi–Bi kinetic model were determined by plot of $1/v$ versus 1/[GA] (Fig. 5). The parameter $K'_{i_{GA}}$ was obtained by plot of $1/v$ versus [GA] (Fig. 6). Different amount of propyl gallate (5 and 10 μmol L^{-1}) was added at the beginning of the reaction, and the parameter $K_{i_{PG}}$ was calculated from the Lineweaver–Burk double-reciprocal plot (Fig. 7). The values of the apparent kinetic parameters and the corresponding equations are shown in Table 1.

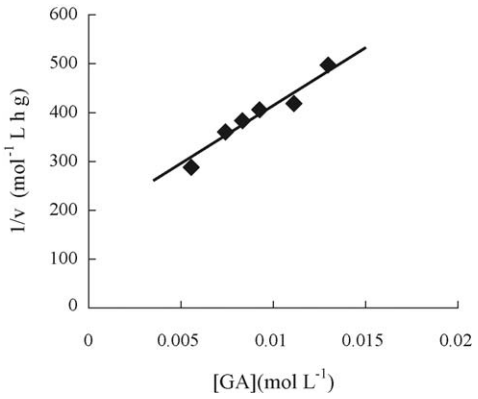


Fig. 6. Reciprocal initial velocity of the reaction versus gallic acid concentration at high gallic acid concentration. Reaction conditions: 5.56–12.96 mmol L^{-1} gallic acid, 7.3% (v/v) 1-propanol and 10 mL benzene, 0.35 g mycelia at 40 °C, pH 3.6, 200 rpm.

The kinetic model of tannase-catalyzed esterification (Eq. (6)) was used to simulate the reaction process. As shown in Fig. 8, a reasonable quality of fit was observed by fitting experimental rate data to the kinetic mode under different gallic acid concentrations. The quality of fit was estimated by correlation analysis and the average correlation coefficient was 0.977. These results indicated that the model could be considered valid for reactor design and for simulation purpose. Additionally, if neglecting the inactivation of tannase, as shown in Fig. 9, the kinetic model fitted the corresponding experimental results poorly. As proved, it is very important to considerate

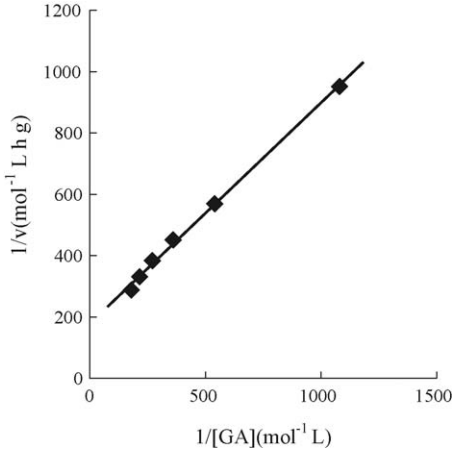


Fig. 5. Lineweaver–Burk double reciprocal plot for mycelium-bound tannase esterification in benzene at low gallic acid concentration. Reaction conditions: 0.926–5.56 mmol L^{-1} gallic acid, 7.3% (v/v) 1-propanol and 10 mL benzene, 0.35 g mycelia at 40 °C, pH 3.6, 200 rpm.

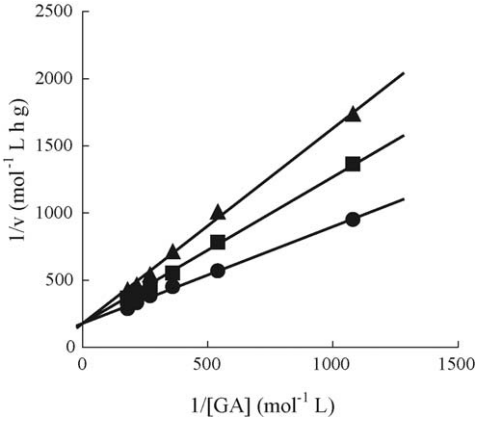


Fig. 7. Lineweaver–Burk double reciprocal plot for mycelium-bound tannase esterification in benzene with propyl gallate present at the beginning of the reaction. The concentration of propyl gallate: (●) 0 μmol L^{-1}, (■) 5 μmol L^{-1} and (▲) 10.

Table 1
Apparent kinetic parameters for mycelium-bound tannase esterification

Apparent kinetic parameter	Value	Equation
V_{max} (mol L^{-1} h^{-1} g^{-1})	0.0056	$1/v = (K_{m_{GA}}/V_{max}) \times (1/[GA]) + (1/V_{max})$
$K_{m_{GA}}$ (mol L^{-1})	0.0041	$K_{m_{GA}}/V_{max} = 0.721$, $1/V_{max} = 177.79$ $R^2 = 0.9976$, standard deviation (S.D.) = 18.59
$K'_{i_{GA}}$ (mol L^{-1})	0.0103	$1/v = 1/(K'_{i_{GA}} K_{m_{GA}}) \times [GA] + (1/V_{max})$, $1/(K'_{i_{GA}} K_{m_{GA}}) = 23,669$, $1/V_{max} = 177.79$ $R^2 = 0.9459$, S.D. = 17.11
$K_{i_{PG}}$ (μmol L^{-1})[a]	9.81	$1/v = \alpha(K_{m_{GA}}/V_{max}) \times (1/[GA]) + (1/V_{max})$, $\alpha = 1 + [PG]/K_{i_{PG}}$, $K_{m_{GA}}/V_{max} = 0.721$, $1/V_{max} = 177.79$ When [PG] = 0, $\alpha = 0$ When [PG] = 10 μmol L^{-1}, $\alpha = 2.02$, $K_{i_{PG1}} = 9.85$ μmol L^{-1}, $R^2 = 0.9966$, S.D. = 30.79 When [PG] = 5 μmol L^{-1}, $\alpha = 1.511$, $K_{i_{PG2}} = 9.77$ μmol L^{-1}, $R^2 = 0.9975$, S.D. = 15.40

[a] The value of $K_{i_{PG}}$ is the average of $K_{i_{PG1}}$ and $K_{i_{PG2}}$.

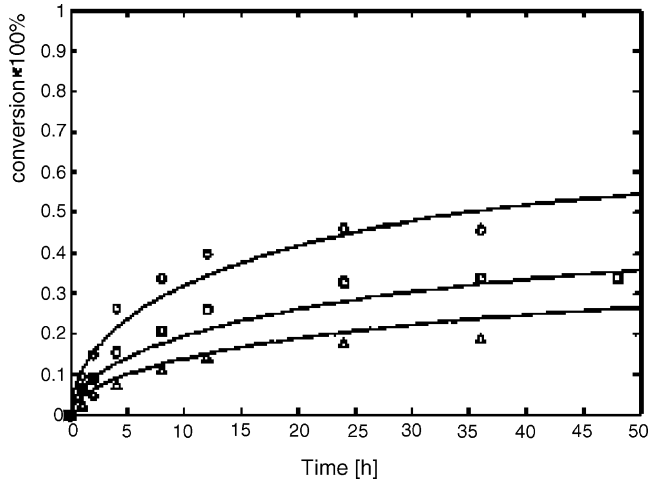


Fig. 8. Simulation of the mycelium-bound tannase esterification in consideration of tannase inactivation (lines: simulated; symbols: experimental). The initial concentration of gallic acid: (◯) 1.85 mmol L^{-1}, (□) 5.56 mmol L^{-1} and (△) 11.1 mmol L^{-1}.

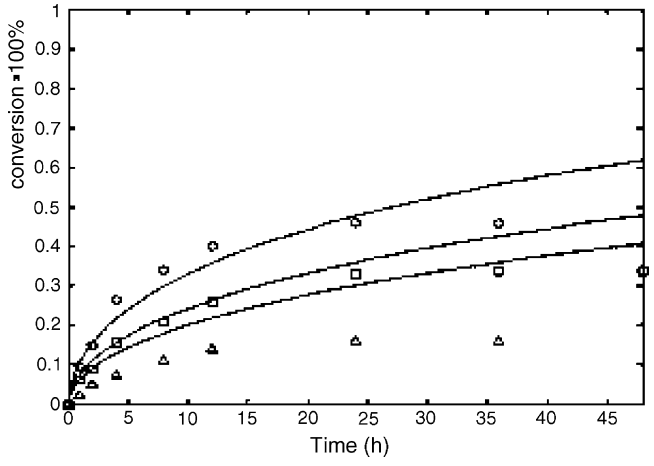


Fig. 9. Simulation of the mycelium-bound tannase esterification without consideration of tannase inactivation (lines: simulated; symbols: experimental). The initial concentration of gallic acid: (◯) 1.85 mmol L^{-1}, (□) 5.56 mmol L^{-1} and (△) 11.1 mmol L^{-1}.

thermodynamics and kinetics simultaneously for simulation of the biological reaction process.

4. Conclusion

A kinetic and thermodynamic study was performed on the synthesis of propyl gallate by mycelium-bound tannase from *A. niger* in organic solvent. The activity of tannase increased with temperature up to a threshold at 47.5 °C. Upon further increasing the reaction temperature a negative effect was observed on the tannase activity. The deactivation of mycelium-bound tannase follows first-order kinetics. Thermodynamic activation parameters were determined. The entropy value of tannase was negative, which is unique in biocatalytic systems, and the mycelium-bound tannase showed an increased stability. In consideration of both the activity and stability of tannase, the optimum reaction temperature for tannase-catalyzed esterification should be 40 °C. A kinetic model of esterification by mycelium-bound tannase was developed based on the Ping–Pong Bi–Bi kinetic mechanism, not only with dead-end inhibition by gallic acid and negative effect by propyl gallate, but also with tannase denaturation. This model was used to simulate the conversion profiles, which are in good agreement with the experimental profiles. Noticeably if neglecting the inactivation of tannase, the kinetic model fitted the corresponding experimental results poorly. Thus, it is important to study the enzyme stability to improve the validity of the kinetic model for reactor design and for simulation purpose. In addition, although the activity and stability of other immobilized or free tannase are different from the mycelium-bound tannase it could be estimated that under a constant water activity the mechanism of esterification in organic solvents by them would also follow the Ping–Pong Bi–Bi kinetic model developed in this study; if the water activity could not be kept constant it is necessary to evaluate the effect of water by adding an extra term in the model [23].

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