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Kinetics of medium-temperature *α*-amylase hydrolyzed *Huai yam* powder

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ABSTRACT

In order to learn the enzymatic characteristics of Huai yam powder with medium-temperature *α*-amylase, effects of substrate concentration, enzyme concentration, pH and temperature were investigated. The Michealis-Menten equation was used to fit the kinetics of the hydrolysis reaction. Experimental results indicate that maximum rate (V_m) is 3.1588 mg/mL·min under the condition of 70˚C, pH 7.0 and 0.0200 mg/mL of enzyme concentration. The Michealis constant (K_m) is 6.6641 mg/mL. The kinetic model, including the factors such as substrate concentration, enzyme concentration and temperature, was established for the hydrolysis reaction under the temperature range from 40˚C - 70˚C.

Keywords: Huai Yam; Medium-Temperature *α*-Amylase; Hydrolysis; Kinetics

1. INTRODUCTION

Yam is the tuber of dioscoreaceae plant widely planted in China, and is very rich in resources. Jiaozuo city, Henan province, is the traditional genuine producing area of yam, commonly called Huai yam, and Huai Yam is also one of four famous Huai drugs. Content of amylum in fresh yam is up to 20% - 30% [1-3] and more than 70% in dried yam [4,5]. At present, studies on amylum are mainly focused on corn and wheat [6,7], and few research pares are involved in the yam, especially Huai yam. Medium temperature *α*-amylase can hydrolyze amylum with high efficiency. Hydrolysis products include dextrin, oligosaccharide, maltose and glucose with the advantages of mild reaction conditions, cost-effective and just simple equipment involved [8-10]. In this work, parameters affecting the reaction rate of medium-temperature *α*-amylase were systematically investigated. The hydrolysis kinetic model of medium-temperature *α*-amylase was deduced, which provides theoretical reference for the hydrolysis and application of Huai yam.

2. MATERIALS AND INSTRUMENTAL

2.1. Materials

Huai yam (Yuecun Countryside, Wen County, Jiaozuo City, China), medium-temperature *α*-amylase (2000 U/g, Fuyuan Biology Technology Co.,Ltd., Zhengzhou, China), glucose (Sinopharm. Chemical Reagent Co.,Ltd, Shanghai, China) were used as received. Other chemicals are all analytical grade. Deionized water was used throughout experiments.

2.2. Instrumental

pH values were measured by a pH meter (Leici Instrument Co., Ltd., Shanghai, China). The UV-Vis spectra were performed on a UV-1900 spectrophotometer (Purkinje General Instrument Co., Ltd., China).

2.3. Hydrolysis

An appropriate amount of Huai yam powder was weighed and to prepare 200 mL slurry. After adjusting pH, medium-temperature *α*-amylase was added to hydrolyze Huai yam.

2.4. Sample Preparation

An amount of Huai yam were dried and ground to 100 mesh. After hydrolysis for a certain time, the medium-temperature *α*-amylase was inactive. After centrifuging, the supertant was diluted to 250 mL. Content of gross sugar was measured by phenol-sulfuric acid method [11].

3. RESULTS AND DISCUSSION

3.1. Effect of Substrate Concentration on the Reaction Rate

In order to investigate the effect of substrate concentration on the reaction rate, a series of 200 mL of substrate aqueous solutions (2.5, 5, 7.5, 10, 12.5, 15, 20

mg/mL) were hydrolyzed by 0.2 mg/mL at pH 7 and 70˚C. The results are shown in **Figure 1**.

Obvious, reaction rate and substrate concentration show typical hyperbolic relationship. Under lower levels, substrate concentration and reaction rate is linear suggesting fist order reaction. Further increase the concentration of substrate, reaction rate reaches maximum, showing zero order reaction. Therefore, the curve of substrate concentration versus reaction rate shows a hyperbola, the typical characteristics of enzymatic reaction [12], suggesting the hydrolysis following the Michaelis-Menten equation.

According to Michaelis-Menten equation, enzymatic reaction can be divided in two steps:

$$E + S \underset{k_2}{\overset{k_1}{\leftrightarrow}} ES \underset{k_4}{\overset{k_3}{\leftrightarrow}} E + P \quad (1)$$

Firstly, enzyme (E) and substrate (S) form intermediate (ES), which decompose to produce products (P) and release enzyme at the same time. k_1, k_2, k_3, k_4 are forward and reverse rate constants, respectively.

At the beginning, the rate of $E + P \rightarrow ES$ is very low and can be neglected due to low level of products. The concentration of intermediate is also much lower than the concentration of substrate being $[S] >> [E]$ and thus can be omitted. Therefore, the reaction rate can be described as follows:

$$v = k_3 \frac{[E][S]}{K_m + [S]} \quad (2)$$

where $[E]$, $[S]$ and K_m are the gross concentration of enzyme, substrate concentration and $(k_2 + k_3)/k_1$, respectively.

Because of $[S] >> [E]$, all the enzyme molecules are surrounded by substrate to form ES, *i.e.*, $[E] = [ES]$, the reaction rate reaches maximum:

$$V_m = k_3[ES] = k_3[E] \quad (3)$$

from **Eqs.2** and **3**, it can be deduced:

$$v = \frac{V_m \cdot [S]}{K_m + [S]} \quad (4)$$

where v, V_m and reaction rat, maximum reaction rate for totally saturated enzyme and Michealis-Menten equation constant.

The value of K_m indicates the substrate concentration when the reaction rate reaches half of the maximum rate. Value of V_m indicates the maximum rate when the enzyme is saturated by substrate within a certain concentration range. Both K_m and V_m are important kinetic parameters of enzyme reaction.

3.2. Lineweaver-Burk Double Reciprocal Plot Method for Solving the Michaelis Constant and the Maximum Reaction Rate [12]

The following equation can be obtained by taking both sides of the **Eq.2** to double reciprocal (**Figure 2**):

$$\frac{1}{v} = \frac{K_m}{V_m} \cdot \frac{1}{[S]} + \frac{1}{V_m} \quad (5)$$

After plotting $1/v$ versus $1/[S]$ and using least square linear method, the maximum reaction rate and K_m can be calculated: V_m = 3.5562 mg/(mL·min), K_m = 8.7219 mg/mL, *i.e.*, Michaelis equation can be expressed:

$$v = \frac{3.5562 \cdot [S]}{8.7219 + [S]} \quad (6)$$

correlation coefficient is 0.9986 indicating good linearity.

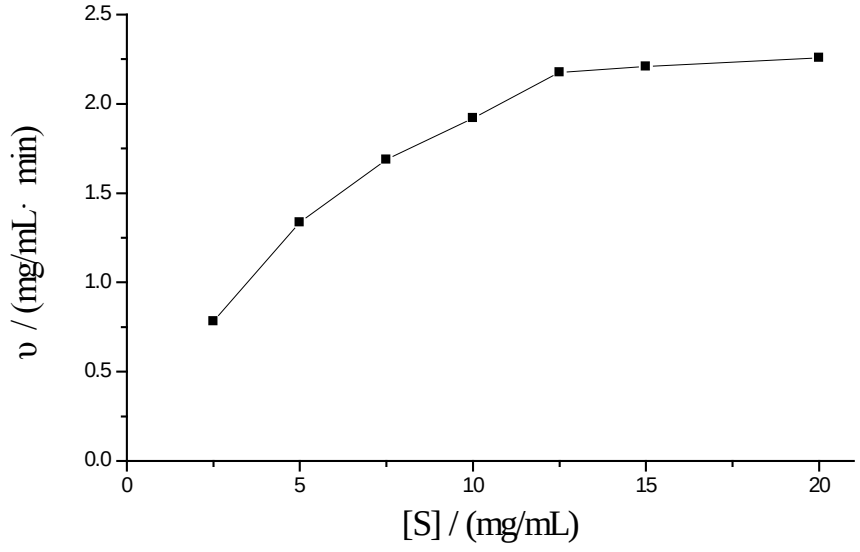

Figure 1. Relationship between reaction rate and substrate concentration.

3.3. Wilkinson Statistical Method for Solving the Michaelis Constant and the Maximum Reaction Rate [13]

Wilkinson statistical method includes two steps: one is estimation of resolutions with nonlinear square method; another is to obtain exact resolutions with Taylor expansion.

a) Estimation: from **Table 1**, it can be obtained:

$\Delta = \alpha\varepsilon - \gamma\delta$ = 4.3131

$$V_m^0 = \frac{\beta\varepsilon - \delta^2}{\Delta} = 3.1724 \text{ (mg/mL·min)};$$

$$K_m^0 = \frac{\beta\gamma - \alpha\delta}{\Delta} = 6.6641 \text{ (mg/mL)}$$

where V_m^0 and K_m^0 are the estimation value of maximum reaction rate and Michaelis constant, respectively.

b) Precision resolution: from **Table 2**, it can be deduced: $\Delta' = \alpha'\beta' - \gamma'^2$ = 2.3216 × 10^5;

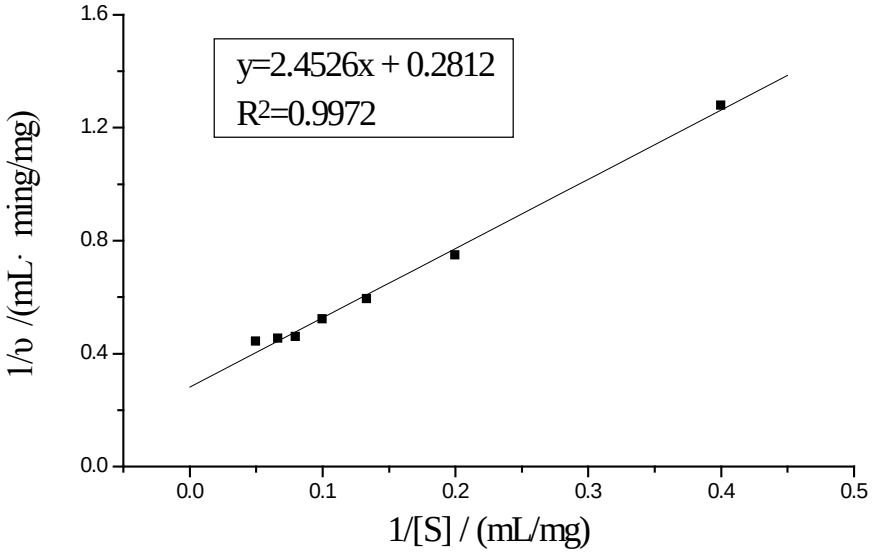

Figure 2. Lineweaver-Burk plot of medium-temperature α-amylase hydrolyzing Huai yam.

Table 1. Estimated resolutions by Wilkinson method.

No.	[S](mg/mL)	υ (mg/mL·min)	$X = \upsilon^2$	$Y = \upsilon^2/[S]$	υX	X^2	υY	XY	Y^2
1	2.5	0.7825	0.6123	0.2449	0.4791	0.3749	0.1916	0.1500	0.0600
2	5	1.3363	1.7857	0.3571	2.3862	3.1887	0.4772	0.6377	0.1275
3	7.5	1.6884	2.8507	0.3801	4.8131	8.1265	0.6418	1.0836	0.1445
4	10	1.9195	3.6845	0.3685	7.0724	13.5755	0.7073	1.3577	0.1358
5	12.5	2.1762	4.7358	0.3789	10.3060	22.4278	0.8246	1.7944	0.1436
6	15	2.2093	4.8810	0.3254	10.7836	23.8242	0.7189	1.5883	0.1059
7	20	2.2569	5.0936	0.2547	11.4957	25.9448	0.5748	1.2973	0.0649
Σ					47.3361	97.4624	4.1362	7.9090	0.7822
Symbol					α	β	γ	δ	ε

Table 2. Accurate resolutions by Wilkinson method.

No.	$[S] + K_m^0$	$V_m^0 [S]$	f	f'	f^2	f'^2	ff'	υf	$\upsilon f'$
1	9.1641	7.931	0.8654	−0.0944	0.7489	62.9008	−0.0817	0.6772	−0.0739
2	11.6641	15.862	1.3599	−0.1166	1.8493	251.603	−0.1586	1.8172	−0.1558
3	14.1641	23.793	1.6798	−0.1186	2.8217	566.1068	−0.1992	2.8362	−0.2002
4	16.6641	31.724	1.9037	−0.1142	3.6241	1006.4122	−0.2174	3.6542	−0.2192
5	19.1641	39.655	2.0692	−0.108	4.2816	1572.519	−0.2235	4.503	−0.235
6	21.6641	47.586	2.1965	−0.1014	4.8246	2264.4274	−0.2227	4.8527	−0.224
7	26.6641	63.448	2.3795	−0.0892	5.662	4025.6487	−0.2122	5.3703	−0.2013
Σ					23.8122	9749.6179	−1.3153	23.7108	−1.3094
Symbol					α'	β'	γ'	δ'	ε'

Note: $f = V_m^0 [S]/([S] + K_m^0)$; $f' = -V_m^0 [S]/([S] + K_m^0)^2$.

$$b_1 = \frac{\beta'\delta' - \gamma'\varepsilon'}{\Delta'} = 0.9957;$$

$$b_2 = \frac{\alpha'\varepsilon' - \gamma'\delta'}{\Delta'} = 3.0671 \times 10^{-8} \quad V_m = V_m^0 \cdot b_1 = 3.1588$$

(mg/mL·min);

$$K_m = K_m^0 + \frac{b_2}{b_1} = 6.6641 \text{ (mg/mL)}$$

where b1and b_2 are the rectified constants of V_m and K_m, respectively, during the calculation process, V_m is the precision resolution of maximum reaction rate, K_m is the precision resolution of Michaelis constant.

3.4. Comparison of Lineweaver-Burk Double Reciprocal Plot and Wilkinson Statistical Methods

There exists differences in the values of V_m and K_m between Lineweaver-Burk double reciprocal plot and Wilkinson statistical methods (**Table 3**). For the former, the experimental data excessively focus under the left of the line. Data of lower substrate concentration have larger errors after reciprocal and thus resulting in larger error. Although Wilkinson statistical method is closer to the actual results, but complex calculation, tedious process, limit its application [14-16].

According to results from experiments, Wilkinson method is adopted to calculate V_m and K_m (V_m = 3.1588 mg/mL·min, K_m = 6.6641 mg/mL).

3.5. Effect of Enzyme Concentration on Reaction Rate

A series of enzyme solutions (0.050, 0.100, 0.150, 0.200, 0.250 mg/mL) were used to hydrolyze abundant substrate at pH 7 and 70˚C. The results are shown in **Figure 3**. Obviously, the concentration of enzyme is linear to the reaction rate. In enzyme reaction, enzyme interacts with substrate to form intermediate complex, which converts to products and releases enzyme. Under a certain system, if the substrate is fully excessive, enzyme totally binds with substrate. The more enzyme molecules, the more resulting products, the more the reaction rate is faster. When [*S*] is more than [*E*], **Eq.3** can be expressed as [17]:

$$v = K[E] \quad (7)$$

Therefore, when the substrate concentration is excessive, reaction rate is linear to the concentration of enzyme, which is consistent with the experimental results.

3.6. Effect of pH on the Efficiency of Hydrolysis

The effect of pH was investigated with 10 mg/mL of substrate and 0.200 mg/mL of enzyme under 70℃ with pH values ranged from 5 to 9. The results are shown in **Figure 4**.

As shown in **Figure 4**, the reaction rate reaches maximum due to extreme acid or base conditions disrupt the conformation of enzyme, which would result in the loss of enzyme, even totally inactivate the enzyme and thus decreases the reaction rate. If pH values do not vary significantly, the dissociative state of substrate is affected by the pH values; enzyme can not bind with substrate or

Table 3. Comparison of Lineweaver-Burk and Wilkinson methods.

Method	V_m/mg/(mL·min)	K_m/(mg/mL)
Lineweaver-Burk	3.5562	8.7219
Wilkinson	3.1588	6.6641

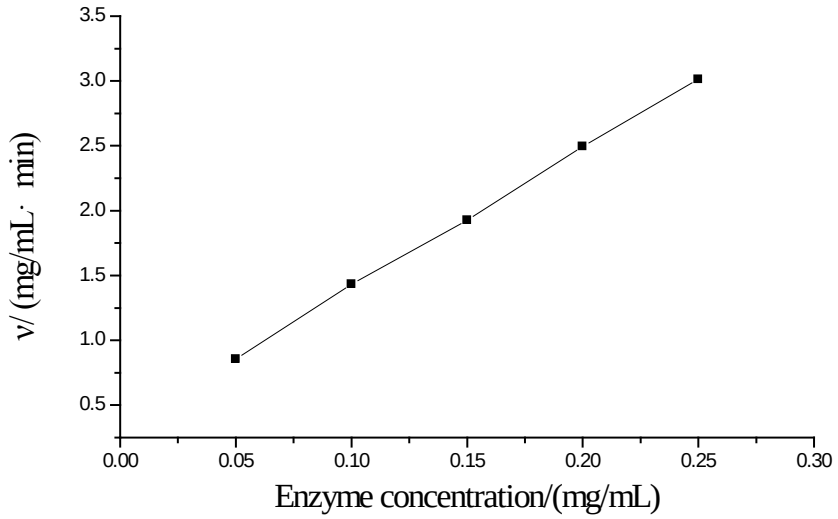

Figure 3. Relationship between α-amylase concentration and reaction rate.

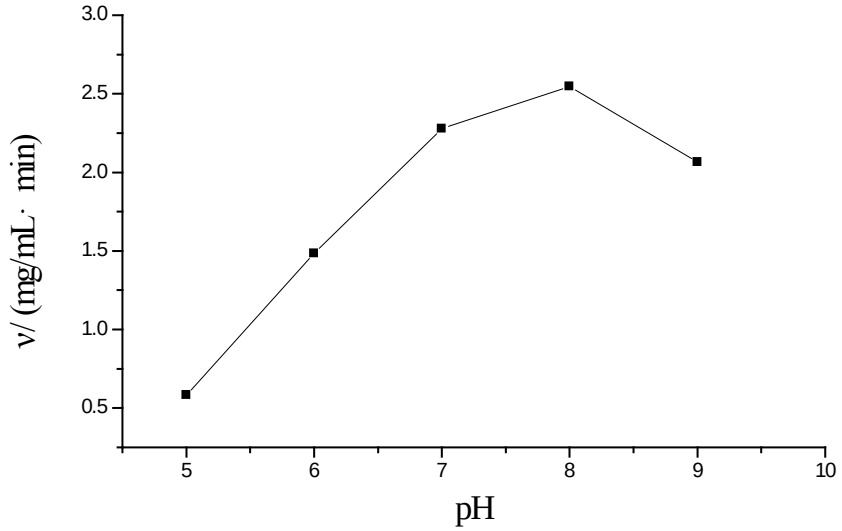

Figure 4. Effects of pH on the hydrolysis of Huai yam.

on products were produced after binding and thus affecting the reaction rate [12]. Reaction rates under different pH values can be fitted with **Eq.8**:

$$v' = \frac{V_m' \cdot [S]}{K_m' + [S]} \quad (8)$$

where v', V_m and K_m indicate reaction rate under different pH values, maximum reaction rate and Michaelis constant, respectively.

3.7. Effect of Temperature on Reaction Rate

The effect of temperature was investigated by fixing pH value, substrate and enzyme concentrations. When the temperatures were selected as 40˚C, 50˚C, 60˚C, 70˚C and 80˚C, the reaction rates are shown in **Figure 5**. It can be seen that the reaction rate increases with increasing temperature in the range of 40˚C to 70˚C. Above this level, the reaction rate decreases due to higher temperature results in the inactivation of enzyme and thus decreases the reaction rate.

Figure 6 shows the relationship between lnv and $1/T \times 10^3$. Obviously, there is a good linearity between $\ln v$ and $1/T \times 10^3$ when the values of $1/T \times 10^3$ range from 2.9 to 3.2, indicating that the hydrolysis rate and temperature follow the Arrhenius equation:

$$\ln k_2 = -\frac{E_a}{R} \cdot \frac{1}{T} + \ln A \quad (9)$$

where A, R and E_a are pre-exponential factor, gas constant (J/mol·K) and activation energy (kJ/mol), respectively.

Values of E_a and A are obtained by linear regression equation. It is found that E_a is 14.774 kJ/mol, A is 454 mg/mL·min and the correlation coefficient is 0.9907, suggesting that the experimental data is good agreement with Arrhenius equation.

If temperature does not vary significantly, the equilibrium constants and temperature (T) follow Van't Hoff equation:

$$\ln K_s = -\frac{\Delta H}{RT} + \ln C \quad (10)$$

where K_s, ΔH, C and R are equilibrium constant, enthalpy, frequency factor and gas constant, respectively. In enzymatic reaction, $k^2 >> k^{-1}$, $K_s \approx K_m$, a linear curve (**Figure 7**) can be obtained by fitting $\ln K_m$ versus $1/T \times 10^3$.

From linear regression equation, it is found that ΔH is 14.641 kJ/mol, C is 1046 and correlation coefficient, r, is 0.9856. Therefore, the kinetic equation of medium-temperature α-amylase hydrolyzing Huai yam can be expressed as:

$$v = \frac{k_3[E][S]}{K_m + [S]} = \frac{A \cdot \exp(-E_a / RT)}{C \cdot \exp(-\Delta H / RT) + [S]}[E][S]$$
$$= \frac{454 \cdot \exp(-14774 / RT)}{1046 \exp(-14641 / RT) + [S]}[E][S]$$

This equation is suitable in the temperature range of 313.15 to 343.15 K (40˚C to 70˚C).

4. CONCLUSIONS

Because starch includes amylose and amylopectin with different molecular weight sizes, and the hydrolysis products by the medium-temperature α-amylase include dextrin, oligosaccharides, glucose and maltose [12]. This work adopts phenol-sulfuric acid method for the determination of total sugars after hydrolysis and investigates the parameters affecting reaction rate. It is found that K_m is 6.6641 mg/mL and maximum reaction rate, V_m, is 3.1588 mg/mL·min. The kinetic model of enzymatic hydrolysis

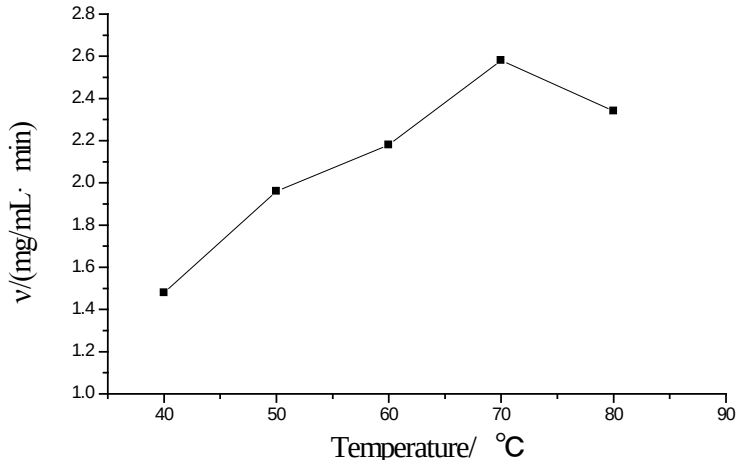

Figure 5. Effects of temperature on the hydrolysis of Huai yam.

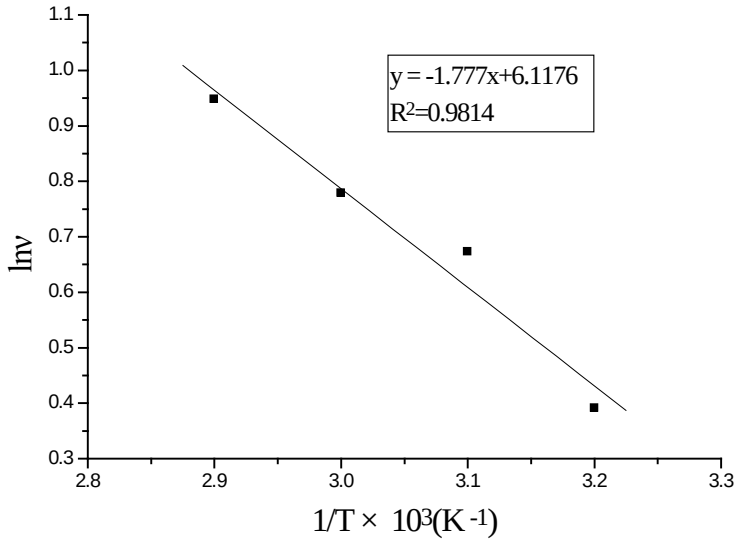

Figure 6. Relationship between ln*v* and 1/T × 10^3.

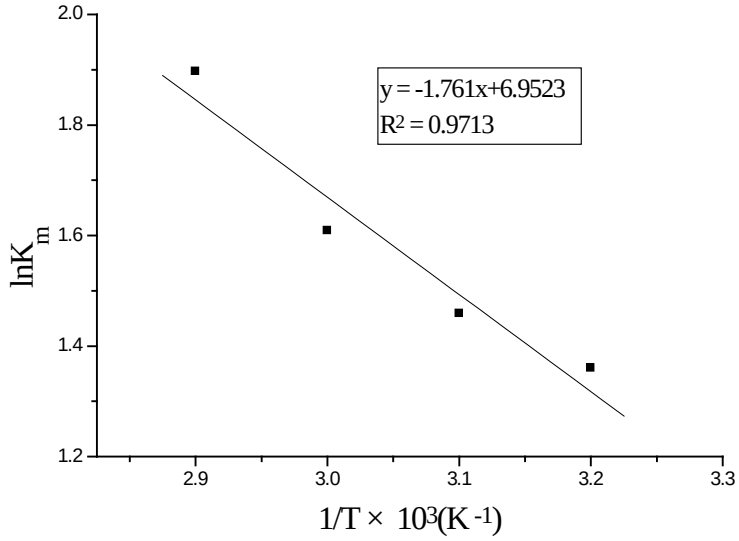

Figure 7. Relationship between lnK_m and 1/T × 10^3.

including the concentrations of enzyme and substrate and temperature is established:

$$v = \frac{454 \cdot \exp(-14774/RT)}{1046\exp(-14641/RT)+[S]}[E][S]$$

The suitable temperature for this model ranges from 40˚C to 70˚C. Every parameter is fitted with high significance suggesting that it is effective with Michaelis-Menten equation simulating the kinetic process of medium-temperature α-amylase hydrolyzing Huai yam.

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