Kinetics of the Thermal Inactivation of *Bacillus subtilis* α-Amylase and Its Application on the Desizing of Cotton Fabrics

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ABSTRACT: The thermal inactivation of *Bacillus subtilis* α-amylase was studied in the presence and in the absence of Ca^{2+} at various temperatures. Inactivation rate constant (k), half-life time ($t_{1/2}$), and activation energy (E_a) were determined to characterize the inactivation of the enzyme. Results obtained showed that the thermal inactivation of *Bacillus subtilis* α-amylase followed a first-order kinetics. The addition of Ca^{2+} had a good thermostabilizing effect on the enzyme. The stabilizing effect of Ca^{2+} is reflected by the increased values of the activation energy, which is about two times higher in the presence than that in the absence of 20 m*M* Ca^{2+}, and the decreased values of the inactivation rate constants. The desizing of the cotton fabrics was performed through steaming at 100°C with *Bacillus subtilis* α-amylase. The desizing efficiency seemed to be dependent on the concentration and pH value of the enzyme solution. It was found that through the steaming process with α-amylase, the desizing ratio of the cotton fabrics could be beyond 98% and little damage happened to the fibers of the fabrics. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 109: 3733–3738, 2008

Key words: α-amylase; thermal inactivation; calcium ion; desizing; cotton fabrics

INTRODUCTION

In the textile process industry, the desizing is one of the important pretreatment processes of cotton fabrics to remove starch-containing size which has served as a protective coating on yarns during weaving. Complete removal of the size coating after weaving is important to ensure optimum results in the subsequent processes, in which the fabric is generally scoured, bleached, and dyed.

In the past, the hydrogen peroxide and the sodium hydroxide were used as desizing agents generally, to cause the environmental pollution, because of high chemical oxygen demand (COD), biological oxygen demand (BOD), pH, and salt content in textile effluents.[1,2] More recently, enzymatic processes have been employed using α-amylase [(1-4)glucan 4-glucanohydrolases, EC3.2.1.1] to catalyze the breaking of α-1,4-glucosidic bonds in amylose and amylopectin molecules.[3,4] This method produces little waste water and is friendly to the environment. But the most of mesophilic α-amylase such as *Bacillus subtilis* and *Bacillus amyloliquefaciens* α-amylase will get inactivated quickly, when the temperature rises to more than 60°C. The desizing of cotton fabrics with this enzyme is performed generally through the means of cold-pad-batch at 45°C or room temperature for 1 to 12 h. The thermostable amylases have been developed and used at relatively high temperatures. But it can only be produced from several scarce bacteria to increase the cost of the treatment of textiles and limits the application of α-amylase on the desizing of cotton fabrics. Mesophilic α-amylases can be stabilized by some appropriate additives to improve their thermostability. So they can be used under higher temperature, which will broaden the application of mesophilic α-amylase on the textile pretreatment.

When the temperature rises above a certain level, enzymes in aqueous solutions undergo partial unfolding caused by heat-induced disruption of the balance of noncovalent interactions maintaining the native conformation. This process, which results in enzyme inactivation due to disintegration of the active center, is fully reversible, i.e., the activity returns if the temperature is promptly lowered to the ambient.[5,6] Upon longer heating, however, only a diminishing fraction of the activity is regained upon cooling thus reflecting another process, irreversible inactivation.[7] This process is responsible for the gradual loss of the enzyme activity with time at elevated temperatures. The kinetics of the reversible thermal inactivation have been extensively studied

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and well known,[5,8,9] but little is known about the kinetics of the irreversible one.

Lecker and Khan[10] believed that the diluted enzyme solution was more prone to inactivation than the concentrated enzyme solution. In this study, we used the means of steaming to treat cotton fabrics, which decreased water content. The thermal inactivation kinetics of *Bacillus subtilis* α-amylase, which was freeze-dried on the freeze drier (LGJ-10, Xiangyi, China), was investigated in the dry ambient. When the incubation time was fixed to 5 min, the residual activity of the dried enzyme was about 63% at 70°C, much higher than that of the undried enzyme, which is about 15%. Therefore, water plays an important role in the inactivation of α-amylase. Reducing the content of water would enhance the thermostability of the enzyme. The reason can be described as follows.

In aqueous solution, the hydrophilic regions of the enzyme locate on the surface of enzymatic molecules and interact with water to form hydrogen bonds, which make the protein of the enzyme form flexible ''open'' style spatial structure. Although the content of water molecules around the enzyme decreased, the hydrophobic regions of the protein will be exposed. Under the interaction of charged groups, active ''close'' conformation of the enzymatic protein comes into being, which enhances the rigidity of the structure of the enzyme molecule and improve its thermostability.

Therefore, the means of steaming not only accelerates the desizing of cotton fabrics, but can be used as one of the means of increasing the stability of *Bacillus subtilis* α-amylase in the following desizing process.

In this study, Ca^{2+} was added to the solution of α-amylase to stabilize the enzyme. The thermal inactivation of *Bacillus subtilis* α-amylase was studied at various temperatures. The stabilizing effect of Ca^{2+} is characterized by the inactivation rate constant, half-life time, and activation energy of the enzyme. The effects of the concentration and pH value of the enzyme solution on the desizing ratio of cotton fabrics were also discussed.

MATERIALS AND METHODS

Enzyme

α-Amylase from *Bacillus subtilis* in this study was obtained from Longda Biotech, China.

Substrate

In this experiment, starch-sized plain-woven 100% cotton fabric, 120 g/cm^2, was used.

Determination of α-amylase activity

The method used to determine the α-amylase activity of the samples was described by De Moraes et al.[11] About 0.2 g of soluble starch was dissolved in 100 mL boiling distilled water. The solution was cooled to 40°C. One milliliter of appropriately diluted enzyme solution was added to 1 mL of starch solution and the mixture was incubated at 40°C in a water bath. To stop the reaction, 0.5 mL of 1*M* hydrochloric acid, 0.5 mL of 10% potassium iodide, and 2 mL of 0.05*M* potassium iodate were added to the mixture. Then the mixture was diluted to 50 mL. The degradation of starch by the enzyme was measured at 660 nm using fluorescence spectrophotometer (UV-3310, Hitachi, Japan) against 0.5 mL of 1*M* hydrochloric acid, 0.5 mL of 10% potassium iodide, and 2 mL of 0.05*M* potassium iodate in 47 mL of distilled water as blank. One unit of α-amylase activity was defined as the quantity of starch, which was hydrolyzed by 1 mg enzyme in 1 min at 40°C when 2 mg starch was present at the start of the reaction.

Thermal inactivation kinetics of α-amylase

The time course of thermal inactivation of α-amylase in the presence and absence of Ca^{2+} was measured. The enzyme was incubated at various temperatures (50–90°C) in a water bath. Samples were removed at intervals, cooled in ice water and assayed for enzyme activity in the conditions previously described. The thermostability of the enzyme was expressed as the percentage of the residual activity relative to the native one, which was defined as 100%.

Data analysis

If the thermal inactivation of α-amylase follows a first-order kinetics, one gets,

$$\ln A = -kt + \ln A_0 \tag{1}$$

where A is residual activity of α-amylase, A_0 is original activity of the enzyme, k is inactivation rate constant (min^{-1}), and t is inactivation time (min).

Arrhenius plot represents the variations of the inactivation rate constant with respect to the reciprocal of the temperature, as follows:

$$\ln k = -\frac{E_a}{RT} + C \tag{2}$$

where E_a is activation energy (J/mol), R is gas constant (8.314 J/(mol K)), T is temperature (K), and C is pre-exponential factor.

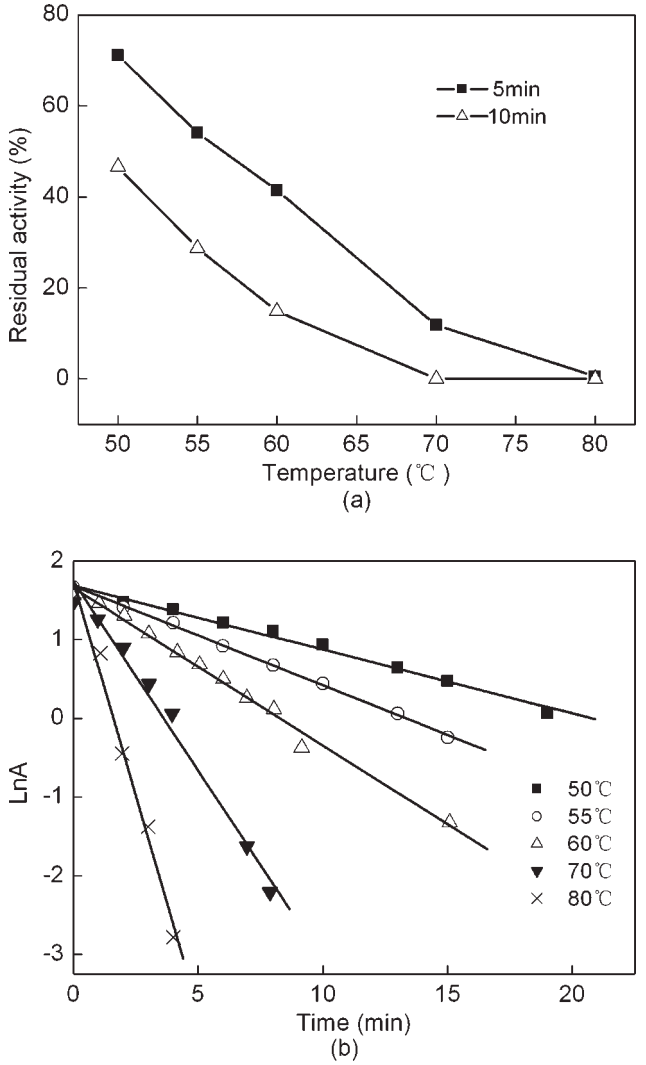

Figure 1 Effect of temperature on the thermostability of native α-amylase (a) when the incubation time was fixed to 5 and 10 min, the residual activity of the enzyme was measured. (b) Kinetics of the thermal inactivation of *Bacillus subtilis* α-amylase at various temperatures.

From eqs. (1) and (2), k and E_a can be obtained. In this experiment, they were used to characterize the inactivation of α-amylase.

Enzymatic desizing

Starch-sized fabrics were treated by the following recipe: α-amylase X% o.w.f (on weight the fabric), JFC 1 g/L, pH 6–9, for Y min in 100°C steam on the universal steamer (DHE, Mathis, Swiss).

Determination of desizing ratio

At the end of the treatment, the fabrics were removed from the steamer and put into 30 mL of 42% perchloric acid for 10 min at room temperature. The mixture was neutralized followed by the filtration. The filtrate was measured by the means which was the same as earlier. Desizing ratio (D%) of the fabric is defined as the following equation.

$$D\% = \frac{W_0 - W_1}{W_0} \tag{3}$$

where W_0 is starch weight on the fabric before desizing, W_1 is starch weight on the fabric after desizing.

RESULTS AND DISCUSSION

The thermostability of native α-amylase was examined in the temperature ranging from 50 to 80°C, fixing the enzyme concentration to 40 μg/mL. As shown in Figure 1(a), when the incubation time was fixed to 5 and 10 min, the residual activity of the enzyme remained 70 and 45% at 50°C, respectively. Although increasing the incubation temperature, the residual activity was reduced rapidly. There was almost no activity remained when the temperature was increased to 80°C.

Arrhenius plots representing the variations of the enzyme activities with respect to incubation time at various temperatures were drawn [Fig. 1(b)]. The thermostability of the enzyme was quite sensitive to the incubation time and temperature. It was found that a linear drop in activity occurred in all cases, that is, the enzyme inactivation followed a first-order behavior as assumed earlier. While increasing the temperature from 50 to 80°C, the inactivation rate constant was increased rapidly from 0.08 to 1.09 min^{-1}.

Effect of Ca^{2+} on the thermostability of α-amylase

It is well-known that addition of Ca^{2+} is required for an enhanced thermal stability of α-amylase.[12] To obtain information concerning the role of this ion in the enzyme stabilization of *Bacillus subtilis* α-amylase, a study of the kinetics of irreversible thermal inactivation of the enzyme in the presence of Ca^{2+} at different temperatures was carried out.

The thermostability of α-amylase was examined in the presence of Ca^{2+}, that is, the supplement of 20 mM Ca^{2+} led to a remarkable enhancement of the thermostability. As shown in Figure 2(a), when the incubation time was fixed to 10 min, the residual activity of the enzyme was almost identical to its initial activity at 60°C. Although over 65°C it was reduced quickly, there was still 50% of the original activity that remained when the temperature was increased to 80°C.

The changes in the enzyme activity of *Bacillus subtilis* α-amylase were also examined as a function of the incubation time [Fig. 2(b)]. In the presence of Ca^{2+}, the inactivation of the enzyme also followed first-order kinetics at various temperatures. With increasing the temperature from 60 to 90°C, the inac-

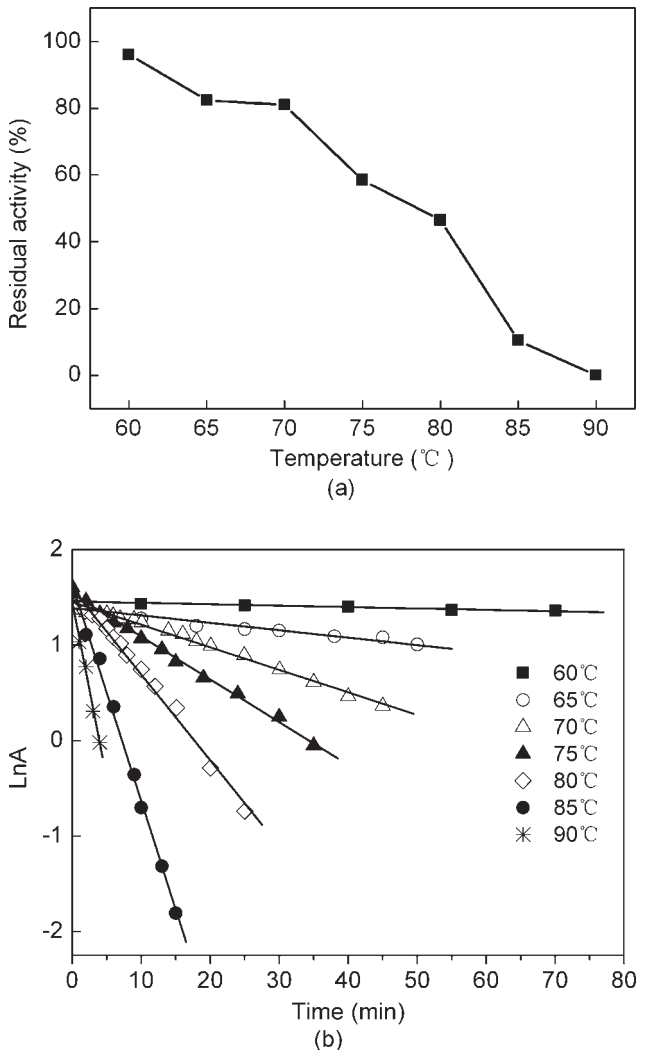

Figure 2 Effect of Ca^{2+} on the thermostability of α-amylase. The thermostability of native α-amylase was examined in the temperature range from 60 to 90°C, fixing the enzyme concentration to 40 μg/mL. (a) When the incubation time was fixed to 10 min, the residual activity of the enzyme was measured in the presence of 20 m*M* Ca^{2+}. (b) Kinetics of the thermal inactivation of *Bacillus subtilis* α-amylase at various temperatures in the presence of 20 m*M* Ca^{2+}.

tivation rate constant was increased from 0.0008 to 0.37 (min^{-1}), which were much less than that in the absence of Ca^{2+} (Table I).

The stabilizing effect of Ca^{2+} on the enzyme seems to be strong, as shown in Table I. To determine the effect of Ca^{2+}, the half-life times of the enzyme in the presence and absence of Ca^{2+} were assayed at various temperatures. One can observe that the inactivation time was prolonged apparently in the presence of Ca^{2+}. For example, when the incubation temperature was 60°C, addition of 20 m*M* Ca^{2+} drastically stabilized *Bacillus subtilis* α-amylase and the half-life time was increased from 3.9 to 800 min, i.e., 200-fold (15- and 10-fold at 70 and 80°C, respectively).

In the presence or in the absence of added Ca^{2+}, the inactivation rate constant followed Arrhenius law (Fig. 3). The stabilizing effect of Ca^{2+} is also reflected by the increased values of the activation energy, which is about two times higher in the presence than that in the absence of 20 m*M* Ca^{2+} (increased from 80.9 to 159.5 kJ/mol).

The three-dimensional structure of pig pancreatic α-amylase has recently been elucidated.[13] It was shown that Ca^{2+} stabilized the active-site cleft by inducing an ionic bridge between the two domain A and B. Although no similar study has been carried out on *Bacillus stabilis* α-amylase, one may consider that Ca^{2+} plays a similar role. In fact, this cation is necessary for the structural integrity of all α-amylases and the conserved sequence regions are involved in the architecture of the Ca^{2+}-binding site and of the active site. The present experiment clearly indicates that Ca^{2+} plays an important role in the enzyme's thermostability. Probably by binding to the protein, it stabilizes the three-dimensional structure and prevents the enzyme from unfolding.[12] The addition of Ca^{2+} could increase the rigidity of the enzyme structure through multipoint electrostatic crosslinks, reducing the protein chain mobility of the enzyme and preserving its active conformation at high temperature.[14]

Desizing of cotton fabric with *Bacillus stubilis* α-amylase

The desizing of the cotton fabrics were performed through steaming at 100°C for a processing time from 1 to 5 min. The concentration of the amylase

TABLE I
Half-Life Times ($t_{1/2}$) and Rate Constants (k) for Inactivation of *Bacillus subtilis* α-Amylase in the Presence/Absence of Ca^{2+}

Incubation temperature (°C)		60	65	70	75	80	85	90
$t_{1/2}$ (min)	Native	3.9	–	2.0	–	0.8	–	–
	Ca^{2+}	800.0	83.0	30.0	14.0	8.6	3.4	1.8
k (min^{-1})	Native	0.2	–	0.48	–	1.09	–	–
	Ca^{2+}	0.0008	0.007	0.024	0.045	0.090	0.23	0.37

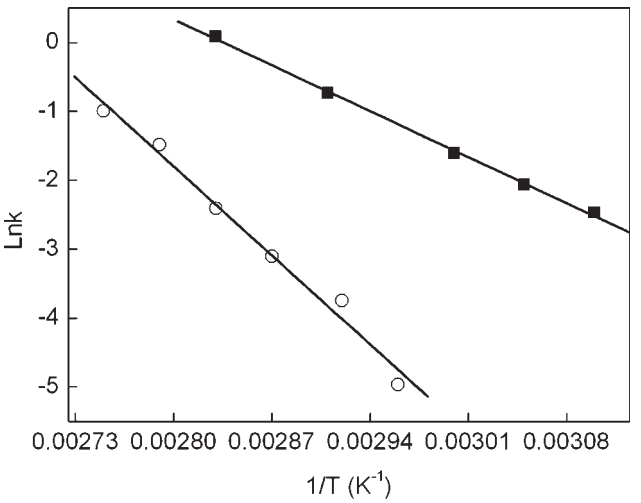

Figure 3 Arrehenius plots for the thermal inactivation rate constant of *Bacillus subtilis* α-amylase. The inactivation rate constants in the absence (■) and in the presence (○) of Ca^{2+} were obtained from Figures 1(b) and 2(b). Activation energies were determined by linear-regression analysis.

was from 0.25% to 2.0% o.w.f and pH value was fixed to 6.5. In the absence of Ca^{2+}, there was no satisfactory effect on the desizing of the cotton fabrics with limited quantity of amylase under steaming condition. A larger quantity of enzyme was needed to obtain better desizing effect, which increased the cost of pretreatment of the cotton. The following experiments were all performed with 20 m*M* Ca^{2+} added to enhance the thermostability of *Bacillus subtilis* α-amylase at 100°C.

The effect of enzyme concentration on the desizing ratio of cotton fabrics using α-amylase produced by *Bacillus subtilis* was investigated. Experimental results are shown in Figure 4. With respect to the

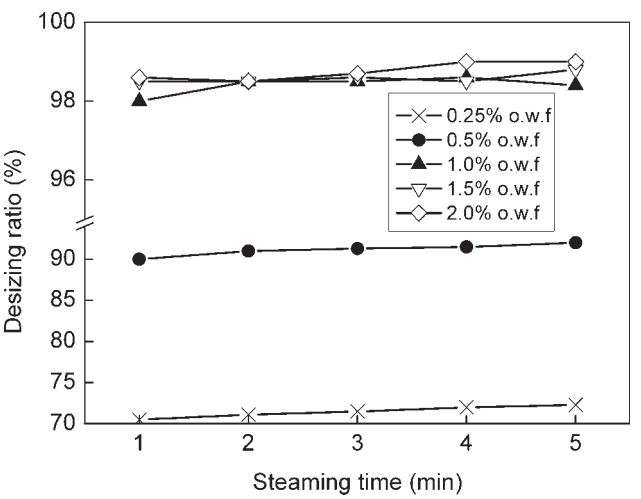

Figure 4 Effect of enzyme concentration on the desizing ratio of the cotton fabrics.

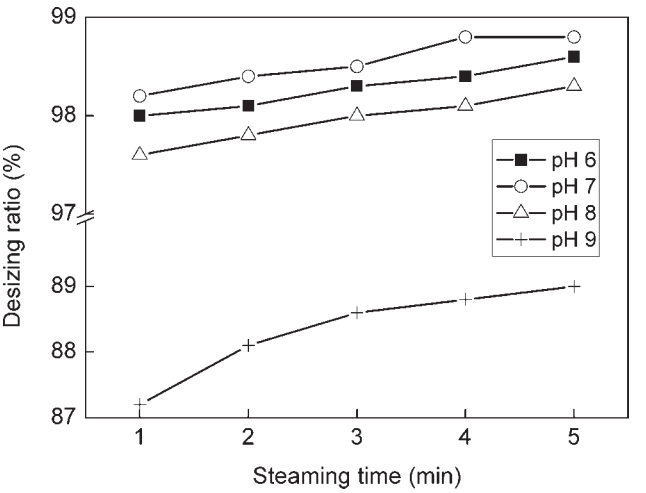

Figure 5 Effect of pH value on desizing ratio of cotton fabrics with *B. stubilis* α-amylase.

increase of enzyme concentration, the desizing ratio was increased. And when the concentration was beyond 1.0% o.w.f, over 98% of the starch on the cotton was hydrolyzed. To our delight, the steaming time was very short. With 1 min treatment, the satisfactory desizing effect was acquired.

The effect of pH value on the desizing ratio of cotton fabrics using *Bacillus subtilis* α-amylase was studied. The concentration of the enzyme was fixed to 1.0% o.w.f. The results are shown in Figure 5. One can see that when pH value was between 6 and 8, the desizing ratio was beyond 97%. When pH value rised continuously, the desizing ratio began to drop.

As shown in Figure 6, the breaking strength of the desized fabrics was measured in Hounsfield H10K-S Universal Strength Tester (English). The pH value was fixed to 6.5. It could not be found that there was

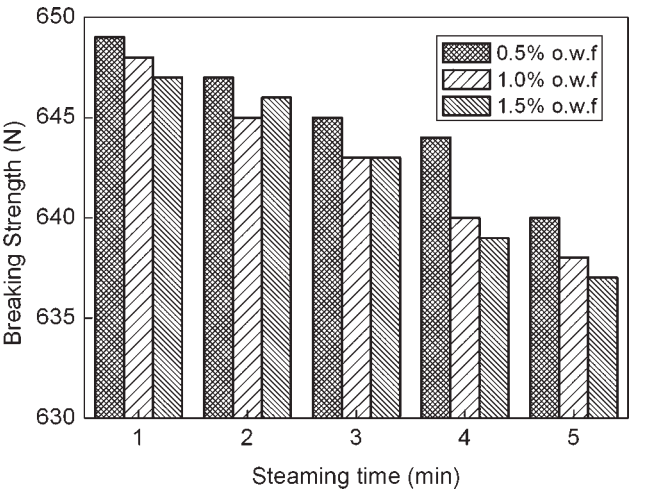

Figure 6 Effect of enzyme concentration on the tensile strength of the cotton fabrics. The native breaking strength of the cotton fabrics was 652 N.

little loss on the strength after 1-min treatment. Then the steaming means of the desizing of cotton fabrics with α-amylase is very friendly to the environment and has little damage to the fibers of fabrics.

CONCLUSIONS

In this study, the thermal inactivation of *Bacillus subtilis* α-amylase was studied at various temperatures from 50 to 90°C. Kinetics parameters such as inactivation rate constant, half-life time, and activation energy were used to characterize the inactivation of the enzyme.

Ca^{2+} had a better stabilizing effect on the α-amylase from *Bacillus subtilis*. After adding 20 m*M* Ca^{2+}, half-life time of the enzyme at 80°C was increased from 0.8 to 8.6 min and the activation energy of inactivation of the enzyme of Ca^{2+} was about 159.5 kJ/mol, which was about two times higher than that in the absence of Ca^{2+}. From the results, we would observe that the thermal inactivation of *Bacillus subtilis* α-amylase followed a first-order kinetics.

Cotton fabrics were desized by the means of steaming at 100°C with the enzyme. When the concentration of amylase was beyond 1.0% o.w.f and pH value was between 6 and 8, over 98% of the starch on the cotton fabrics was removed and there was little damage on the cotton fibers. The results reported in this article show that this approach of desizing with *Bacillus subtilis* α-amylase may be efficient and promising.

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