Development and Testing of Fast Responsive Bi-Enzyme Time - Temperature Indicator

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Abstract. In order to directly indicate the change of the quality of temperature sensitive products, such as food and medicine, during transportation and storage, the time-temperature indicator (TTI) was researched. It has cumulative effect on the time and temperature in the transport process and also can be used to monitor the environmental temperature changes. Based on the novel glucose oxidase and horseradish peroxidase double enzyme reaction system, a new time-temperature indicator was developed. The suitable storage conditions and the composition of double enzyme as of the TTI reaction system were studied. In conclusion, the time-temperature indicator with obvious color change is applied to the packaging of cold products during the transportation and storage process.

1. Introduction
Recently, people pay more attention to the food quality and safety as the improvement of their living standard, which has boosted the rapid development of the food quality and safety monitoring technology, especially the way to identify the food quality visibly. Easily, quickly and safety issues has gained such a lot of attention from the researchers that it has become gradually the hot spot in the food research sector [1]. The quality of temperature-sensitive products like chilled products, can be affected by many factors such as the pressure, humidity, sunshine and other outside factors during transportation, yet the most critical one lies in storage temperature [2]. Temperature monitoring in the whole process from food production to cold chain transportation, storage, sales and use is quite necessary to ensure the freshness of the chilled products. In order to present an intuitive and convenient variation for the quality of temperature-sensitive food and drugs with the change of time and temperature, the time-temperature indicator/integrator (TTI) applicable to food thus came into being. With the cumulative effect on time and temperature during product transportation, the indicator can detect the change of the ambient temperature during product transportation and storage, thus can indicate the food quality and safety status.

With its important practical value in the packaging for temperature-sensitive products and others, TTI has been widely researched in foreign countries. For example, Check Point [3] has developed TTI based on the color change reaction of the system due to the color change of the acid-base indicator resulting from pH decrease caused by the hydrolysis of lipase and substrate. Rani and Abraham [4] catalyzed the decomposition of hydrogen peroxide substrate by using the purified anionic peroxide
isoenzymes extracted from the feverfew Fragrant Eupatorium Herb. An enzymatic TTI was developed based on the color change resulting from the isoenzymes and the chromogen substrate used for the peroxidase determination. Tucker and Kim [5, 6] carried out researches respectively on the enzymatic TTI for monitoring food quality and predicting remaining shelf life of food based on the color change of the amylase and laccase indicators.

The TTI research in China, however, is with a late start and less quantity, most of the researches are still in the theoretical stage. For example, Cai [7] developed an amylase TTI with amylase, starch and iodine as raw materials, boosting the hydrolysis of starch to reflect the cumulative effect of time-temperature based on the color change of the indicator – iodine [8]. Lu [9] developed a TTI system based on lipase reaction diffusion and measured 4 kinds of TTI activation energy with different formula parameters. TTI can be activated by laminating the upper cover with gelatinous reaction substrate and the bottom plate with gelase substrate, forming a yellow band, and the cumulative effect on time-temperature can be judged by the diffusion length of the yellow band. Zhou [10] developed the patent of CN 101349599[P], which made use of the hydrolysis reaction of the alkaline lipase with the substrate triacetin.

With respect to the fact that it has been a long history in the foreign countries for the research and development of the shelf life indicator, many indicators have been developed and applied, the shelf life indicator, as a tool effectively monitoring the food quality and shelf life, has been accepted and recognized by more and more people. In China, however, the status quo is that research on shelf life indicator is still rare. The research launches the attempts and exploration on the shelf life indicator development by using new bi-enzyme system in an aim to bring forward a new type of shelf life indicator.

The shelf life indicator works to indicate the mechanic, chemical, electrochemical, enzymatic or microbial irreversible changes by the visible reaction in the form of mechanical deformation, color change or color movement, the rate of change varies with temperature, and the higher the temperature, the higher the reaction rate [11]. The shelf life indicator proposed in this thesis is based on the reaction of glucose oxidase (GOX) with horseradish peroxidase (HRP).

GOX is a kind of healthy and safe food additive, which can exclusively catalyze β-D glucose (GLU) for the consumption of oxygen in food and produce H$_2$O$_2$ with bactericidal effect, therefore, it is crucial for a lot of food preservation as well as a natural and non-toxic preservative [12]. HRP is a plant peroxidase extracted from the perennial herb horseradish that are planted in temperate regions of the world, with vast sources. Therefore, HRP is also the enzyme preparation with vastest sources and relatively inexpensive price. As the representative of peroxidase, it has been applied in many fields such as synthesis, biology, environmental chemistry and food industry. In this research, β-D-glucose and 3, 3', 5', 5' - tetramethylbenzidine (TMB) were used as substrates for the enzymatic time-temperature indicator, because β-D-glucose is the most widely distributed and most important monosaccharide in the natural world, soluble in water and the best substrate for glucose oxidase. TMB is the most sensitive color former of HRP and a new and safe chromogenic agent, which is often used as a chromogenic agent for such immunoassay as ELISA and others, with the advantages of high sensitivity, good stability and safety for use. Animal experiments and Ames bacterial autovariation test results show that TMB is non-carcinogenic and non-mutagenic. At present, TMB has gradually replaced strong carcinogenic benzidine and other carcinogenic benzidine derivatives, and has been applied in clinical testing, forensic testing, criminal detection and environmental testing, especially in clinical biochemical tests. Now the commercial TMB chromogenic agent produced by many companies are for sale on the market.

Glucose oxidase acts as the donor of H$_2$O$_2$. The addition of glucose instead of direct addition of H$_2$O$_2$ can eliminates the drawbacks of horseradish peroxidase inactivation caused by too high local concentration of H$_2$O$_2$. Under the catalytic action of the glucose oxidase, glucose is first oxidized by dissolved oxygen, resulting in gluconic acid and H$_2$O$_2$. Under the catalysis of HRP, the generated H$_2$O$_2$ oxidizes TMB to generate a colored azo compound [11], therefore, it is unnecessary to add an indicator to indicate the color change, and the degree of color change is used to analyze the
temperature change course in which the experiment is conducted. In the reaction, the reaction rate of bi-enzyme catalyzed decomposition is closely related to the temperature, within a certain temperature range, the higher the temperature, the faster the catalytic reaction rate. Due to the different amounts produced during the reaction, visible color change will present. The bi-enzyme reaction complies with general TTI work principle. Therefore, the development of TTI by the use of horseradish peroxidase combined with glucose oxidase bi-enzyme catalytic reaction is theoretically feasible.

2. Materials and Methods

2.1. Materials
Glucose Beijing Kehua Jingwei Technology Co., Ltd. Glucose oxidase (10KU) Beijing Kehua Jingwei Technology Co., Ltd. Horseradish catalase (10KU) AMRESCO (U.S.).

TMB AMRESCO (U.S.), Anhydrous ethanol Beijing Chemical Plant. Dihydrogen Phosphate Dihydrogen Phosphate Chemical Group Beijing Chemical Co., Ltd., Sinopharm Chemical Reagent Beijing Co., Ltd., Disodium hydrogen phosphate dodecahydrate Sinopharm Chemical Reagent Beijing Co., Ltd.,

2.2. Method

2.2.1. Determination of double enzyme storage conditions. Selection of storage buffer solutions. Three solutions, i.e. disodium hydrogen phosphate (Na₂HPO₄) and citric acid (C₆H₈O₇) buffer solution (PCB), disodium hydrogen phosphate (Na₂HPO₄) and sodium phosphate (NaH₂PO₄) buffer solution (PBS), and disodium hydrogen phosphate (Na₂HPO₄) and monopotassium phosphate (KH₂PO₄) buffer solution (PBS), were used to prepare the buffer solutions with a pH value at 8.0 respectively. The effect of buffer solution on the stability of HRP and GOX was studied by UV-Vis spectrophotometry with oxidation of TMB by HRP and GOX as the experimental system and the initial reaction rate as the test parameters.

Selection of the pH value of the buffer solution. Buffer solutions were prepared with different pH values at pH 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 respectively, with accurately weighed certain amounts of GOX and HRP dissolved in the prepared buffer solutions. The activity of the enzyme was measured after a week of storage.

2.2.2. Determination of the optimum parameters of TTI. The enzyme and substrate concentrations were adjusted separately to obtain the most noticeable color change, enabling the system to get the most obvious color change at different temperatures, selecting the appropriate enzyme and substrate concentrations for the TTI system.

Determination of Glu concentration. 0.025 mg/ml, 0.25 mg/ml, 2.5 mg/ml and 25 mg/ml Glu solutions were prepared, and added to 4 clean 5 ml centrifuge tubes according to the ratio determined in the above experiment for 15 min reaction at room temperature after even shaking, then 10% sulfuric acid solution was added to terminate the reaction, and the absorbance at 450 nm was measured. Three groups of parallel experiments were conducted for each concentration.

Determination of TMB concentration. TMB solutions with concentrations at 0.025 mg/ml, 0.05 mg/ml, 0.075 mg/ml, 0.1 mg/ml, 0.25 mg/ml and 0.5 mg/ml respectively were prepared (high concentration initial solution can be prepared with anhydrous ethanol, and low concentration ones can simply be diluted with deionized water), and added in sequence to 6 clean 5 ml centrifuge tubes according to the concentration ratio determined in the above experiment for 15 min reaction at room temperature after even shaking, then 10% sulfuric acid solution was added to terminate the reaction, and the absorbance at 450 nm of each tube was measured. Three groups of parallel experiments were conducted for each concentration.
2.2.3. **Determination of HRP concentration.** HRP solutions with concentrations at 1 μg/ml, 5 μg/ml, 10 μg/ml, 15 μg/ml and 25 μg/ml were prepared respectively (the original enzyme solution to be stored at low temperature), and added in sequence to 5 clean 5 ml centrifuge tubes according to the concentration ratio determined in the above experiment for 15 min reaction at room temperature after even shaking, then 10% sulfuric acid solution was added to terminate the reaction, and the absorbance at 450 nm of each solution was measured. Three groups of parallel experiments were conducted for each concentration. The above experiment was repeated at 30°C. (Substrate solution and enzyme solution need to be preheated for 5 min).

2.2.4. **Determination of COX concentration.** GOD solutions with concentrations at 1 μg/ml, 3 μg/ml, 5 μg/ml, 10 μg/ml and 15 μg/ml were prepared respectively and added in sequence to 5 clean 5 ml centrifuge tubes according to the concentration ratio determined in the above experiment for 15 min reaction at room temperature after even shaking, then 10% sulfuric acid solution was added to terminate the reaction, and the absorbance at 450 nm of each solution was measured. Three groups of parallel experiments were conducted for each concentration.

2.2.5. **The optimum volume ratio between GOX and HRP enzymes.** According to references, the optimum substrate volume ratio was firstly determined as glucose (hereinafter referred to as Glu): tetramethylbenzidine (hereinafter referred to as TMB) = 5:1, then the study on the ratio of glucose oxidase (hereinafter referred to as GOD) and horseradish Oxidase (hereinafter referred to as HRP) was carried out. Certain amounts of GOD and HRP were weighed and prepared as 5 μg/ml (tentative) enzyme solution. 1500 μL Glu (0.5 mg/ml tentative) solution and 300 μL TMB (0.1 mg/ml tentative) solution were added into 5 clean 5 ml centrifuge tubes, then the prepared enzyme solution was added into the tubes according to the volume ratio GOD: HRP = 1:1, 3:1, 1:2, 1:3 respectively, with the sequence from Glu to TMB, HRP and GOD. After reaction at room temperature for 15 min, 10% sulfuric acid was added to terminate the reaction and the absorbance was measured at 450 nm. Three groups of parallel experiments were conducted for each ratio.

3. **Determination of the optimum conditions for the TTI enzyme reaction**

3.1. **Effect of temperature on the enzyme reaction system**

33 pieces of clean 5ml centrifuge tubes were arranged in groups for 3 pieces each and numbered from 1-11, corresponding respectively to the reaction time of 2 min, 5 min, 8 min, 10 min, 15 min, 20 min, 25 min, 30 min, 40 min, 50 min, 60 min. Based on the optimal reaction concentration determined in the aforesaid experiment, certain amounts of substrate solution and enzyme solution were prepared, and then the same were added to 33 centrifuge tubes respectively in accordance with the best reaction ratio, shaking evenly and placing in ice water mixture (0°C) for reaction. After reaching the reaction time, they were taken out and placed in the cuvette for full wavelength scanning until the completion of all reactions. It has repeated the above steps at 4°C, 20°C (room temperature), 30°C, 40°C, and compared the results of similarities and differences.

3.2. **Effect of pH value on the enzyme reaction system**

1 L of 0.2 mol/L sodium dihydrogen phosphate mother solution and 1 L of disodium hydrogen phosphate mother solution were prepared respectively for the preparation of the buffer solutions with different pH values.

Buffer solutions were prepared with pH=5, pH=6, pH=8 and pH=10 respectively (pH=5 buffer solution can use 0.2 mol/L NaH2PO4 solution directly, buffer solution with pH=10 can get by 0.2 mol/L Na2HPO4 solution dipped with NaOH solution. Based on the newly prepared buffer solutions, substrate solution and enzyme solution under different pH conditions were prepared, and added to a 5ml centrifuge tube in accordance with the best proportion, shaking evenly for full wavelength
scanning at 2 min, 5 min, 8 min, 10 min, 15 min, 20 min, 25 min, 30 min, 40 min, 50 min, 60 min respectively, and the analysis results were recorded.

4. Results and Analysis

4.1. Determination of double enzyme storage conditions

4.1.1. Selection of storage buffer solutions

The experiment found that the type of buffer solution also has some impact on the stability of HRP in solution. As shown in Figure 1A, the absorbance (A) of the product at the initial stage of the HRP catalysis is plotted against time (t) and the slope (dA / dt) of the resulting line is the initial reaction rate k, the size of which represents the catalytic activity of HRP. The dA / dt of oxidation of TMB by H₂O₂ catalyzed by HRP in PBS was larger than that of HRP immobilized in PCB, indicating HRP was more stable in PBS. Therefore, HRP solution should be prepared with PBS for preservation, and the enzyme activities of PBS in sodium and potassium salt are quite similar, there is no much difference.

4.1.2. Effect of buffer solution pH value on the stability of double enzymes

The experiment found that the type of buffer solution also has some impact on the stability of HRP in solution. As shown in Figure 1A, the absorbance (A) of the product at the initial stage of the HRP catalysis is plotted against time (t) and the slope (dA / dt) of the resulting line is the initial reaction rate k, the size of which represents the catalytic activity of HRP. The dA / dt of oxidation of TMB by H₂O₂ catalyzed by HRP in PBS was larger than that of HRP immobilized in PCB, indicating HRP was more stable in PBS. Therefore, HRP solution should be prepared with PBS for preservation, and the enzyme activities of PBS in sodium and potassium salt are quite similar, there is no much difference.

4.1.2. Effect of buffer solution pH value on the stability of double enzymes

Figure 1B. Effect of pH on enzyme stability in solution

(Double enzyme stored in PBS at pH 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 for 7 days)
Enzyme is a valuable reagent, the concentration of the enzyme solution used are generally very small, so each time the amount of preparation cannot be too small, otherwise it will bring greater error. Therefore, the preparation of solution for once should be stored for use for a period of time, and the said stability refers to the change of enzyme activity in the solution. It can be seen from Fig.1B that the HRP catalytic reaction rate was 0 after being kept for 7 days in the solution at pH 2.5 to 5, indicating that the HRP was completely inactivated after being preserved for 7 days in this condition. In a pH range of pH 6.0 ~ 8.0, the initial rate of HRP catalysis reaction increases with the increase of pH value. When the pH reached 8.0, the reaction rate tended to be stable, indicating that HRP is more stable in alkaline PBS. However, HRP is easily inactivated under acidic conditions. The reason may be that the structure of enzyme is relatively stable under alkaline conditions and the enzyme activity is dormant.

4.2. Determination of the optimum parameters of TTI

4.2.1. Determination of substrate GLU concentration

| Serial number | GLU concentration (mg/mL) | Absorbance (A) |
|---------------|--------------------------|----------------|
| 1             | 0.025                    | 0.108          |
| 2             | 0.25                     | 0.15           |
| 3             | 2.5                      | 0.173          |
| 4             | 25                       | 0.24           |

As shown in the Table, the absorbance at 450 nm of the reaction system gradually increased following the increase of the glucose concentration. It was found during reaction that when the GLU concentration was lower, i.e. 0.025 and 0.25 mg/mL respectively, the reaction rate was slightly slower, of which the color change at the concentration of 0.025 mg/ml was the slowest, the color was changing from the initial light blue to brown red and finally to yellow during reaction. When the concentration of GLU was higher at 2.5 and 25 mg/mL, the reaction speed of the system was faster than that at lower than that of the lower concentrations, and the color changed from brown to yellow directly. Therefore, it determined that at a concentration of 0.25 mg/mL, the absorbance was slightly higher, but color change was obvious and the reaction speed was slightly slow.
4.2.2. Determination of substrate TMB concentration

![Graph showing the effect of TMB concentration on absorbance](image1)

**Figure 2A.** Effect of TMB Concentration on the Absorbance of Reaction System

In general, the intensity of the color change is proportional to the chromogenic material concentration when the other conditions remain the same in the chromogenic reaction. Absorbance increased with TMB concentration at 450 nm. However, as the TMB concentration continued to increase, the absorbance decreased, because more TMB has limited solubility in ethanol. When the amount of TMB was larger than its solubility in absolute ethanol, excess TMB would precipitate, therefore the absorption would drop rapidly at 450 nm. Thus, the best TMB concentration was measured at 0.1 mg/mL.

4.2.3. Determination of HRP concentration

![Graph showing the effect of HRP concentration on absorbance](image2)

**Figure 2B.** Effect of HRP Concentration on the Absorbance of Reaction System

According to Figure 2B, the absorbance at 450 nm of the system did not increase with the increase of HRP concentration at either room temperature 20°C or in water bath at 30°C. Because in the case of a determined amount of substrate, the amount of product was also determined. Throughout the reaction, the color changed from the initial light blue to blue-green, then to red-brown and finally to yellow. With the increase of HRP enzyme concentration, the color change gradually accelerated.
When at 30°C, the absorbance value was generally lower than that at 20°C, which might be due to that, the temperature increase caused the decomposition by heat of hydrogen peroxide - the intermediate product produced in the system, leading to the decrease of the product and the decrease of the absorbance of the system. While we could see from the absorbance that when the HRP was at a lower concentration, the system absorbance increased rapidly with the increase of concentration, when the concentration was increased to 0.25 μg/ml, the absorbance value did not increase with the increase of enzyme concentration. In the reaction, however, it was found that when the concentration was at 0.25 μg/ml, the reaction speed of the system was fast, so the time for changing between colors was short, it was not easy to observe, and the enzyme was relatively expensive, considering from the prospect of economy and the effect in the reaction process, enzyme concentrations at 0.1 μg/ml was selected as the best option.

4.2.4. Determination of GOX concentration

According to Fig.2D, the system absorbance value at 450 nm increased somewhat with the increase of GOX concentration, and reached the maximum absorbance value at 5 μg / ml, thereafter the
The absorbance of the system remained basically unchanged, indicating that the amount of enzyme was small when the concentration was at 1 μg / ml, the system reaction was not complete. When the amount of enzyme increased from 1 μg / ml to 3 μg / ml and then increased to 5 μg / ml, the system reaction speed accelerated and the absorbance increased with the increase of enzyme amount. However, when the enzyme amount was increased from 5 μg / ml to 15 μg / ml, the absorbance did not show significantly change but with slight decrease. When the amount of enzyme was at 5 μg / ml, the reaction was fast with rapid color change, the obvious stage color change was hard to be visualized, and in an economic point of view, when there was little difference in absorbance change between the two, it was most suitable to select the enzyme concentration at 3 μg / ml which was with small enzyme amount.

4.2.5. Effect of GOX: HRP (volume ratio) on the reaction

According to the figure, when the GOX: HRP (V / V) was adjusted from 1:1 to 3:1, following the increase of glucose oxidase concentration, the absorbance at 450 nm was gradually increased. When the V / V was 2:1, the maximum absorbance value reached. When the GOX: HRP ratio was further increased to 3:1, the absorbance value decreases somewhat. Therefore, it was determined that V / V at 2:1 to be the best response ratio.

4.2.6. Gradient changes of system color at different temperatures

![Figure 3A. The System Color of Glu, TMB, HRP](image)
Figure 3B. The System Color after 1 min of Glu, TMB, HRP, GOX

Figure 3C. The System Color after 5 min of Glu, TMB, HRP, GOX

Figure 3D. The System Color after 10 min of Glu, TMB, HRP, GOX
During the experiment, the system showed obviously different color changes under different temperature conditions, which were changed from light blue and green when first exposed to bi-enzyme and double substrates respectively to light yellow, light red and yellow. Under low temperature conditions, enzyme activity was limited, the enzyme reaction process in the system was slow. For example, the system reaction was slow under 0°C, the color was in light blue all the time. When the temperature was at 4°C, the reaction was faster compared to that under 0°C condition. After enzyme was added for 5 min, the color turned from light blue to green, gradually to dark green and finally to brown yellow. While under 20°C, the reaction was faster than under 4°C, the system color turned directly from light blue to brown yellow and then light red. When under 30-40°C with relatively higher enzyme activity, the reaction was faster, the system color swiftly passed green and turned to brown yellow and finally yellow during reaction.
4.3. Determination of the optimum conditions for the TTI enzyme reaction

4.3.1. Optimum temperature for GOX and HRP reaction system

The table showed that at different temperatures, the reaction absorbance values were different, the absorbance increased with the increase of temperature. Absorbance at 30°C was rather abrupt, and the absorbance at 40°C was further increased than that at 20°C. The color was also the visible blue but somewhat decreased than under 30°C, indicating that the optimum temperature for the bi-enzyme was about 30°C. The whole reaction system appeared blue after adding bi-enzyme and double substrates, and the color of reaction system deepened. The reaction was done at different temperatures respectively for 40 minutes, the overall reaction color was deepened, at the optimum temperature, the color change was the most obvious, the color change also reflected the changes in absorbance.

**Figure 4.** Changes of Reaction System Absorbance (A) and Color (B) at Different Temperatures (from left to the right 0, 4, 20, 30, 40°C respectively) after 45 Minutes
4.3.2. The optimum pH of GOX and HRP reaction system

As shown in Figure 5, when pH was at 5, the enzyme activity was the highest. Therefore, when pH was at about 5, it was suitable for the reaction of the bi-enzyme system. Regarding the principle that the pH value of the phosphate buffer solution affects the sensitivity of the TMB reaction, it was thought that this was reflected on two aspects: on one hand, the pH affected the weak electrostatic interaction between the chromogen (TMB) and the amino acid component of the HRP during TMB reaction, and thus affecting the combination of chromogen and HRP, further affecting its oxidation. On the other hand, it was speculated that the oxidized TMB polymer might remain stable in an acidic environment and soluble in water due to the formation of polar groups in a neutral or slightly acidic environment.

Figure 5. Effect of pH Value of Buffer Solution on Enzyme Activity

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References
[1] Shen L, Xu Y, Zhan JC, Zhu YF, Wang J, Wang HS. Application and research progress of smart labels in food packages. Science and Technology of Food Industry. 05: 377-383 (2015).
[2] Zheng GG, Qian J, Feng Q. Preparation of glucoamylase TTI reaction system. Food Sci. 12: 82-85 (2013).
[3] Bobelyn E, Hertog M L, Nicolaï B M. Applicability of an enzymatic time temperature integrator as a quality indicator for mushrooms in the distribution chain. Postharvest Biol Tec. 42(1): 104-114 (2006).
[4] Rani DN, Abraham T E. Kinetic study of a purified anionic peroxidase isolated from Eupatorium odoratum and its novel application as time temperature indicator for food materials. J Food Eng. 77(3): 594-600 (2006).
[5] Tucker GS, Brown H M, Fryer P J, et al. A sterilisation time–temperature integrator based on amylase from the hyperthermophilic organism Pyrococcus furiosus. Innov Food Sci Emerg. 8(1): 63-72 (2007).
[6] Kim K, Kim E, Lee S J. New enzymatic time - temperature integrator (TTI) that uses laccase. J Food Eng. 113(1): 118-123 (2012).
[7] Cai HW, Ren FZ, Zhang HT et al. Development of Amylase Time-Temperature Indicator. Food Sci. 27(11): 60-63 (2006).

[8] Wan ZG, Chen YZ, Zhang ZJ. Application of time - temperature indicator in vaccine transport packaging. Packaging Engineering. 37(3): 100-102 (2016).

[9] Lu LX, Cai Y, Zheng WZ. Preparation Method and Application of Time-Temperature Indicator based on Lipase Reaction Diffusion: CN 102175677 B [P]. 2012.

[10] Zhou GH, Ning P, Zhang N, et al. Indication method and product of alkaline lipase type time and temperature: CN, CN 101349599 A [P]. 2008.

[11] Wu QM. Research on the development of Shelf Life Indication System by the Application of Urease. Master’s thesis. Hangzhou: Zhejiang University (2005).

[12] Chen PC. Synthesis study of chiral benzyl sulfoxide catalyzed by co-immobilization of glucose oxidase and horseradish peroxidase. Zhejiang University of Technology. (2010).