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Enzymatic time temperature indicators: A review

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Abstract
Due to budding demand for safety, quality and convenience of food products, intelligent packaging particularly enzymatic Time Temperature Indicators (TTI) are in demand since decades due to irretrievable and distinct colour change properties. The colour change in TTI seems to have good correlation with physicochemical and microbiological parameters. Number of enzymes *viz.* amylase, lipase, laccase, urease, etc. has been used for different categories of food to monitor the quality and safety during supply chain as a result of thermal abuse conditions. These enzymatic TTIs have specific kinetic energies and activation energies (Ea) and can be used for various categories of food systems by manipulating the concentration of substrate and enzymes.

Keywords: Intelligent packaging, time temperature indicator, enzymatic, thermal abuse

1. Introduction
In the era of fast altering regime and the increased demand for triflingly processed foods, studies for intelligent/smart packaging of foods had gained admiration. The intelligent packaging system is one of the innovative techniques which have been used since decades for quality and safety pledge of the foods systems, a social concern for healthy and responsible consumers. These packaging strategies will monitor the conditions of packaged foods to give information about shelf life and the quality of the food during transport and storage conditions. To accomplish the desired task, indicators/integrators and sensors (easy to use small/simple devices) are used instead of measuring because of time consuming, expensive quality parameters for determining the shelf life and food quality. The increased demand for quality food has augmented consumer anticipations, leading to a grander significance for retaining quality between production and consumption [1].
Nowadays, peoples are impending more and more on monitoring and controlling critical parameters throughout the entire life cycle of food product, instead of focusing on testing and verification of final products [2]. To fulfil the consumers demand of close monitoring of temperature exposure during distribution and an optimized stock rotation in retail level based on the temperature of each product, instead of an often meaningless expiration date, can lead to better control of quality and a significant decrease in food waste. Temperature fluctuations can cause a lessening of the duration of perishable products [3], resulting in an incongruity between the final date shown on the label consumption and legitimate food quality conditions. Time temperature indicator (TTI) can serve as prospective tool for attaining these goals. Time temperature indicators (TTIs) are devices used for recording thermal antiquity and specifying the remaining shelf life of perishable food products during their storage, supply and consumption. A TTI can be defined as a small measuring device that shows a time temperature reliant, easily, accurately and precisely measurable irretrievable change that impersonates the changes of a target attribute undergoing the same variable temperature exposure [4]. In others words, TTI is a simple, cost-effective, and consumer friendly device for monitoring, recording, and translating quality information for consumers [5]. This can be achieved by the irreversible change of colour resulting from the collective effects of time and temperature. Furthermore, it can display remaining shelf life information of food [6].

2. TTIs available for food industry
Time temperature indicators have mainly been applied to reflect the time temperature exposure of chilled and frozen food products such as marine products [7, 8], horticultural products [9], dairy products [10], and meat and poultry products [11]. Furthermore, TTIs have also been applied to assess the pasteurization and sterilization process [12], and to estimate the remaining

shelf life of food products [2]. In general, to successfully apply a TTI to a food product, the activation energies (Ea) of both food changes relating to shelf life and TTI's response to temperature must be similar [13].

3. Enzymatic Time Temperature Indicators

Enzymatic TTIs are based on enzyme chemistry and have many benefits over other types of TTIs due to unwavering performance, low production cost and easy control. Ramstad and Volz developed the first enzymatic indicator based on the reaction between an oxidizing enzyme and a colourless phenolic compound [14]. An enzymatic TTI enables the development of a TTI with various activation energy values, as it can be used by conjoining different enzymes and substrates. Thus, it has an advantage of being applied as a tailored type of TTI for various food products undergoing lot of quality changes during supply chain. Its working principle is to produce an acid/base by enzymatic hydrolysis to increase or decrease pH value and then to dynamically display the cumulative effect of time and temperature by colour change of an acid–base indicator. The system can be calibrated to match the rate of spoilage of a designate product by altering several parameters: enzyme type and concentration, substrate type and concentration, presence of activators (e.g. coenzymes) or inhibitors, pH and presence of a buffer. Theoretically, it is possible to produce indicator devices whose response life ranges from a few minutes to several years. Various enzymatic TTIs using lipase, esterase, β-glucosidase, amylase, or laccase were developed based on enzymatic hydrolysis or conversion of their substrates [15].

3.1 Types of enzymatic TTIs

TTIs can be classified in terms of working principle, type of response, origin, application in the food material and location in the food [16]. Depending on the working principle, TTIs are mainly classified as biological, chemical, physical, diffusion based, enzymatic, polymeric, solid state reaction, photosensitive and microbial [13]. The first commercially available TTI was developed by Honeywell Corporation (Minneapolis, MN) and was described in detail by Renier and Morin [17].

3.1.1 Amylase based enzymatic Time temperature indicator

An amylase based enzymatic TTI involves the reaction between amylase and starch to form a clathrate compound, whose colour change was according to the degree of polymerization and the relative molecular weight; colour changes from blue to colourless with a continual decrease in degree of polymerization from 34.7 to 30.8 [18]. Usually, TTIs uses amylases from bacterial sources such as *Bacillus amyloliquefaciens* or *licheniformis* and the feasibility of extending its useable range upwards into sterilisation temperatures was demonstrated by drying amylases to precise moisture levels [19]. The new amylase based enzymatic TTI containing 60% of amylase was developed for monitoring quality during the poultry supply chain [20]. An amylase type TTI had also been used for enzymatic validation of pasteurization of meat products [1]. Grönqvist *et al.* developed an amylase TTI based on inactivation of α-amylase to assess the thermal pasteurization of fried fish burgers [21]. An isolated extrinsic enzyme based time temperature integrator (TTI) was developed based on reaction enthalpy measurement of heat inactivation of *Bacillus amyloliquefaciens* α-amylase to monitor in-pack pasteurization processes [22]. Raviyan *et al.* developed an amylase based TTI for validitation of dielectric pasteurization process by immobilizing α-amylase enzyme obtained from *Aspergillus oryzae* in polyacrylamide gel in phosphate buffer, mashed potatoes, and minced shrimp [23]. For the immobilization of α-amylase obtained from *Bacillus licheniformis* on porous glass beads, three linking agents *viz.* glutaric dialdehyde, benzoquinone and s-trichlorotriazine were used for amylase based TTI [24]. The shelf life of chilled fresh pork could be indicated through the colour evolution of the TTI from mazarine (safety) to colourless (spoilage). The solid-state glucoamylase TTI can be applied to more time-temperature–sensitive food products by changing the formulation [25].

3.1.2 Laccase based Time temperature indicator

A TTI based on laccase enzyme with guaiacol as substrate has been developed which upon oxidation changes its colour from colourless to brown [26]. The main components of the system are laccase solution, guaiacol (substrate for laccase), bovine serum albumin (enzyme stabilizer) and sodium acetate buffer (pH=5). Laccase oxidizes guaiacol causing discolouration to indicate the cumulative time temperature exposure history of the food products. If laccase is applied to enzymatic TTI, there will be some advantages such as: (1) the possibility of developing a TTI with a wide range of activation energy. Differences in the oxidation rate of various substrates are reported to be attributed to redox potential differences between laccase and substrates [27]. The colour response to time–temperature variation, especially the hue value, was linearly proportional to the rate of oxidized guaiacol production, allowing direct and intuitive determination of the remaining shelf-life of food quality losses occurring within Ea in the range of 18.9 - 71.9 kJ/mol.

Park *et al.* adjusted Ea of laccase based TTI by using sodium azide, which has an inhibitory effect on laccase. The relationship between Ea of TTI and sodium azide concentration was measured, so a series of TTI can be designed to match the rates of spoilage of different foods [28]. The higher sodium azide quantity leads to a higher Ea of the system, and the Ea of the system could be expanded to 48–110 kJ/mol with sodium azide.

3.1.3 Lipase based enzymatic Time temperature indicator

Lipase based TTI shows an irreversible colour change induced by a pH decline resulting from the controlled enzymatic hydrolysis of a lipid substrate [29, 30]. VITSAB TTI was the first commercial TTI based on lipase enzyme. The indicator consists of two separate compartments containing an aqueous solution of a lipolytic enzyme such as pancreatic lipase and another containing the liquid substrate suspended in an aqueous medium and a pH indicator mix [30]. Lipases can be of animal (pancreatic, hepatic and gastric), microbial (bacterial, fungal and yeast) or vegetable origin, with variations in their catalytic properties [31]. Among bacterial lipases being exploited, those from Bacillus exhibit interesting properties that make them potential candidates for biotechnological applications. *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus coagulans*, *Bacillus stearothermophilus*, and *Bacillus alcalophilus* are the most common bacterial lipases. In addition, *Pseudomonas* sp., *Pseudomonas aeruginosa*, *Burkholderia multivorans*, *Burkholderia cepacia*, and *Staphylococcus caseolyticus* are also reported as bacterial lipase producers. Most

commercially important lipase producing fungi are recognized as belonging to the genera *Rhizopus sp.*, *Aspergillus sp.*, *Penicillium* sp., *Geotrichum sp.*, *Mucor sp.*, *and Rhizomucor sp* [32]. *Burkholderia cepacia* lipase displays high stability at different temperatures and pHs and can tolerate various organic solvents [33]. The range of uses is because lipase shows activity with regard to hydrolysis, esterification, transesterification, interesterification, alcoholysis and acidolysis [34]. As substrates of lipase, various triacylglycerols such as tricaproin, tripelargonin, and tributyrin can be used [29]. The short-chain tributyrin (TG(4:0) substrate offers several advantages as a substrate for lipases compared to natural long-chain TGs. It is readily dispersed without the need for emulsifiers like gum Arabic used with olive oil, the products formed on hydrolysis are water-soluble and can be titrated directly in a large range of pH [35]. The use of tricaprylin (TG(8:0)) as a totally insoluble medium-chain TG substrate is thus more appropriate to detect and assay a true lipase activity as demonstrated with various microbial and mammalian lipases [36]. When using these substrates, it is typically difficult to obtain stable substrate emulsion at low temperature, and thus selection of proper emulsifier is critical in the process [37].

Wu *et al.* developed an enzymatic time temperature indicator based on the reaction between *Aspergillus niger* lipase and glycerol tributyrate [38]. TTIs ranged from 31.60 to 42.12 kJ/mol with calculated activation energies, would be suitable for fresh products in accordance with the Ea of 31.60 to 42.12 kJ/mol, for example, some fruits and vegetables some fish and shell fish, that quality losses due to lipid oxidation, enzymatic reactions, etc. The lipases obtained from the genus *Aspergillus* have remarkable importance in biotechnological applications [39]. Glycerol tributyrate is usually used for lipase activity determination and with the application of glycerol tributyrate as a test substrate, the lipase is more stable and its activity is two or three times higher than with glycerol trioleate and olive oil [40]. An alkaline lipase TTI based on the reaction between alkaline lipase and glycerol tributyrate has been developed [41]. Additionally, developing a TTI using this lipase would be economical and could be considered a great commercial utilization value, because this lipase is secreted outside of the cell and is able to create various catalytic reactions in liquefied or non-liquefied states.

3.1.4 Urease based enzymatic Time temperature indicator

A TTI based on urease was developed whose colour change depends on the increase in pH while hydrolizing carbamide [42]. The main components of the system were urea substrate, phenol red indicator, urease solution and disodium hydrogen phosphate–potassium dihydrogen phosphate as a buffer solution. The buffer solution is added in order to ensure catalytic reaction occurs in a certain range of pH. Urease catalyses the decomposition of urea to produce ammonia, which causes pH to change and consequently brings about the colour change of the phenol red indicator.

4. Miscellaneous enzymatic Time temperature indicator

Son *et al.* developed enzymatic TTI based on the reaction between porcine esterase and tripropionin, which causes a pH decline that leads to change in colour of the pH indicator from blue to light green [43]. A new enzymatic TTI based on enzyme reaction, diffusion and enzyme demobilization technology is developed [44]. Another novel TTI using phospholipid-phospholipase was developed to monitor quality change of frozen foods during storage [37].

Gogou *et al.* developed an enzymatic TTI prototype that can be used in the temperature range of 100–130 °C, and it is suitable for the monitoring of sterilization processes, such as milk pasteurization [40]. The TTI prototype takes advantage of xylanases, whose thermostability can be improved at a low level of water activity. These xylanase solutions are prepared by *Thermomyces lanuginosus*, which belongs to the thermophilic fungus. The thermophilic fungus grows optimally at a temperature of 48–52 °C and can secrete a variety of thermozymes including xylanases.

Rani and Abraham reported a method to extract and purify peroxidase from *Eupatorium odoratum*, which belongs to the Asteraceae family [45]. The enzyme is an anionic peroxidase isoenzyme with an optimum pH of 4.5. Its optimum temperature is 55 °C, so it is very stable at room temperature and maintains more than 90% activity even after a period of 2 months and is even stable for more than 6 months at 4 ± 1 °C without other additives. An enzymatic TTI prototype was developed on the basis of this peroxidase. The TTI consists of three layers. The upper layer is H_2O_2, which serves as the substrate; the middle zone is solid oil, which plays a role as the partition segment; and the enzyme and 2,2′- azinobis-(3-ethyl benzthiazoline-6-sulfonic acid) make up the lowest layer. The metal ions such as Hg^{2+}, K^+ and Ca^{2+} were found to increase the activity of this enzyme. From a practical point of view, a series of TTI can be derived from the prototype by adding metal ions into it. Qian *et al.* developed a glucoamylase-type TTI in 2011, in which glucoamylase catalyses the hydrolysis of dextrin and an iodine solution works as the indicator [46]. The cumulative time–temperature effect is indicated by the extent of colour change. Similarly, CheckPoint™ is an acid–base reaction-based commercial enzymatic TTI developed by Vitsab (Vitsab A.B., Sweden).

5. Conclusion

Although a countless number of researches on enzymatic TTI have been carried out by research institutions and many patents are granted all around the world, commercially available TTI are very few. Therefore, to develop easy-to-read enzymatic TTI with a stable performance for commercial application is the goal in future research, which can be achieved in four ways as follows. First, it is necessary to develop different kinds of enzymes that can be applied to TTI. Second, expand the application scope of the enzymatic TTI by utilizing the feature of easy control of enzymatic TTI. The reaction rate and apparent active energy Ea of enzymatic TTI can be adjusted by changing concentrations of substrate or enzyme solution to expand the scope of TTI. Third, it is feasible to change the TTI system performance by altering formulations of coenzymes, inhibitors and buffers. Based on this principle, a number of TTI systems can be designed to suit certain food products. Last but not least, it is necessary to intensify applied research in the areas of expanding calibration range, sharpening the chromatic change and avoiding the influence of parameters except time and temperature.

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