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Preparation of a Microbial Time-temperature Indicator by Using the Vegetative Form of *Bacillus amyloliquefaciens* for Monitoring the Quality of Chilled Food Products

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Abstract

Background and Objective: Time-temperature indicators are used in smart packaging, and described as intelligent tools attached to the label of food products to monitor their time-temperature history. Since the previous studies on microbial time-temperature indicators were only based on pH-dependent changes, and they were long-time response indicators, in the present work, a new microbial time-temperature indicator was designed by using the alpha-amylase activity of *Bacillus amyloliquefaciens* vegetative cells.

Material and Methods: The designed time-temperature indicator system consists of *Bacillus amyloliquefaciens*, specific substrate medium and iodine reagent. The relation of the time-temperature indicator' response to the growth and metabolic activity (starch consumption and production of reduced sugars) of *Bacillus amyloliquefaciens* was studied. In addition, the temperature dependence of the time-temperature indicator was considered at 8 and 28˚C. Finally, in order to adjust time-temperature indicator endpoint, the effect of the inoculum level was investigated at 8ºC.

Results and Conclusion: In the designed system, a color change of an iodine reagent to yellow progressively occurs due to the starch hydrolysis. The effect of the inoculum level showed the negative linear relationship between the levels of *Bacillus amyloliquefaciens* inoculated in the medium and the endpoints of the time-temperature indicators. The endpoints were adjusted to 156, 72 and 36 hours at the inoculum levels of 10^2, 10^4 and 10^6 CFU ml^{-1}, respectively. The main advantages of the time-temperature indicator is low cost and application for monitoring the quality of chilled food products.

Conflict of interest: The authors declare no conflict of interest.

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1. Introduction

An essential parameter for monitoring the quality of packaged chilled products is temperature. Time-temperature indicators (TTIs) are defined as simple, affordable and accessible devices attached to the package of food products, which interpret the overall impact of temperature and time on the quality of the products. According to the guidelines of protecting perishable foods, TTIs can be designed such that manufacturers, distributors and customers will be able to check quickly these products for proper transportation and storage conditions [1]. In these TTI systems, extreme exposure to prolonged or heightened temperature exposure is recorded, usually calorimetrically. The primary requirement for design and manufacture of TTIs is a visual continuous irreversible change, which increases with temperature [2,3]. Based on the working principles, TTI systems are classified as chemical, biochemical, enzymatic, physicochemical, biological or microbiological systems [2,3].

Some Commercially available TTIs are based on the principles of (a) molecular diffusion (the 3M Monitor Mark indicator), (b) polymerization reactions (the Lifelines Fresh-Check and Freshness Monitor indicators) and (c) enzymatic changes (the Vitsab TTI) [2,4,5]. Since microbial TTI systems' response is directly related to the bacterial growth and metabolism, they take an advantage among all TTI types. Up to now, three types of marketable microbial TTIs have been developed by Cryolog (Traceo, Traceo restauration, and eO) based on the growth and metabolic activities of patent microbial strains (according to pH dependent changes) [2,4,5].

Since the previous studies on microbial TTIs were only based on pH dependent change and they were long-time response indicators, the present work aimed to design a microbial TTI by combining the microbial growth and enzymatic activity of the vegetative form of *Bacillus* (*B.*) *amyloliquefaciens*. The purposes of this research were to investigate the relation between the TTI response (color change) and the growth and enzymatic activity (starch consumption and production of reduced sugars) of *B. amyloliquefaciens* in the TTI substrate, and to evaluate our ability to adjust the TTI endpoint according to the recommended shelf life of the food product of concern by changing the inoculum level of *B. amyloliquefaciens*.

2. Materials and Methods

2.1. Preparation of bacterium

B. amyloliquefaciens PTCC 23350 was prepared from the Persian type culture collection.

2.2. Preparation of bacterial suspension

Bacterial suspension was prepared according to [6-8]. In brief, a fresh pre-culture of the bacterium in nutrient broth was added to the nutrient broth plus yeast extract (3 g l^{-1}) and grown at 37°C with 200 rpm shaking for an overnight. Bacterial cell was harvested by centrifugation at 5000 rpm for 20 min. The pellet was washed with physiological NaCl (0.9 %) solution and re-suspended in distilled water.

2.3. Preparation of the TTI system

An introductory study was carried out to recognize a proper combination of the components of the designed TTI system (the suitable bacterial strain, the medium, and the chemical reagent). *B. amyloliquefaciens* was selected among several *Bacillus sp.* such as *B. cereus*, *B. subtilis* ATCC 12711, *B. pumillus* M13 and *B. pumillus* Z17 based on the high ability of starch hydrolysis on agar medium supplemented with soluble starch. The substrate medium consisted of Basal Saline Medium (BSM) plus Soluble Starch (BHD Co.). Typical iodine reagent and povidone iodine were tested as reagents to be added to the medium at the time of TTI-response observation.

In order to prepare a bacterial based TTI, the growth curve along with the enzymatic activity of the bacterium in the TTI medium was investigated. In the next stage, in order to achieve the desired inoculum level, the prepared bacterial suspension was properly diluted by normal saline, and a volume of the proper dilution was inoculated into the final medium. For the experiments on the effect of inoculum size, the inoculum level used ranged between 10^2 and 10^6 CFU ml^{-1}, whereas for the study of the effect of temperature on the TTI response, the initial inoculum level in the growth medium was adjusted to 10^6 CFU ml^{-1} [4,5].

The response of the TTI system was verified at two distinct storage temperatures (8 and 28°C) using low-temperature, high-precision (±0.2°C) incubators at an initial inoculum level of 6 log CFU ml^{-1}. The samples were tested in duplicate at proper time intervals to allow for the efficient analysis of microbial growth and enzymatic activity (starch consumption and production of reduced sugars) simultane-ously as described below at each tested temperature [4,5].

2.4. Microbiological analysis

Samples of the TTI system were analyzed at different time intervals for plotting the growth curve. Volume of 1 ml of the medium was transferred aseptically into cuvette and read by spectrophotometer at 600 nm. Some samples were randomly streaked into the nutrient agar plate and observed for morphological characteristics of the colony types. They were also checked for possible contamination.

2.5. Biochemical analysis

The relation of the TTI response (color change) to the growth and metabolic activity (starch consumption and production of reduced sugars) of *B. amyloliquefaciens* was studied. In order to obtain cell-free samples, 1 ml of each sample was centrifuged (Sigma, Germany, 4°C, 3354 ×g, 5 min) at different time intervals. The prepared samples were measured by Spectro-photometer (Shimadzu, Japan) for determination of the remaining starch and production of reduced sugars in the medium with iodine reagent and DNS (3,5-Dinitrosalicylic acid), respectively [3,9-11].

2.6. Adjustment of the endpoint of the TTI system

With the purpose of relating the endpoint of the TTI system with the initial level of the inoculated micro-organism, the effect of different inoculums levels on the TTI response at 8°C was studied. *B. amyloliquefaciens* was inoculated in the TTI medium at different levels (2, 4 and 6 log CFU ml^{-1}), and the tests were carried out based on the procedures explained above [4,5].

2.7. Statistical analysis

The data were assessed using the analysis of variance (ANOVA) at $p \leq 0.05$ level of significance using the SPSS software (ver. 22). Graphs were plotted by Excel 2016, and all of treatments were done in triplicate.

3. Results and Discussion

The selection of *B. amyloliquefaciens* because of showing more extensive zone of clearance due to high ability of starch hydrolysis on the starch agar medium was in accordance with the screening results of Deb et al. [12].

In contrast to typical iodine reagent, povidone iodine exhibited an intense, distinct, and approximately irreversible color change after being added to the medium. So, in this study, typical iodine reagent and povidone iodine were used for quantitative and qualitative proposes, respectively.

Previous studies on microbial TTIs focused on the acidification of the TTI medium by selected lactic acid

bacteria, which induced a color change in the indicator [2-5]. Moreover, a new enzymatic-type TTI was recently developed based on the enzymatic hydrolysis of starch by an α-amylase [13]. In this research, the combination of the specific properties of previous studies (bacterial growth and enzymatic activity) was used to create a new microbial enzymatic TTI. The microbial TTI system consists of *B. amyloliquefaciens*, specific substrate medium and iodine reagent. Figure 1 shows the growth curve and starch consumption of *B. amyloliquefaciens* grown in TTI medium incubated at 28°C. As it can be seen, since the bacterium grown at environmental temperature showed lower growth rate in the TTI medium, it is suitable for selecting as a TTI medium because it allows the bacterium to consume starch slowly depending on the time and temperature. According to the time of sampling and concentration of the remaining starch in the medium, different colors can appear after adding iodine reagent (Fig. 2). The final color change to the distinct yellow happened when the concentration of starch in the medium was below 0.02 g l^{-1}, so this time was accepted as the endpoint of the TTI system.

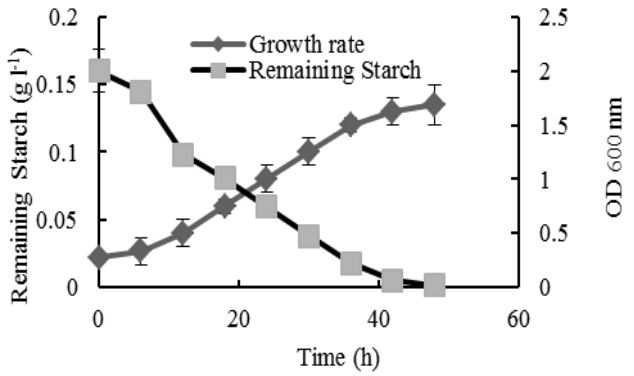

Figure 1. The growth curve and starch consumption of *Bacillus amyloliquefaciens* grown in TTI medium and stored at 28°C.

Figure 2. Reaction of different concentrations of starch with iodine reagent can generate various ranges of color.

Figures 3 and 4 show the changes in the level of starch consumption and production of reduced sugars monitored throughout the storage of the TTI system at 8 and 28°C, respectively by the initial inoculum level of 10^6 CFU ml^{-1}. These figures also indicate the temperature dependence of the TTI response. As shown, in low temperatures, biochemical reactions occur more slowly than higher temperatures; this is mainly due to the low activity of enzyme (here amylase) in low temperatures. However, other studies imply that in low-storage-temperature conditions, ATP requirements increase for the metabolic functions of cell conservation that is strongly growth rate-dependent (e.g., the conservation of proton gradients across the cell membrane, the synthesis of further proteins, and the turnover of macromolecules such as mRNA could all decrease ATP accessibility for growth) [4,14]. It can be concluded that at low growth rates, biomass formation becomes energetically more expensive (e.g., higher protein content of cells), resulting in yield decrease, and consequently, low protein (enzyme) production [15].

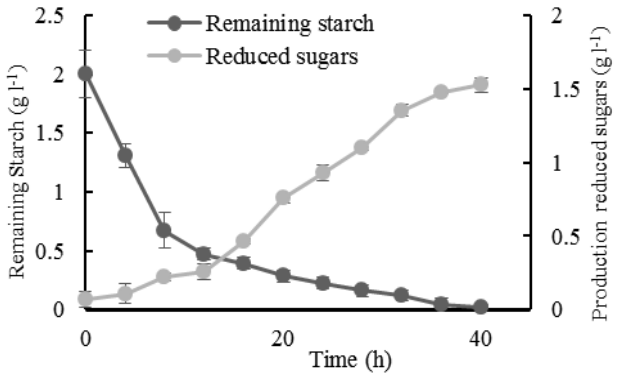

Figure 3. Biochemical changes occurring in the TTI system by the initial inoculum level (10^6 CFU ml^{-1}) stored at 8°C. The experiment was carried out in triplicate.

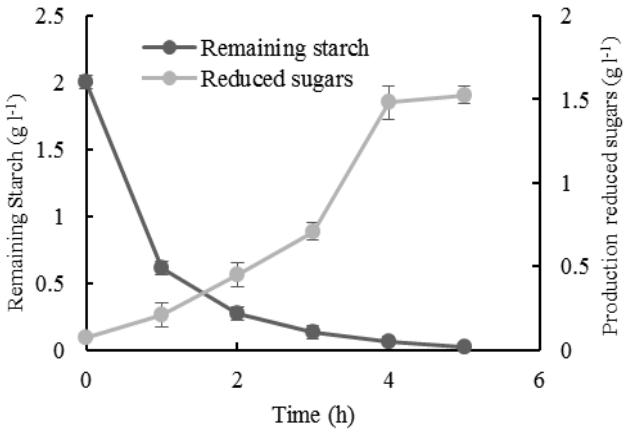

Figure 4. Biochemical changes occurring in the TTI system by the initial inoculum level (10^6 CFU ml^{-1}) stored at 28°C. The experiment was carried out in triplicate.

According to previous studies, one of the important requirements that determine the effective application of a TTI is that its endpoint should correspond to the end of the product's shelf life at a chosen reference temperature. The TTI system, as presented above, can be used for monitoring the quality of food products with a shelf life of about 1 week at 8°C. However, the shelf life of products at a certain temperature can vary significantly depending on various factors [16]. Thus, a successful TTI system should provide the ability to easily adjust the endpoint according to the shelf life of the product of concern. So, the initial inoculum level can be set in relation to the end of the shelf lives of many food or medical products stored at 8°C.

Changing the level of the bacterial strain into the TTI system is an alternative approach for the adjustment of the TTI endpoint. In the present work, the effect of different initial inoculum concentrations of *B.amyloliquefaciens* (10^2, 10^4 and 10^6 CFU ml^{-1}) on the TTI response at 8°C was examined. As it was predictable, when the inoculum level was increased, starch consumption occurred at shorter time leading to shorter endpoints. Indeed, the effect of the inoculum level showed a negative linear relationship between the level of *B. amyloliquefaciens* inoculated in the medium and the endpoint of the TTI. In particular, the endpoints were 156, 72 and 36 h at the inoculum levels of 10^2, 10^4 and 10^6 CFU ml^{-1}, respectively.

Figure 5 shows the effect of the initial inoculum level on the starch consumption of the TTI system stored at 8˚C. As it can be seen, the lower is the inoculum level, the longer is the time of starch consumption. In comparison with the results of Vaikousi et al. that developed long-time response microbial TTI [4,5], the designed TTI in this work has shorter response time, which is suitable for monitoring the chilled food products that should preserve at a specific temperature (8˚C) for certain hours or days.

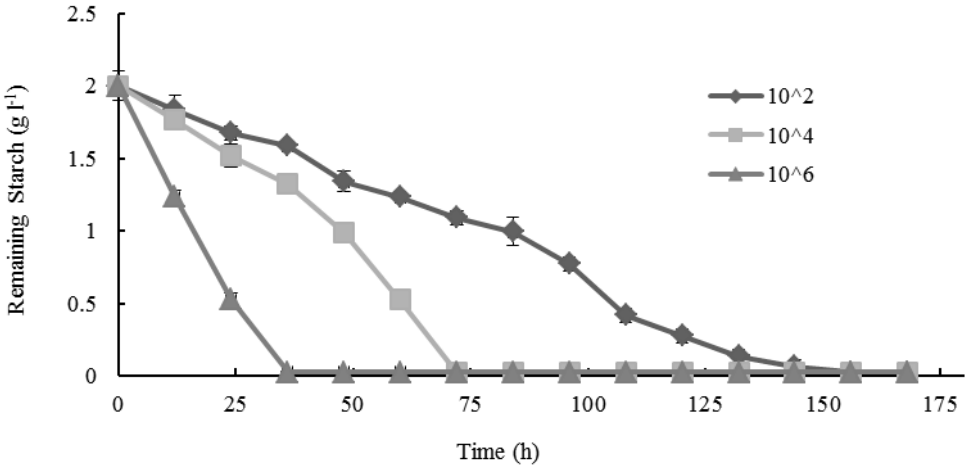

Figure 5. Effect of the initial inoculum level on the starch consumption of the TTI system stored at 8°C.

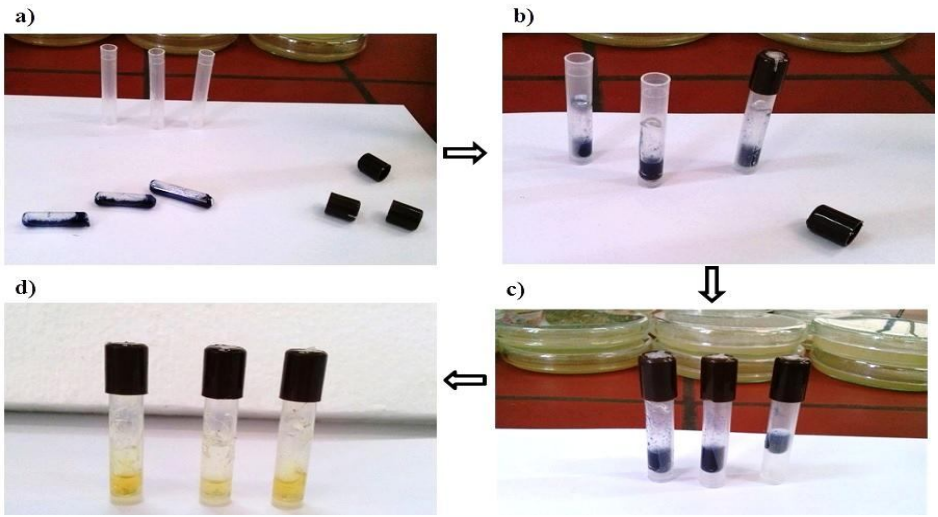

Figure 6. Initial form of the practical TTI: The TTI system contains a plastic tube, an ampoule medium, and a bacterial suspension at the bottom of the plastic tube (a, b). The ampoule medium (TTI medium with iodine reagent) crushes by squeezing the sides of the plastic tube (c). Then it exposes to the bacteria. The response of TTI can be read when needed. If the product is out of the permitted temperature, the TTI color will be yellow (d).

A commercial TTI should have some properties such as being small, integrating easily as part of the product package, being compatible with a high-speed packaging process, activating easily, etc. It also should have a long shelf life before activation [4].

A preliminary work was carried out to convert the designed TTI system presented in this study into a practical TTI product. It can be used with the product of concern, and activation is required before use. As shown in Figure 6, the TTI system contains a plastic tube, an ampoule medium, and a bacterial suspension at the bottom of the plastic tube.

Further research should be taken to investigate the effect of different concentrations of starch on TTI response and preservation of bacteria.

4. Conclusion

The designed microbial TTI in the present work is based on the growth and amylase activity of *B. amyloliquefaciens*. The endpoint at a specific temperature can be adjusted according to the shelf life of the food product of concern by varying the inoculum level of *B. amyloliquefaciens*. Apart from the low cost, the main advantage of the proposed TTI is that its application for monitoring the shelf life of the products during the storage and distribution.

5. Acknowledgements

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6. Conflict of Interest

The authors report no conflicts of interest.

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تهیه نشانگر میکروبی دما-زمان با استفاده از فرم رویشی *باسیلوس آمیلولیکوفسینس* برای ارزیابی کیفیت محصولات غذایی سرد

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چکیده

سابقه و هدف: نشانگرهای دما-زمان در بسته‌بندی هوشمند مورد استفاده قرار می‌گیرند و به عنوان ابزاری متصل شونده به برچسب محصولات غذایی به منظور نظارت بر تاریخچه دما و زمان توصیف می‌شوند. از آنجا که مطالعات قبلی بر روی نشانگرهای میکروبی دما- زمان بر اساس تغییرات وابسته به pH بوده است و زمان پاسخ دهی این نوع نشانگرها طولانی می‌باشد، هدف از این مطالعه، طراحی یک نشانگر دما- زمان میکروبی جدید با استفاده از فعالیت آلفا- آمیلازی سلول‌های رویشی *باسیلوس آمیلولیکوفسینس* می‌باشد.

مواد و روش‌ها: این سیستم نشانگرهای دما- زمان متشکل از *باسیلوس آمیلولیکوفسینس*، محیط کشت سوبسترای اختصاصی و معرف ید می‌باشد. ارتباط پاسخ نشانگرهای دما-زمان با رشد و فعالیت متابولیکی باکتری *باسیلوس آمیلولیکوفسینس* (مصرف نشاسته و تولید قندهای احیاء) مطالعه شد. به علاوه، وابستگی دمایی TTI در دماهای $8^{\circ}C$ و 28 بررسی و سرانجام به منظور تنظیم نقطه پایانی نشانگرهای دما- زمان، اثر میزان تلقیح اولیه در $8^{\circ}C$ ارزیابی گردید.

یافته‌ها و نتیجه‌گیری: در سیستم طراحی شده، در اثر آبکافت نشاسته، به تدریج رنگ شناساگر ید به زرد تغییر می‌کند. اثر سطح تلقیح یک رابطه خطی منفی را بین میزان تلقیح *باسیلوس آمیلولیکوفسینس* در محیط کشت و نقاط پایانی نشان داد. نقاط پایانی به صورت 156، 72 و 36 ساعت به ترتیب در سطوح تلقیح 10^{2}، 10^{4} و 10^{6} $CFU\ m\ l^{-1}$ تنظیم شدند. برتری اصلی این نشانگرهای دما-زمان، هزینه کم و کاربردی بودن آن برای پایش کیفیت محصولات غذایی سرد می‌باشد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ تعارض منافعی وجود ندارد.

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واژگان کلیدی

- فعالیت آمیلاز
- *باسیلوس آمیلولیکوفسینس*
- بسته بندی کوچک
- شناسگر زمان-دما

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