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Empirical manipulation of the thermoinactivation kinetics of *Bacillus amyloliquefaciens* and *Bacillus licheniformis* α-amylases for thermal process evaluations

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ABSTRACT

In an attempt to obtain more accurate and stable time and temperature integrators (TTIs) for the evaluation of commercial pasteurisation process, the thermoinactivation kinetics (D and z value) of amylases contained in the TTIs from two sources, *Bacillus amyloliquefaciens* (BAA) and *Bacillus licheniformis* (BLA) were determined under different TTI conditions (*i.e.*, pH, Ca^{2+}, and enzyme concentration). Under 21 different TTI conditions tested for BAA, D_{70} value is found to vary from 5.9 to 159.9 min, D_{85} value vary from 4.5 to 40.5 min, and z value vary from 6.2 to 30.4°C, respectively; Under 14 different TTI conditions tested for BLA, D_{90} value is found to vary from 2.5 to 9.9 min, and z value vary from 9.3 to 17.4°C, respectively. The thermoinactivation kinetics of TTI has been successfully modified to match the thermoinactivation kinetics of target microorganisms in commercial mild pasteurisation processes through these manipulations. We believe this empirical approach of manipulation has provided practical data and a better understanding of how to obtain desired thermoinactivation kinetics. This makes the enzymes and therefore TTIs better candidates for quantifying the thermal impact on both safety and quality parameters in commercial pasteurisation process.

Industrial relevance: Quantification of the thermal process is of paramount importance to ensure the safety and quality of the heat-preserved food. *Bacillus* amylase-based time and temperature integrators (TTIs) have been developed as an alternative tool for thermal process evaluations in food industries, where conventional thermal sensors can not apply.

Based on the results of this study, it is possible to manipulate the thermoinactivation kinetics of both *Bacillus amyloliquefaciens* and *Bacillus licheniformis* amylases to match the thermoinactivation kinetics of the target microorganisms in commercial pasteurisation process. Manipulation of the D and z value may be required when the supplied enzyme batch changes.

The ability to undertake this manipulation contributes significantly to producing more accurate and consistent time and temperature integrators for its commercial use (*e.g.*, thermal process validation). Moreover, being able to manipulate amylase activity to achieve target D and z values may also make it a potential candidate for the development of TTIs to monitor quality and nutritional markers. Consequently, TTIs can be developed to quantify the thermal impact on quality and nutritional parameters in pasteurised products.

1. Introduction

Pasteurisation is now one of the most extensively used methods for food preservation. The thermal process is used to reduced target microorganisms to an acceptable level, and other hurdles (such as pH, aw, salt, preservatives, storage temperature, and pack atmosphere) are used to suppress the growth of the surviving microorganisms in order to achieve a safe and stable product (Gaze, 2006). Quantification of the thermal process is of paramount importance to ensure the safety and quality of foods. Since conventional evaluation methods have limitations with regard to their applicability in some processes (*e.g.*, continuous processing) (Van Loey, Arthawan, Hendrickx, Haentjens, & Tobback, 1997), time and temperature integrators (TTIs) have been developed as an alternative tool for thermal process evaluations (Grauwet, Plancken, Vervoort, Hendrickx, & Van Loey, 2009; Guiavarc'h, Deli, Van Loey, & Hendrickx, 2002; Hendrickx, Weng, Maesmans, & Tobback, 1992; Mehauden et al., 2007; Tucker, 1999; Tucker et al., 2007;

Abbreviations: TTIs, time and temperature integrators; BAA, *Bacillus amyloliquefaciens* amylase; BLA, *Bacillus licheniformis* amylase; D value, the decimal reduction times, the time required to effect one log reduction in the initial microbial or enzymic population at reference temperature; z value, the temperature change required to effect one log reduction in the rate of microbial or enzymic destruction; *P*-value, lethality, refers to the accumulation of lethal rates during thermal process; Lethal rate, a measure of the lethal effect of heat on microorganisms or enzyme at a given temperature (*i.e.*, reference temperature, denoted as T_{ref}).; MAP, modified atmosphere packaging.

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Tucker, Hanby, & Brown, 2009; Van Loey, Hendrickx, Ludikhuyze, et al., 1996; Van Loey, Arthawan, et al., 1997; Van Loey, Haentjens, Hendrickx, & Tobback, 1997). The term 'TTIs' in this study refers to a small silicone tube containing an enzyme system having similar thermoinactivation kinetics to a target attribute, *e.g.*, microorganisms. In other words, the enzyme responds to the thermal process in a manner that is very similar to the target attribute. By measuring the enzyme activity before and after the thermal process, the delivered lethality (or *P*-value) can be calculated to give an indication of the level of thermal process that the TTIs experienced.

The major advantage of TTIs is the ability to quantify the integrated thermal impact on a target attribute without information on the actual time and temperature history of the product (Van Loey, Arthawan, et al., 1997). TTIs can also be used in large numbers to obtain multiple measuring points and due to the small size, can enable access and to take measurements in many places, even in the most complicated processes (such as steam capping and sachet hot filling) and pack configurations (Luo & George, 2015). TTIs have been successfully applied in a number of batch and continuous thermal processes in the food industry, for example, retorting (Tucker et al., 2007), steam-jacketed vessels (Mehauden, Bakalis, Cox, Fryer, & Simmons, 2008; Tucker, 1999), vaccum pack processing (Tucker, 2004), tubular heat exchangers (Tucker, Lambourne, Adams, & Lach, 2002), ohmic heater (Tucker, 1999), hot-filling process (Luo & George, 2015; Tucker et al., 2009), cook-quench-chill process (Tucker et al., 2009) and high pressure processing (Grauwet, Plancken, Vervoort, Hendrickx, & Van Loey, 2010).

There are specific thermoinactivation kinetic requirements for TTIs to be used as a tool for thermal impact quantification. The temperature sensitivity of the rate constant (z value) should ideally be the same as the target attribute (Hendrickx et al., 1995). This is hard to achieve in practice so a minimal acceptable difference may need to be defined. Acceptable differences in the z value between TTIs and the target attribute should result in a conservative evaluation *i.e.*, underestimation of the thermal process (Van Loey, Ludikhuyze, Hendrickx, De Cordt, & Tobback, 1995). The decimal reduction times (D value), are likely to differ between TTIs and the target attribute. This is because thermal processes are designed to reduce microbiological populations by typically between six and twelve log reductions (Tucker, 2008), whereas it is unlikely that TTIs systems based on colour or enzyme activity changes will have such high log reductions in the measured parameter. Therefore, the D value of TTIs should ideally be several times larger than the D value of the target attribute, to allow a detectable response to the temperature history (Tucker, 2008; Van Loey, Hendrickx, Smout, Haentjens, & Tobback, 1996).

In practice, the enzyme source has an impact on the enzyme thermoinactivation kinetics along with purification and manufacturing processes (such as extraction and drying process). As a result, it is not uncommon to find different batches of nominally the same enzyme have noticeable differences in the thermoinactivation kinetics, even when obtained from the same supplier. Such variation, either from natural or manufacturing sources, can severely hinder the commercial use of TTIs. The practical application of TTIs would be greatly improved by the capability to easily manipulate the enzyme system contained in the TTIs to achieve the desired thermoinactivation kinetics, especially if an enzyme batch is found to behave differently from previous batches.

Considerable efforts have been made to manipulate the thermoinactivation kinetics of *Bacillus* enzymes. The influence of different loading density (De Cordt, Hendrickx, Maesmans, & Tobback, 1994a; De Cordt et al., 1992), different pH and calcium concentration (De Cordt et al., 1992), different hydrophobic solvents (Saraiva, Oliveira, & Hendrickx, 1996), different water content (Guiavarc'h et al., 2002; Saraiva, Oliveira, Hendrickx, Oliveira, & Tobback, 1996), different added sugars (Guiavarc'h, Sila, Duvetter, Van Loey, & Hendrickx, 2003), and different added polyalcohols and carbohydrates (De Cordt, Hendrickx, Maesmans, & Tobback, 1994b) have been studied. However, all of these studies investigated thermoinactivation kinetics under severe thermal processes (typically higher than 100°C), none were applied to the common mild pasteurisation targets shown in Table 1.

In this paper, the thermoinactivation kinetics (D and z value) of amylases from two sources, *Bacillus amyloliquefaciens* (BAA) and *Bacillus licheniformis* (BLA) were manipulated empirically by altering the enzyme system contained in the TTI (*i.e.*, the initial enzyme concentration, calcium concentration, and pH) to match the thermoinactivation kinetics of mild commercial pasteurisation targets. Information in the published literature related to thermoinactivation mechanisms and structural differences between the enzymes from the two sources are discussed.

We believe this empirical approach of manipulation has provided practical data and a better understanding of how to obtain desired thermoinactivation kinetics. This makes the enzymes and therefore TTIs better candidates for quantifying the thermal impact on both safety and quality parameters in commercial pasteurisation process.

2. Materials and methods

α-amylase powder from *B. amyloliquefaciens* source (A6380, lyophilized powder, Type II-A, ≥1500 units/mg protein) and α-amylase powder from *B. licheniformis* source (A4551, lyophilized powder, 500–1500 units/mg protein, >93%) were purchased from Sigma. The enzyme concentration (mg/ml) used in this study refers to mg lyophilized powder per ml of final enzyme solution. Trizma base (T6066, bioperformance certified grade, ≥99.9%), hydrolytic acid (258,148, ACS reagent grade, 36.5–38%), sodium acetate trihydrate (236,500, ACS reagent grade, ≥99%), acetic acid (320,099, ACS reagent grade, ≥99.7%) and calcium chloride dehydrate (223,506, ACS reagent grade, ≥99%) were all purchased from Sigma. Amylase substrate solution (AY1582) was purchased from Randox. AlteSil high strength silicone tubing (01–93-1410, 2.0-mm bore, 0.5-mm wall) was purchased from Altec Products Ltd., Silicone elastomer (Sylgard 170) was purchased from Farnell UK Ltd.

2.1. Heating trials and measurement of enzyme activity

A series of heating trials (at specific times and temperatures) were performed to determine the thermoinactivation kinetics (D_{ref} and z values) of amylases from two different sources, from different batches, and at different levels of pH, calcium and initial enzyme concentration. A 'TTI condition' refers to a specific batch of enzyme at a specified level of pH, calcium and initial enzyme concentration. The objective was to find a specific TTI condition under which the z value of TTIs match the z value of the target microorganisms in the commercial pasteurisation process; and the D value of the TTIs is sufficiently large to encompass the recommended pasteurisation time (see Table 1).

Preparation of buffers, enzymes and the encapsulation in a TTI were as described by Tucker et al., (2009). TTIs were heated in a well stirred water bath (Grant Instruments, Cambridge) for different combinations of times and temperatures (Tables 2a–2c). Three calibrated Ellab Tracksense dataloggers were used to measure the actual temperature in the water bath.

Immersion times were chosen to ensure that the final amylase activity was within 3 log reductions of the initial activity in order to maximise the accuracy of the activity measurement. TTI tubes were held in stainless steel tea strainers (IKEA, UK) whose immersion position was next to the sensor of the dataloggers. Temperature readings were taken every 15 s and an average bath temperature was calculated, having first taken into account any offset in temperature from the most recent probe calibration certificates.

Initially three independent TTIs (replicates) were subject to each combination of heating time and temperature, and six unheated TTIs used to measure the initial enzyme activity. Variation in the enzyme activity after heating between the three independent TTIs exposed to the same time and temperature conditions were small (<10%) and showed