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THE ACCELERATION OF YOGHURT FERMENTATION USING PEPTIDES DERIVED FROM PROTEOLYTIC PRE-TREATMENT OF MILK

Aceleração da fermentação do iogurte usando peptídeos derivados do pré-tratamento proteolítico do leite

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ABSTRACT

In this study, yoghurt fermentation time was accelerated by using the proteolytic enzyme Flavourzyme to produce sufficient peptides. Flavourzyme was added directly to the milk used for fermentation at different concentrations (0.1 g/L, 0.01 g/L, and 0.001g/L), and before heat treatment (90˚C for 15 min) where Flavourzyme was inactivated. The liberation of peptides during Flavourzyme pre-treatment was confirmed by RP-HPLC peptide profiling. It was possible to accelerate the fermentation time by 13.33% with Flavourzyme. The yoghurt made with Flavourzyme pre-treated milk did not demonstrate any significant quality changes. However, consumers were able to detect a difference between yoghurt made with Flavourzyme pre-treated milk and yoghurt made with untreated milk (control). Yoghurt made with Flavourzyme pre-treated milk scored lower in taste, texture, appearance, and overall acceptability, but was generally not rejected. Accelerated fermentation times could lead to economic advantages in the yoghurt manufacturing industry.

Keywords: Flavourzyme; protease; consumer satisfaction.

RESUMO

Neste estudo, o tempo de fermentação do iogurte foi acelerado usando a enzima proteolítica Flavourzyme para produzir peptídeos suficientes. Flavourzyme foi adicionado diretamente ao leite utilizado para fermentação em diferentes concentrações (0,1 g/L, 0,01 g/L e 0,001 g/L) e antes do tratamento térmico (90˚C por 15 min), onde o Flavourzyme foi inativado. A libertação de peptídeos durante o pré-tratamento com

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Flavourzyme foi confirmada pelo perfil peptídico de RP-HPLC. Foi possível acelerar o tempo de fermentação em 13,33% com Flavourzyme. O iogurte feito com leite pré-tratado Flavourzyme não apresentou alterações significativas na qualidade. No entanto, os consumidores conseguiram detectar uma diferença entre o iogurte feito com leite pré-tratado Flavourzyme e o iogurte feito com leite não tratado (controle). O iogurte feito com leite pré-tratado Flavourzyme obteve menor pontuação em sabor, textura, aparência e aceitabilidade geral, mas geralmente não foi rejeitado. Tempos de fermentação acelerados podem levar a vantagens econômicas na indústria de produção de iogurte.

Palavras-chave: Flavourzyme; protease; satisfação do consumidor.

INTRODUCTION

Yoghurt is a renowned and increasingly popular cultured dairy product, which is produced from milk through bacterial fermentation (SHIBY; MISHRA, 2013). The term yoghurt is used to describe milk ferments in the presence of symbiotic lactic acid bacteria comprising *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* (PENNA *et al*., 1997). The fermentation stage is the most essential phase of yoghurt manufacture. Lactic acid bacteria are responsible for several biochemical conversions of natural milk constituents to produce yoghurt. Metabolites produced during fermentation lead to the development of the distinctive flavor and texture of yoghurt (SFAKIANAKIS; TZIA, 2014). The ability of lactic acid bacteria to acidify milk is the most crucial role of these microorganisms in the fermentation process (QUIBERONI *et al*., 2003).

Due to an increase in yoghurt consumption, manufacturing procedures require constant improvement in process technology and starter cultures to keep up with demand. It is, therefore, not surprising that studies have been concerned with the supplementation of milk with the necessary nutrients for lactic acid fermentation (ZHANG *et al*., 2011). For instance, Smith *et al*. (2014) found that yeast extract supplements significantly decreased fermentation time, but resulted in quality compromises, especially flavour. The authors identified components in the yeast extract responsible for the acceleration of lactic acid fermentation and concluded that short peptides were the leading accelerators (SMITH *et al*., 2014).

Zhang *et al*. (2011) examined the influence of casein hydrolysates produced by proteolytic enzymes on the growth and performance of yoghurt starter bacteria in an artificial medium. The authors noted that casein hydrolysates acted as a stimulator and that the ultra-filtrated fraction (<3000 Da) appeared to stimulate yoghurt bacteria. It was concluded that the supplementation with hydrolysis fractions promoted the viability of *S. thermophilus*. In contrast, it did not affect *L. bulgaricus* counts to a great extent and the rise in lactic acid yield was mainly as result of an increase in the metabolism of *S. thermophiles* (ZHANG *et al*., 2011).

This study aimed to liberate peptides (capable of acceleration of yoghurt fermentation) from native milk proteins using varying loads of the commercial enzyme Flavourzyme (EC number: 3.4.11.1). The effect of Flavourzyme derived peptides in the milk used for yoghurt fermentation to reduce fermentation time and impact on the product quality was investigated.

MATERIALS AND METHODS

Preparation of the yoghurt starter culture

A thermophilic freeze-dried commercial starter culture mixture, YF-L812 Yo-Flex (Chr. Hansen, Regal Fruits, 1620, South Africa) was used. The culture was ground to powder before experimentation, and the same batch was used during all experiments. Yo-Flex contained the lactic acid starters *S. thermophilus* and *L. bulgaricus*, which was ideal for a direct vat set (DVS). The final starter culture concentration of 0.104 g/L milk was used during all yoghurt fermentation experiments.

The manufacture of the yoghurt

Yoghurt was manufactured as described by Tamime; Robinson (1996), but with the exclusion of stabilizers. The yoghurt was prepared using the flow diagram in Figure 1 and served as a standard for comparison in all experiments. A control was prepared using only reconstituted milk (pH control).

Triplicate batches of all yoghurt fermentations were prepared for each independent experiment. The term percentage increase was used in this study to refer to the difference in the times taken to reach pH 4.6 between a particular sample and the standard.

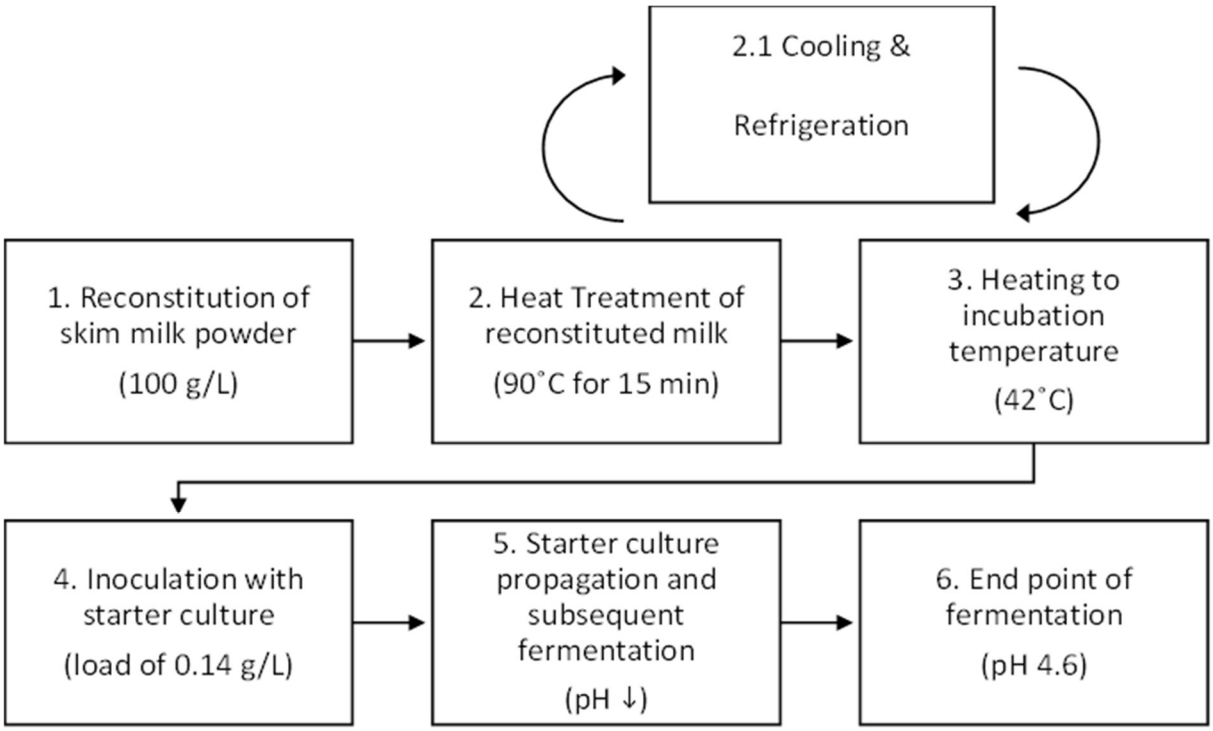

Figure 1. Procedure for the manufacture of yogurt.

Reconstituted skim milk powder with a more constant composition and longer shelf-life than fresh milk was rehydrated and used throughout the study. A 100 g/L solution of skim milk powder (Nestlé, Bryanston, 2021, South Africa), was prepared using distilled water and rehydrated by heating to 50°C for 60 minutes (step 1 in Figure 1). Reconstituted milk was heat treated at 90°C for 15 min (step 2 in Figure 1) before refrigeration until use (step 2.1 in Figure 1). Before the commencement of fermentation, the milk was heated to 42°C in a water bath (step 3 in Figure 1). Once the temperature reached 42°C, 1 mL of the 0.104 g/L starter culture batch solution was added to the milk to obtain a final reaction volume of 15 mL (step 4 in Figure 1). The fermentation process was monitored through pH measurement during the incubation period (step 5 in Figure 1). A calibrated (standard pH buffers of pH 7.0 and pH 4.0.) portable pH Spear (Eutech Instrument Co., Oakton, USA) was used for all pH measurements. The first pH measurement in all experiments was taken directly after inoculation, where the pH was monitored at 15-minute time intervals until the final pH of 4.6 was reached.

Pre-treatment of yogurt milk with Flavourzyme

A commercial proteolytic enzyme preparation, Flavourzyme (EC number: 3.4.11.1) (Novozymes, Krogshoejvej 36, 2880, Bagsvard, Denmark) was added to yoghurt milk to perform protein hydrolysis of the reconstituted milk before and during heat treatment (90˚C for 15 min) until subsequent heat inactivation of the enzymes (step 2 in Figure 1). Flavourzyme dilutions of 0.1 g/L, 0.01 g/L and 0.001 g/L were prepared using distilled water and the Flavourzyme granules and 1 ml of each of the respective dilutions were then added to 13 mL of the reconstituted milk before heat treatment (90˚C for 15 min). An adjustment was made to the skim milk powder concentration to compensate for the addition of water during enzyme addition. The treated milk samples were incubated in a refrigerator for 18 h

before use to establish any observable changes to the yoghurt milk (no coagulation). The rest of the yoghurt procedure was done as described previously (Steps 3-6 in Figure 1).

Sample preparation and reverse phase high performance liquid chromatography (RP-HPLC) analysis.

In preparation for RP-HPLC analysis, milk samples with differing concentrations of Flavourzyme as well as one control sample (not treated with Flavourzyme before heat treatment) were treated with 5 % trichloroacetic acid (Merck, RSA - 1, Friesland Drive, Longmeadow Business Estate, Modderfontein, 1645, South Africa) to precipitate the proteins. Centrifugation (Table Top Eppendorf Centrifuge, Harmonic series, model PLC-012, Gemmy Industrial Corporation, Taiwan) was done at 9 838.4 g for 30 min in Eppendorf centrifuge tubes and 1 mL of the clear supernatant was collected for RP-HPLC analysis.

A Fisher Scientific Surveyor HPLC system, equipped with a quaternary pump, autosampler, and photodiode detector, was used for RP-HPLC analysis. A 150 mm Phenomenex Jupiter C18 column (Thermo Scientific) with an internal diameter of 4.6 mm was maintained at a temperature of 40°C. An automatic sampler (capable of injecting 20 µL) was used to inject the samples into the column and HPLC grade solvents were used exclusively. Acetonitrile (Merck, RSA) was used as the elution solvent, and the concentration thereof ranged from 0 to 65 % over a time interval of 90 min, with a flow rate of 1mL/min at 200 bar. Spectrophotometric detection was performed at a UV detection wavelength of 214 nm. The data obtained were processed using software capable of measuring peak areas (International Standard, 2005). The hydrolysis profiles of the samples treated with different enzyme concentrations as well as the untreated sample were determined using this method.

Consumer sensory evaluation

Fifty (50) regular yoghurt consumers (untrained), consisting of both staff and students from the Bloemfontein campus of the University of the Free State, South Africa, were asked to participate in the sensory evaluation of two yoghurt variants. The sensory test was conducted in the sensory analysis facility of the University of the Free State. Evaluations were performed in individual sensory booths, and white lights were used because there was no color difference to be masked. The yoghurt samples were served to the panelists in transparent plastic containers with lids (to avoid the container influencing of the panelists' perceptions of the color of the yoghurt samples), and each container contained a volume of 15 mL yoghurt per variant. The refrigerated yoghurt samples were kept at room temperature for 30 min before serving. Each of the panelists was presented with two different samples, one of which was the yoghurt made with milk that was pre-treated with the 0.1 g/L Flavourzyme dilution and one of which was the control yoghurt. Due to limited resources a worst-case scenario approach was taken and only the 0.1 g/L concentration was tested, although it is conceded that lower concentrations should be investigated in future research These two samples were presented to the panelists at the same time. The samples were coded with randomized 3-digit codes and rotated to prevent bias.

Bottled water was provided as a palate cleanser to minimize the effects of sensory carryover. The panelists were instructed to smell the samples before tasting them. The questionnaire consisted of a simple written 9-point hedonic scale (STONE; SIDEL, 1992; LAWLESS; HEYMANN, 2010), which was amended to include the following attributes: taste, texture, appearance, and overall acceptability. The amended hedonic scale was anchored with 1 = "Dislike extremely" and 9 = "Like extremely" and with a neutral center point of 5 = "Neither like nor dislike". Ethical clearance was obtained from the Ethics Committee of the University of the Free State (UFS-ESD2020/0006) and the study was conducted accordingly.

All the data were collected in spreadsheets using Microsoft Excel 2007 and statistical analyses were done using NCSS (2007). Analysis of variance (ANOVA) was used to explore significant differences in mean (± SD) hedonic scores of the yoghurt.

RESULTS AND DISCUSSION

Reports have stated that supplementation of milk with peptides shortens the fermentation phase

of yoghurt manufacture (DAVE; SHAH, 1998; LUCAS *et al*., 2004). Previous studies have indicated that Yeast extract (SMITH *et al*., 2014) could provide the necessary peptides to decrease the fermentation time of yoghurt manufacture. However, the preparation of accelerants is expensive and to simplify the supplementation process, enzyme addition before heat treatment (90˚C for 15 min) was assessed as an alternative.

The influence of Flavourzyme treatment on the physical properties of the milk

There was no observable whey syneresis (separation of the whey fluid) or obvious viscosity changes in comparison to the control. At the start of fermentation, it was also noted that the pH of the yogurt milk was not greatly affected by the Flavourzyme pre-treatment. At the start of fermentation (0 min), the average pH of the control yogurt samples was 6.64; the average pH of the yogurt milk that was pre-treated with the 0.1 g/L Flavourzyme batch dilution was 6.64; the average pH of the yogurt milk that was pre-treated with the 0.01 g/L Flavourzyme batch dilution was 6.64; the average pH of the yogurt milk that was pre-treated with the 0.001 g/L Flavourzyme batch dilution was 6.65. This relates to a standard deviation of approximately 0.004 across the population, which indicates that the initial pH is not greatly affected due to the Flavourzyme pre-treatment of yogurt milk.

These observations indicated that the enzyme load was low enough to avoid excessive protein damage. This was the desired outcome because excessive protein damage could have led to premature coagulation of the milk proteins and resulted in a sweet yoghurt.

RP-HPLC analysis

It was evident from Figure 2 that peptides of different sizes were liberated in abundance by Flavourzyme treatment of milk (between 55 – 70 min retention time). The divergent sizes of the liberated peptides were attributed to the complexity of the Flavourzyme enzyme preparation, which consisted of at least seven distinct protease enzymes, some were endoproteases and exoproteases (MERZ *et al*., 2015). The amount of peptides liberated by means of protein hydrolysis appeared to be dependent on the concentration of the Flavourzyme added.

Flavourzyme treated milk on yogurt fermentation.

Figure 3 depicts the average acidification curves of the standard yoghurt fermentation as well as Flavourzyme treated yoghurt. In comparison to the standard yoghurt, which completed fermentation by 225 min, the yoghurt treated with Flavourzyme, fermented in a considerably shorter time. All Flavourzyme samples completed fermentation by 195 min (13.33% decrease in the fermentation time), mainly because of the decrease in the lag phase.

Although various concentrations of Flavourzyme were added to the milk before heat treatment, the fermentation profiles were similar. The similarity between fermentation curves indicated that the acceleration of yoghurt fermentation by the addition of Flavourzyme enzyme dilution solutions ranging from 0.1 g/L to 0.001 g/L was not dependent on the concentration of the enzyme. None of the enzyme-treated samples coagulated prematurely (i.e., before pH 5.2) during fermentation. The results of this study are comparable with work done by Smith *et al*. (2014) on yeast extract as an accelerant of yoghurt fermentation. However, Smith *et al*. (2014) determined yeast extract resulted in unpalatable bitter-tasting yoghurt and undesirable texture changes, which was not the case during the current research. Even though Figure 3 indicated that the enzyme concentrations did not affect the degree of acceleration, Figure 2 suggested that the extent of peptides liberated from milk proteins increased as the concentration of the proteolytic enzyme (Flavourzyme) increased. A plausible explanation could be that the peptide concentration required to initiate the growth of the inoculated starter culture (*S. thermophilus)* might have been so low that the peptides liberated by Flavourzyme were in excess, (even at the lowest dilution used during this study), however as this was beyond the scope of this study, further studies are required to confirm this.

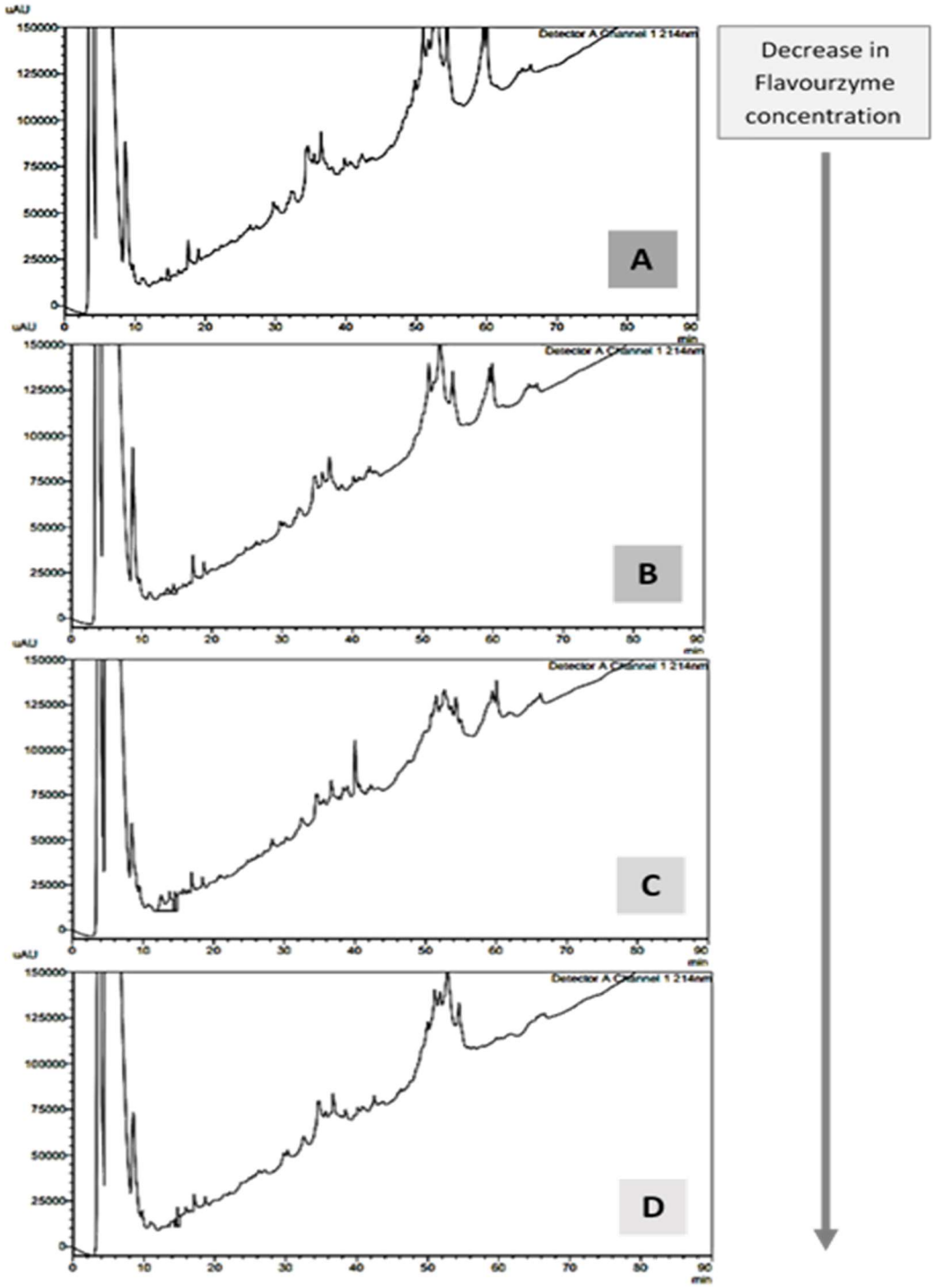

Figure 2. The RP-HPLC for A) Skim milk treated with 0.1 g/L Flavourzyme batch solution, prior to heat treatment (90˚C for 15 min); B) Skim milk treated with 0.01 g/L Flavourzyme batch solution, prior to heat treatment (90˚C for 15 min); C) Skim milk treated with 0.001 g/L Flavourzyme batch solution, prior to heat treatment (90˚C for 15 min); D) Untreated pasteurized milk (control).

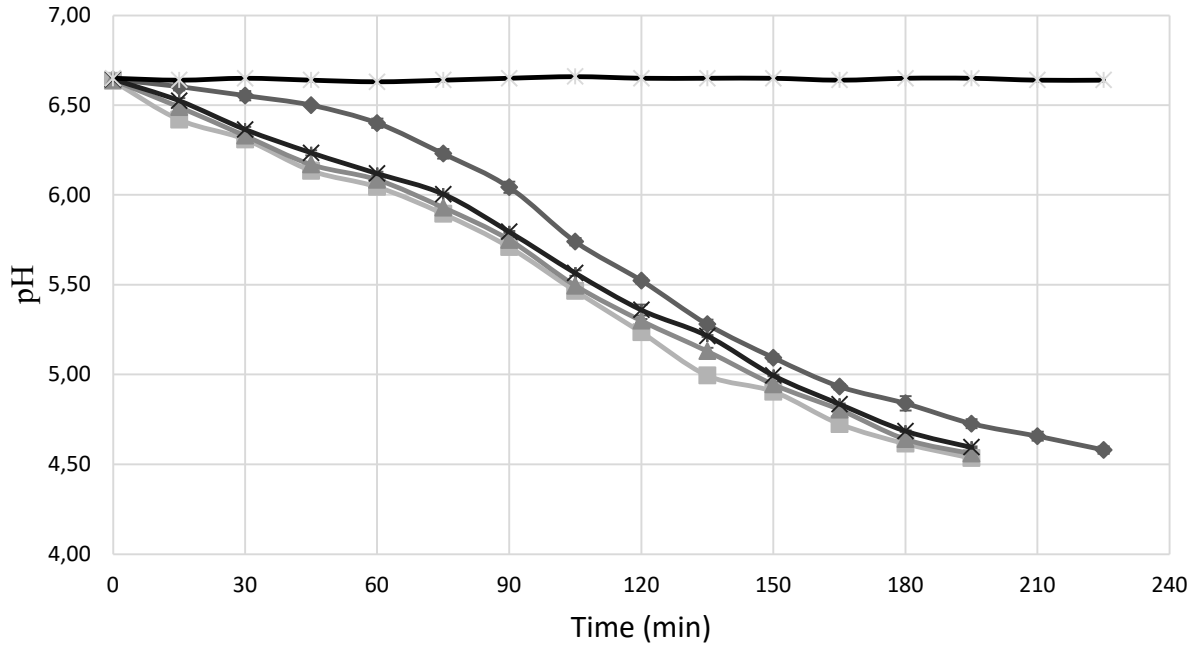

Figure 3. The average acidification curves of: A) standard yogurt fermentations; B) Average yoghurt fermentations using milk treated with 1ml of the 0.1 g/L batch dilution of Flavourzyme; C) Average yoghurt fermentations using milk treated with 1ml of the 0.01 g/L batch dilution of Flavourzyme; D) Average yoghurt fermentations using milk treated with 1ml of the 0.001 g/L batch dilution of Flavourzyme; E) Control containing only milk.

Consumer sensory evaluation

The panel comprised 37 women and 13 men, with the ages ranging from 20 to > 60 years. The percentage split for the age profile of the panelists was as follows: 20–29 years (52%); 30–39 years (22%); 40–49 years (12%); 50–59 years (12%); and older than 60 years (2%). The majority of panelists consisted of students in the younger age group from 20 to 39 years (64%), while only 26% were 40 years to older than 60 years, representing older staff members. The t-test results are summarized in Table 1. The control yoghurt received significantly higher scores ($P<0.001$) for all attributes (taste, texture, appearance, and overall acceptability) than the experimental yoghurt. Except for texture, the mean sensory ranking for the other attributes was between 5 and 6, meaning that all attributes for the experimental products were considered as neutral ("neither like nor dislike").

Table 1. Analysis of Variance (ANOVA) of the consumer panel data on the yogurt made with Flavourzyme pre-treated milk and control yoghurts

Sample	Control yoghurt	Experimental yoghurt	Significance level
Taste	6.80^b	5.64^a	$P < 0.001$
Texture	6.88^b	4.50^a	$P < 0.001$
Appearance	7.10^b	5.50^a	$P < 0.001$
Overall acceptability	6.78^b	5.22^a	$P < 0.001$

Means with different superscripts in the same row differ significantly.

Notably, no stabilizers, added milk powder or, flavorings were used during the study. Therefore, any variations that occurred in the texture or flavor were a direct result of the accelerating treatment. Most of the sensory changes such as a less acceptable flavor, texture, and appearance could likely be overcome if

simple industrial practices are applied. Flavorings such as fruit, synthetic Flavors, and sweeteners generally increase consumer acceptance of yoghurt (ARDING, 2015). Sugar (BAKER, 1981; TAMIIME; DEETH, 1980) and fruit flavorings are also known for masking the sharp, acidic taste of unsweetened (plain) natural yoghurts (BAKER, 1981; SCHVED; HASSIDOV, 2008). Fructose sugar is known for being a masking agent, and masks bitterness and metallic aftertaste (SCHVED; HASSIDOV, 2008). The use of stabilizers and the addition of fruit pulps can also help improve the flavor of the final yoghurt product (TAMIIME; DEETH, 1980).

Neither the control nor the experimental yoghurt had a taste or overall acceptability of more than 7 ("Like moderately"). The low scores for taste and overall acceptability of both the control and the experimental yoghurt could have been because no sugars were added to the yoghurt. According to Vickers *et al*. (2001), consumers prefer yoghurt with high sugar content.

The textural change can be addressed using stabilizers (or adjustment of the stabilizer concentration). During the industrial manufacture of yoghurt, stabilizers are used for various purposes. Some of the common functions of stabilizers are to obtain a more uniform consistency and to reduce the possible variation between batches (LEE; LUCEY, 2010), reduce syneresis (KUMAR; MISHRA, 2004; LEE; LUCEY, 2010), improve the texture and body of the yoghurt (KUMAR; MISHRA, 2004; SODINI *et al*., 2005), and improve the structure, appearance, and mouthfeel of the yoghurt (KUMAR; MISHRA, 2004). Different stabilizers are common in the yoghurt industry, for example, modified starches, gelatine, pectin, and gums (CHANDAN; O'RELL, 2006; LEE; LUCEY, 2010; LI; GUO, 2006). Many stabilizers are hydrocolloids, which have two main functions in yoghurt. Firstly, the binding of water and secondly the improvement of texture (KUMAR; MISHRA, 2004). Milk proteins can also mimic the thickening functionality of stabilizers; consequently, the addition thereof before manufacture improves the final texture of the yoghurt (LI; GUO, 2006). Therefore, it is common practice to increase the milk protein content through fortification with other dairy products such as milk powder or whey protein (ISLETEN; KARAGUL-YUCEER, 2006; SODINI *et al*., 2005). The protein concentration is often "boosted" to 40–50 g protein/kg (SODINI *et al*., 2005).

CONCLUSIONS

Supplementation with short peptides results in acceleration of yoghurt fermentation time. Such peptides can be liberated from naturally occurring milk proteins by adding a proteolytic enzyme such as Flavourzyme to the milk prior to heat treatment at 90˚C for 15 min (which leads to subsequent thermal inactivation of the enzyme). During this study, it was possible to accelerate the yoghurt fermentation process using Flavourzyme. The peptides liberated from natural milk proteins reduced the lag phase of yoghurt fermentation dramatically. Although by Flavourzyme treatment of the milk before heat treatment caused significant protein degradation, it did not lead to coagulation of milk after incubation or premature coagulation (i.e., coagulation before pH 5.2) during fermentation at low concentrations. The extent of hydrolysis, but not the fermentation times appeared to be concentration-dependent, although further investigations should include lower concentrations.

The load of enzyme added to a batch of milk to result in the drastic acceleration of yoghurt fermentation was exceptionally small, and the method did not involve expensive changes in infrastructure. Furthermore, the use of this method did not appear to affect the quality of the yoghurt (i.e., flavor and texture). Thus, supplementing yoghurt milk with Flavourzyme before heat treatment has industrial advantages since it would not be exceedingly expensive to improve the production rates.

Consumers were able to detect a difference between yoghurt made with Flavourzyme and untreated milk. The most pronounced sensory difference was the texture of the yoghurt, which was perceived negatively by consumers. It is, however, possible to improve sensory flaws of the process with simple techniques, which are already standard practice in yoghurt manufacturing facilities. For example, the texture can be improved through the addition of stabilizers and/or milk powder while the taste and flavor can be enhanced through the addition of sugars and/or flavorings.

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