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Evaluation of Time-Temperature Integrators (TTIs) with Microorganism-Entrapped Microbeads Produced Using Homogenization and SPG Membrane Emulsification Techniques

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A comparative study was conducted to evaluate precision and accuracy in controlling the temperature dependence of encapsulated microbial time–temperature integrators (TTIs) developed using two different emulsification techniques. *Weissela cibaria* CIFP 009 cells, immobilized within 2% Na-alginate gel microbeads using homogenization (5,000, 7,000, and 10,000 rpm) and Shirasu porous glass (SPG) membrane technologies (10 μm), were applied to microbial TTIs. The prepared micobeads were characterized with respect to their size, size distribution, shape and morphology, entrapment efficiency, and bead production yield. Additionally, fermentation process parameters including growth rate were investigated. The TTI responses (changes in pH and titratable acidity (TA)) were evaluated as a function of temperature (20°C, 25°C, and 30°C). In comparison with conventional methods, SPG membrane technology was able not only to produce highly uniform, small-sized beads with the narrowest size distribution, but also the bead production yield was found to be nearly 3.0 to 4.5 times higher. However, among the TTIs produced using the homogenization technique, poor linearity (R^2) in terms of TA was observed for the 5,000 and 7,000 rpm treatments. Consequently, microbeads produced by the SPG membrane and by homogenization at 10,000 rpm were selected for adjusting the temperature dependence. The E_a values of TTIs containing 0.5, 1.0, and 1.5 g microbeads, prepared by SPG membrane and conventional methods, were estimated to be 86.0, 83.5, and 76.6 kJ/mol, and 85.5, 73.5, and 62.2 kJ/mol, respectively. Therefore, microbial TTIs developed using SPG membrane technology are much more efficient in controlling temperature dependence.

Keywords: Microbial TTIs, microencapsulation, temperature dependency, homogenization method, Shirasu porous glass (SPG) membrane technology

Introduction

A time–temperature integrator (TTI) is a simple device that allows manufacturers, distributors, retailers, and consumers to check, at a glance, whether perishable foods have been exposed to harmful temperatures during transportation or storage, by expressing an irreversible visual color change determined by the time–temperature history. During the last 30 years, numerous TTI systems have been proposed, of which only a few reached the prototype stage, with even fewer reaching the market; this is commonly due to issues of cost and precision [10].

Among the various types of TTIs developed so far, microbial TTIs are considered to be the most advanced because their response is directly related to microbial food spoilage, which is an indication of the growth and metabolism of bacteria present in the TTI system itself [35]. In order to apply TTIs successfully in monitoring food quality, the difference in Arrhenius activation energy (E_a), (which indicates the temperature sensitivity, determines

the accuracy of food safety measurements, and ensures quality) of the TTI and food should not be more than 25 kJ/mol [33, 35]. However, adjusting the shelf-life of TTIs with that of the particular food to be monitored at all temperatures is not a simple task. In particular, adjusting the shelf-life for microbial TTIs is very difficult [38, 40] because, although a given concentration of microorganisms is present when a TTI is produced, their concentration changes as a result of growth. Very recently, an interesting experimental study has been carried out to overcome the above-mentioned prevailing drawback of classic microbial TTIs through developing a new microbial TTI based on microencapsulation technology [6]. The authors entrapped a very high initial lactic acid bacteria cell concentration within microbeads, with this level being constant over time. This treatment has not only solved the difficulties in adjusting the shelf-life of microbial TTIs but it has also resulted in a wide range of TTI response (pH change) rates in zero-order reactions with linearity. It is known that zero-order or pseudo-zero order reaction kinetics are the optimum choice for describing temperature dependence through the Arrhenius relationship [39].

Microencapsulation is a technology by which small solid particles, liquid droplets, or even gases may be enclosed in a coating that forms a microcapsule with a size between 1 and 1,000 μm [23, 28]. Microbeads can be produced by either the extrusion (droplet)- or emulsion (two-phase system)-solidification method. Although the extrusion process is the most popular owing to its ease, simplicity, low cost, and gentle formulation conditions, which ensure high retention of cell viability, it can be difficult to produce beads smaller than 1 mm as the bead size is limited by the syringe needle bore size and by the viscosity of the solution. The size of these large beads imposes diffusion limits on the transfer of substrate and product to and from the entrapped microbial cells [9]. Consequently, smaller beads are needed to better facilitate both internal and external mass transfers, enhancing fermentation performance and minimizing bead rupture due to gas formation and accumulation. However, small bead sizes can be produced using the emulsion technique, where at least two insoluble liquids are employed to produce microspheres [42]. An emulsion can be formed by either conventional methods, using colloid mills, rotor stator systems, high-pressure homogenizers, ultrasonic homogenizers, and magnetic stirrers [14], or by a relatively new technique termed "membrane emulsification" [13].

Highly uniform and small-sized microbeads can be produced using Shirasu porous glass (SPG) membrane technology, which was first demonstrated by Nakashima and Shimizu [19]. The size of the resulting emulsion droplet can be easily controlled by using a membrane with the desired pore size, and by adjusting the operating parameters (especially the transmembrane pressure). However, the technique does have disadvantages, including low permeability of the SPG membranes, which occurs because they are quite thick (0.45–0.75 mm) and are homogeneous in structure, which could result in a low flux of the disperse phase; additionally, membranes are expensive and require a long time for regeneration (3 days). Conversely, high-pressure homogenization is a simple and commonly used method in the food industry for producing finely dispersed emulsions by homogenizing oil and aqueous phases [29]. The sizes of the emulsion droplets are dependent on the speed of the homogenizer. Increased homogenization speed reduces the size of the emulsion droplets or *vice versa* [7]. However, difficulties in controlling droplet size and the droplet size distribution, along with high-energy inputs, are still major concerns for employing this method. In general, the formation of the initial emulsion droplet is very influential because controlling the subsequent final solid microparticle (after solidification of the emulsion droplets by applying an adequate solidifying procedure) size and size distribution depends on this previous step. However, here it is hypothesized that good control of bead size, shape, and size distribution are the crucial key factors for developing a more sophisticated TTI system in which the reaction kinetics can be controlled accurately. Consequently, a comparative evaluation of the accuracy and precision in controlling the reaction kinetics of encapsulated microbial TTIs produced using the above two methods is necessary.

The aim of this study was to conduct an extensive comparison of the precision of microbial TTIs developed on the basis of the homogenization and SPG membrane emulsion techniques. Each technique has its own advantages and disadvantages and this study can provide information to food companies regarding which methods could be the best choice for developing a microbial TTI specifically tailored to their intended use. *Weissela cibaria* CIFP 009 cells were immobilized within alginate gel microbeads using homogenization and SPG membrane emulsion technologies; these were then applied to microbial TTIs. A comparison of the microbial TTIs was made under no cell growth conditions as a function of different temperatures, with the TTI responses (pH change, total titratable acidity (TA)) and the Arrhenius parameters of the TTIs being determined. The prepared microbeads were characterized with respect to their size and size distribution, shape and morphology,

microorganism entrapment efficiency, and bead production yield. In addition, fermentation process parameters, including the growth rate, were investigated.

Materials and Methods

Materials

Polyethylene glycol sorbitan monolaurate (Tween 20; hydrophile-lipophile balance value (HLB) = 16.7) and paraffin oil were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Calcium chloride, to be used as a crosslinking agent, was obtained from Ajax Chemicals Co. (Sydney, Australia). Sodium alginate, sorbitane monooleate (Span 80; HLB = 4.3), to be used as a nonionic surfactant, and all other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The SPG membrane with an average pore size of 10 μm was purchased from Ise Chemical Co. (Japan). A hydrophobic silane-coupling agent, KP-18C, used to modify the membrane, was provided by Shin-Etsu Chemical Co. (Japan).

Fabrication of TTI

Bacterial strain and culture preparations. The strain *W. cibaria* CIFP 009 used in this study (provided by the Center for Intelligent Agro-Food Packaging, Dongguk University–Ilsan, Korea) was subcultured three times for use in the preparation of cell suspensions. For each cell suspension, 100 μl of cell suspension was inoculated into 50 ml centrifuge tubes containing 25 ml of de Man, Rogosa, and Sharpe (MRS; Difco, Detroit, MI, USA) broth; these were then incubated at 37°C for 18 h anaerobically, using a BBL Gas Pak Plus Anaerobic System (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA). Finally, the liquid culture was harvested by centrifugation at 6,860 ×*g* for 10 min at 4°C (Combi-514R; Hanil Science Industrial Co. Ltd., Korea). After discarding the supernatant, the precipitated cells were washed three times with sterile distilled water by centrifugation (6,860 ×*g* for 10 min at 4°C) for use in the microencapsulation process [11].

Preparation of the Dispersed (Bacterial–Alginate Mixture) and the Continuous Phase

The dispersed and the continuous phases were prepared according to the preparation procedures described previously with minor modification [4, 31]. Briefly, the dispersed aqueous phases were prepared by mixing sodium alginate powders in 100 ml of sterile double distilled water. This was stirred at a controlled temperature of 80°C until the mixture became clear to produce a 2% (w/v) solution. The solution was then sterilized by autoclaving at 121°C for 15 min before being cooled to 38–40°C. Subsequently, the concentrated cell suspension was mixed with the alginate mixtures under aseptic conditions. The continuous phase (98 ml of paraffin oil in a 500 ml beaker) containing 2% (v/v) surfactant (Span 80) was also autoclaved at 121°C for 15 min. Four samples were prepared according to the same conditions of production.

Microencapsulation by Homogenization Emulsification Technique

All solutions and glassware used in this study for the microencapsulation process were autoclaved and the entire work was performed under a laminar airflow hood using standard microbiological practices. The method used for microencapsulation of *W. cibaria* CIFP 009 was a modified version of a previously reported method [31]. Briefly, 100 ml of the suspension of sodium alginate and cells was gently dispensed using a pipette into the continuous phase (100 ml). Emulsions were then formed by homogenization using the WiseTis homogenizer (HG-15D; Daihan Scientific Co., Ltd., Wonju, Korea) operated at 5,000, 7,000, and 10,000 rpm for 10 min. Following each of the homogenization treatments, each emulsification was transferred into a beaker and a 0.1 M $CaCl_2$ solution was added gently along the wall of the rotator of the Eyela Mazela Z (Z-2310; Tokyo Rikakikai Co. Ltd., Japan) system while stirring at 300 rpm for 10 min to break the emulsion [30]. The emulsions were transferred into separating funnels and were rinsed with 1% Tween 20 solution with vigorous shaking (C-SKR; Chang Shin Scientific Co., Busan, Korea) at 350 rpm for 10 min. The beads were then precipitated by centrifugation at 2,000 ×*g* for 5 min. Following the removal of the supernatant, the bead suspensions were washed five times with double-distilled water for 5 min each time to remove the oil phase completely; the samples were then stored at 4°C.

Microencapsulation by the SPG Emulsification Technique

The microencapsulation process was performed according to a previously published method with slight modifications, using the SPG membrane [2]. An internal pressure-type micro-kit (IMK-40M1; SPG Techno Co. Ltd., Miyazaki, Japan) was used for the membrane emulsification/solidification process. This kit was equipped with a hydrophobic SPG membrane (SPG Techno Co. Ltd., Miyazaki, Japan) with an outer diameter of 10 mm, a thickness of 1 mm, a length of 50 mm, and a pore size of 10 μm. Before emulsification, the membrane module was immersed into the oil phase and was treated with an ultrasonicator for 30 min prior to use, so that its surface was entirely wetted by the continuous phase [15]. This process has been reported to increase the flux rate, and a W/O (Water in Oil) emulsion with controlled dispersed droplet size could be produced [16]. After being wetted with the oil phase, this module was installed in the membrane system. At least 2 h prior to the start of the experiment, the UV light was turned on to sterilize the inside of the SPG device. Recently, the SPG device used in this study had been upgraded by introducing a UV light source in order to make this study as accurate as possible. The dispersed (100 ml) and continuous phases (100 ml) were then stored in the phase reservoirs. The dispersed phase was then extruded through the SPG membrane under the critical nitrogen gas pressure (15 kPa) into the continuous phase with stirring (60 rpm) to form the W/O emulsion. The emulsion was then transferred into a beaker; the solidification stage and the subsequent procedural steps for microbead collection were performed as described in the previous

section. The used SPG membrane was treated with KP-18C to render the SPG membrane hydrophobic.

Quantitative Analysis of Microbeads

Size distribution analysis. The size distribution of the microencapsulated beads (1 g in wet status) was determined using a laser light diffraction particle size analyzer (S3500; Microtrac Inc., FL, USA).

Microstructure of Microbeads

An optical microscope (Nikon Eclipse E200; Nikon Co., Japan) was used to observe the shape and uniformity of the emulsion system as well as the microbeads following solidification using $CaCl_2$. The shape and size of the alginate microbeads were also observed with a scanning electron microscope (S-3000N; Hitachi, Japan).

Enumeration of *W. cibaria* CIFP 009 Within Calcium Alginate Microbeads

Viability counting of the microbeads-entrapped *W. cibaria* CIFP 009 was performed according to a method reported previously [31]. Viability counts were performed immediately following separation and washing, and after incubation of 1 g of each form of microbead by suspending in a 50 ml centrifuge tube containing 10 ml of MRS broth at 20ºC, 25ºC, and 30ºC for 24 h. For the freshly prepared microbeads, 1 g of particles was suspended in 9 ml of phosphate-buffered saline (PBS, pH 7.4) before being gently shaken for 30 min in order to achieve the destruction of the microbeads. The PBS is utilized to weaken the crosslinks in the calcium alginate beads by sequestering calcium ions at neutral pH; this results in the release of the entrapped microorganisms. Once released, dilutions (10^{-1} to 10^{-6}) of the microorganisms were plated (100 µl) on MRS (Difco, Detroit, MI, USA) agar with an automatic spiral-plater (EasySpiral; Interscience, St. Nom la Bretèche, France) using 90 mm petri dishes. Following this, the petri dishes were incubated anaerobically at 37ºC for 48 h. In order to determine microbeads-entrapped cell viability after incubation, microbeads and precipitate were harvested by centrifugation (6,860 ×*g* for 10 min at 4ºC) before being washed three times in distilled water. After the final wash, the precipitate was suspended in 9 ml of PBS (pH 7.4) before being subjected to gentle shaking for 30 min to destroy the microbeads. The rest of the procedure for the viability count was the same as for the freshly prepared microbeads. All microbial counts were performed in triplicate. The average of the results was expressed as colony-forming units per gram of sample (CFU/g). Subsequently, the results calculated as CFU/g were transformed to log CFU/g to facilitate better comparison.

Microorganism Entrapment Efficiency and Bead Production Yield

The microencapsulation process was monitored by both microorganism entrapment efficiency and alginate bead production yield. Eq. (1) was used to evaluate the efficiency of the two processess:

$$\text{Entrapment efficiency } (\%) = \frac{y}{x} \times 100 \quad (1)$$

where x is the logarithmic number of the initial viable population of LAB (log colony forming units) prior to homogenization and SPG membrane treatments, and y is the logarithmic number of viable population of LAB (log colony forming units) entrapped within the calcium alginate microbeads.

Microbead production yield was measured as the wet weight of calcium alginate beads (in g) obtained from 100 g of alginate and was expressed as a percentage.

Measurement of TTI Function

Prototypes of microbial TTIs were prepared by adding 1 g of each form of microbead to 10 ml of MRS broth. The TTI responses (pH) due to lactic acid production by *W. cibaria* CIFP 009 were measured over time at 20ºC, 25ºC, and 30ºC using a pH meter (S20 SevenEasy™ pH; Mettler-Toledo International Inc., Seoul, Korea). Titratable acidity was determined using the following procedure: 5.0 g of the TTI mixture was diluted with 20 ml of sterilized distilled water and was homogenized for 10 sec with 0.5 ml of 1.0% phenolphthalein (w/v) added. It was finally titrated with 0.1 N NaOH solution to pH 8.2. TA was expressed as a percentage of lactic acid, determined using Eq. (2):

$$\text{Lactic acid } (\%) = \frac{0.1\text{ N NaOH required} \times 0.1\text{ N NaOH factor} \times 0.009}{\text{sample weight}} \times 100 \quad (2)$$

Characteristics of the microbial TTI response were analyzed in terms of kinetic and Arrhenius parameters. The TTI response variable counted was pH, which directly influences TTI color change. The TTI response was modeled with zero-order kinetics in Eq. (3).

$$Y = -kt \quad (3)$$

where Y is the TTI response (TA), k is the TTI response rate (TA/h), and t is the time. The temperature dependence of the TTI response rate was modeled using the Arrhenius relationship in Eq. (4).

$$k = A \exp\left(-\frac{E_a}{RT}\right) \quad (4)$$

where A is a pre-exponential factor (TA/h), E_a is the activation energy (kJ/mol), R is the ideal gas constant (8.314×10^{-3} kJ/mol K), and T is the temperature (K).

Statistical Analysis

All experiments were performed in triplicate, and each replicate was quantified in duplicate. The quantitative data were averaged and standard deviations were calculated using the Microsoft Excel software (Microsoft Corporation, Redmond, WA, USA). Error bars represent standard error.

Results and Discussion

Effects of the Two Emulsification Methods on the Characterization of Prepared Beads: Size, Morphology, Microorganism Entrapment Efficiency, and Microbead Production Yield

It can be observed from Fig. 1 that emulsions and microbeads (produced after solidification of emulsions with $CaCl_2$) formed by homogenization at 10,000 rpm were the only uniform beads among the three homogenization treatments. This is in accordance with earlier work [7], which has shown that increasing the homogenization speed from 5,000 to 10,000 rpm reduces the emulsion droplet size and increases the surface area. In the case of the homogenization method, the size of the emulsion droplet depends on the speed of the homogenizer. Homogenization speeds of 5,000, 7,000, and 10,000 rpm were chosen to represent speeds that are commonly used [7]. Therefore, in the present study, three different homogenization treatments were performed (5,000, 7,000, and 10,000 rpm) to optimize the best operating conditions (keeping the alginate concentration and the operation time constant) for producing microbeads for the purpose of developing efficient microbial TTIs. Conversely, the SPG membrane emulsification technology also produced highly uniform emulsions and microbeads. In general, the formation of the initial emulsion droplet is very influential, as the control of the subsequent final solid microparticle (after solidification of the emulsion droplets by applying an adequate solidifying procedure) size and size distribution is dependent on this previous step. Some rod-shaped objects within the microbeads were clearly observed on both the light microscopic and SEM images, which indicated the presence of microorganisms. The SEM photographs also show that the microbeads produced by homogenizing at 5,000 and 7,000 rpm were non-spherical. In contrast, beads made through homogenization at 10,000 rpm and beads produced by the SPG membrane emulsification technique were spherical in shape.

From Figs. 2A, 2B, and 2C, it is apparent that the beads produced by homogenizing at 5,000 and 7,000 rpm have multiple overshoots, with varied peak magnitudes; conversely, beads produced by homogenization at a speed of 10,000 rpm exhibited only a single peak with a greater overshoot magnitude, indicating a wide and narrow particle size distribution, respectively. These data are in

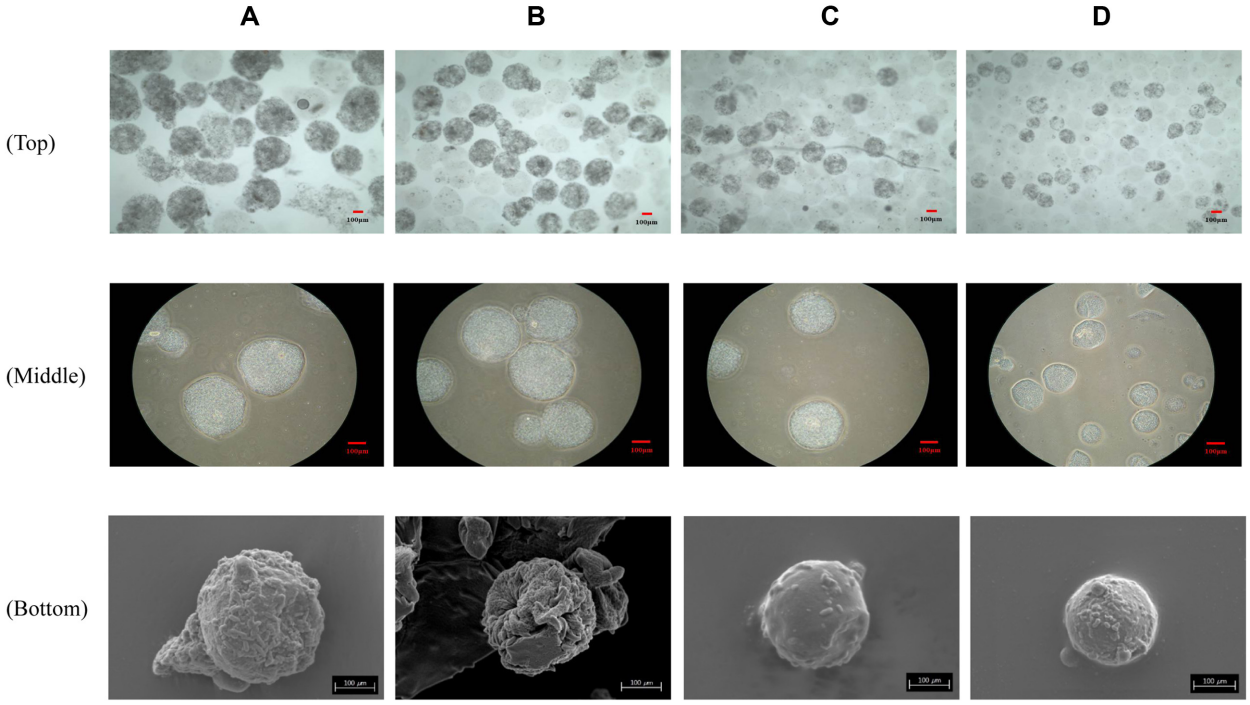

Fig. 1. Light microscope images of alginate emulsion droplets (top) and Ca-alginate beads (middle), and scanning electron micrograph (bottom) of Ca-alginate beads containing entrapped lactic acid bacteria.
(**A**) formed by emulsification method using the homogenizer at 5,000 rpm, (**B**) formed by homogenization at 7,000 rpm, (**C**) formed by homogenization at 10,000 rpm, and (**D**) formed by membrane emulsification method using SPG membrane pore size of 10 μm.

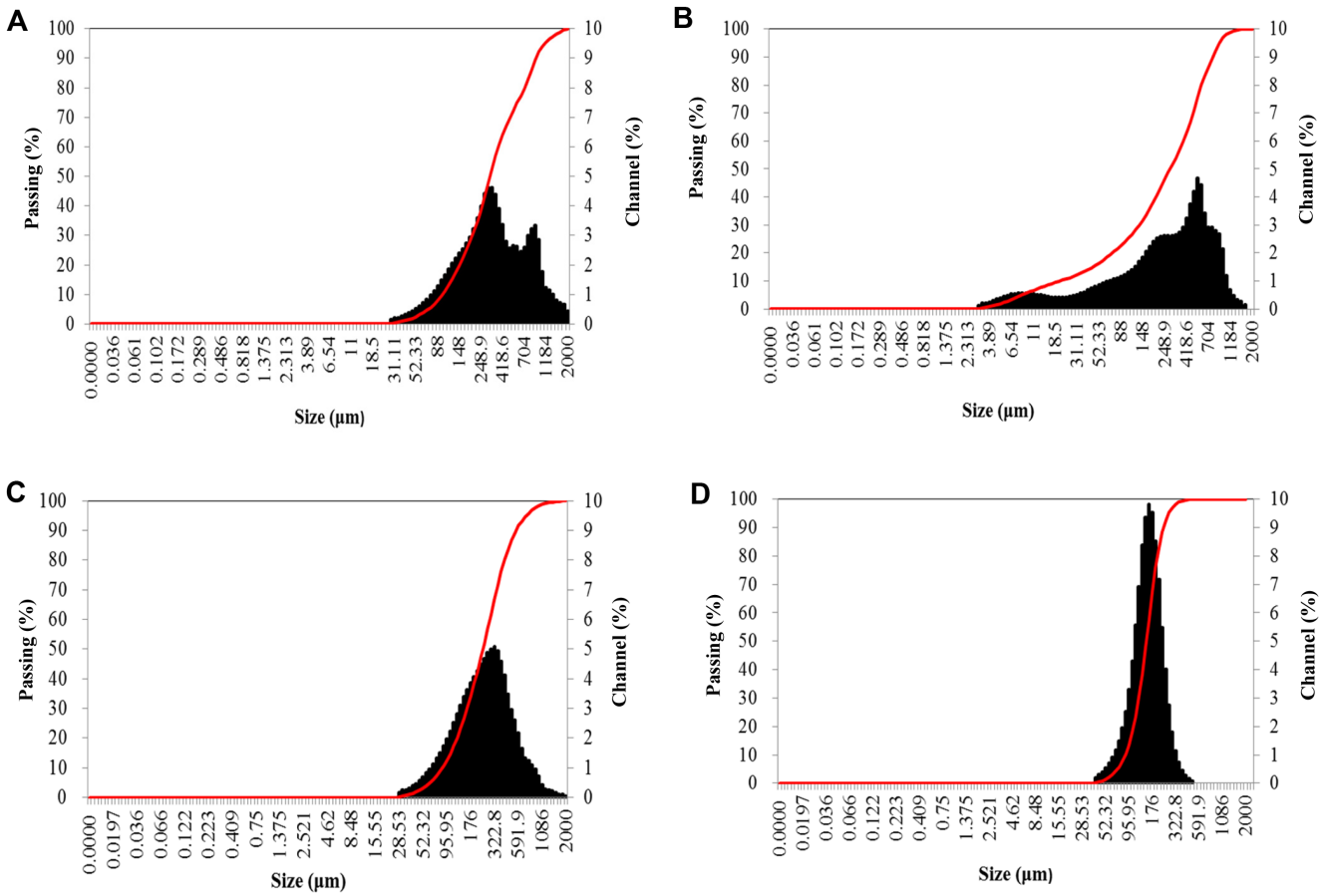

Fig. 2. Size distributions of 2% alginate beads prepared using the homogenization (5,000 rpm (**A**), 7,000 rpm (**B**), and 10,000 rpm (**C**)) and SPG membrane (10 μm) emulsification methods (**D**).

agreement with previous reports that have shown that increasing the homogenization speed from 5,000 to 10,000 rpm reduces the emulsion droplet size [7]. Conversely, beads produced using the SPG membrane technology exhibited the greatest overshoot magnitude, which confirms the best uniformity and smallest bead sizes with the narrowest size distribution.

The ranges in the sizes of beads produced in the 5,000, 7,000, and 10,000 rpm and 10 μm conditions were 99.28–1995, 20.59–1662, 85.62–733.1, and 87.41–268.4 μm, respectively. Additionally, the mean diameters of the beads were 308.7, 283, 241.5, and 150.6 μm, respectively. The mean diameters and ranges of the sizes of beads were found to be in the following rank order: 5,000 rpm > 7,000 rpm > 10,000 rpm > 10 μm (Table 1). Consequently, it is clear from these data that the SPG membrane emulsification method produced more uniform and smaller beads, with the narrowest size distribution, between the two methods. Previous studies have also reported the superiority of SPG membrane technology over conventional mechanical methods in regards producing uniform, small spherical beads with a narrow size distribution [20, 37]. This occurs owing to the size of the emulsion droplet being controlled by the choice of the membrane and not by the generation of turbulent droplet break-up. Conversely, the mean particle size and size distribution of alginate beads prepared using homogenization at 10,000 rpm were the smallest and exhibited the narrowest range among the three homogenization techniques. The conventional homogenization emulsification method could generate emulsions with relatively small droplet sizes, but wide droplet-size distributions were found [5]. In fact, the size and size distribution of beads were dependent on the homogenizing speed. Particle size and size distribution can be decreased at a high homogenization speed, whereas a low speed generates larger beads with a wide size distribution [18, 26].

The inoculum count of *W. cibaria* for bead preparation was 11.00 log CFU/ml. The entrapment efficiency of the

Table 1. Comparison of the sizes, microorganism entrapment efficiency, and production yield of microbeads produced using the homogenization and SPG membrane emulsification methods.

Emulsification methods	Conditions	Mean diameter (μm)	Range of bead sizes (μm)	Count of freshly prepared microbeads (log CFU/g)	Entrapment efficiency (%)	Bead production yield (%)
Homogenization	5,000 rpm	308.7	99.28–1995	8.45	76.82	13
	7,000 rpm	283	20.59–1662	8.66	78.72	15
	10,000 rpm	241.5	85.62–733.1	8.97	81.55	20
SPG	10 μm	150.6	87.41–268.4	10.5	95.45	60

SPG membrane emulsification method applied was higher by 95.45% in comparison with the three homogenization treatments for 5,000 (76.82%), 7,000 (78.72%), and 10,000 rpm (81.55%). The increased entrapment efficiency of the SPG membrane emulsification technique is probably due to an increased surface-volume ratio of the smallest beads, as well as by the very mild conditions of the procedure applied and the uniformity of the beads [6]. However, the percentage of entrapment efficiency and microbead production increased with increasing the speed of the homogenizer. This is because increasing the homogenization speed from 5,000 to 10,000 rpm increases the surface area of the produced beads and, subsequently, an increase in entrapment efficiency is observed. This is in accordance with earlier work [26], which has shown that the mean particle size and entrapment efficiency of the microbeads were found to increase as the speed of homogenization increased from 5,000 to 10,000 rpm.

One of the major disadvantages of the emulsion technique is the difficulty in collecting or to remove the oil phase from the resulting microparticles. The production yield of microbeads produced using SPG membrane technology was found to be nearly 3.0 to 4.5 times higher (60%) in comparison with the three homogenization treatments for 5,000 (13%), 7,000 (15%), and 10,000 rpm (20%). This is due to the differences in the emulsion generation process of the two methods that have already been described in the Materials and Methods section. The bead collection process by centrifugation depends on the size, shape, density of beads, and rate of rotation. Liquid paraffin oil, as a continuous phase used in this study for preparing emulsion, has a lower relative density (0.86). This oil has the nature to make beads buoyant. The floating behavior depends on the amount of liquid paraffin entrapped in the beads [36]. The emulsion droplets formed by the SPG membrane technique deposited at the bottom of the continuous phase reservoir. This may be explained by the fact that emulsions produced by the SPG membrane technique have low oil content and, consequently, have high density. This increases the rate of sedimentation, where the beads are easily pulled to the bottom by gravity [3]. Conversely, homogenization is a two-step process, which involves the formation of primary coarse emulsion that is then subject to size reduction, creating fine secondary emulsion by machines that supply higher energy density [34]. These treatments may produce emulsions having high oil content and, therefore, have low density. Consequently, the formation of creaming resulted in the floating of the microbeads, which may be lost during the collection process. Consequently, SPG membrane technology is advantageous in view of the uniformity, lowering shear stress, energy output, high entrapment efficiency, and bead production yield; in addition, as it has high reproducibility and suitability for industrial applications [32].

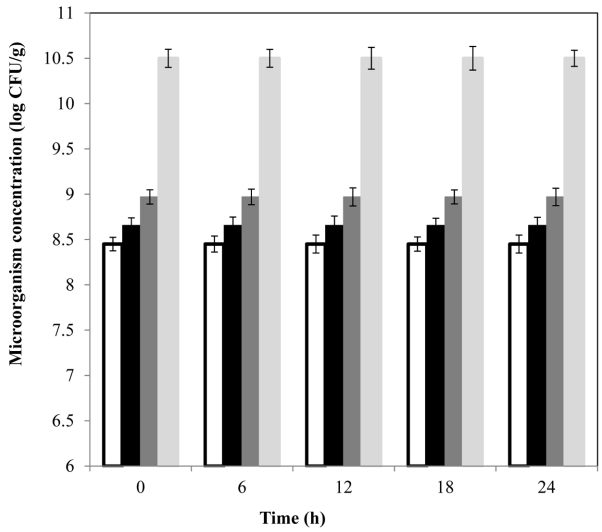

Fig. 3. Changes of the viable cell counts with time in the 2% alginate microbeads prepared using the homogenization and SPG membrane (10 μm) emulsification methods at 30°C.
□ 5,000 rpm, ■ 7,000 rpm, ■ 10,000 rpm, ■ SPG membrane (10 μm).

Effects of the Two Emulsification Methods for Producing Alginate Microbeads on Microbial Growth Kinetics

The viable cell counts of all the different microbeads did not change over time following incubation at 30°C for up to 24 h (Fig. 3). This is because the growth of cells is affected by the inner space of the microbeads available for cell growth [24]. Therefore, it is assumed that the initial level might be sufficiently high to reach the N_{max} within the microbeads. Consequently, the viable cells could be maintained at a constant by micro-beading, resulting in simple kinetics based on only the lactic acid production and not influenced by microorganism growth. However, if the initial microbial levels of the microbeads were not at N_{max}, then changes in the level of microorganisms would have occurred, especially in the early stages of TTI use [6].

Effect of Micro-beading on the Kinetics of TTI pH Response

Fig. 4 clearly illustrates that the change in pH level (decreasing pH at different temperatures) of the TTIs decreased with increasing temperatures. However, the rate of pH change of the TTIs was found to follow the rank order 10 μm > 10,000 rpm > 7,000 rpm > 5,000 rpm. The time it took to reach the lowest pH level of 3.9 by the TTIs was found to be 32, 40, 56, and 64 h at 20°C; 16, 28, 32, and 36 h at 25°C; and 10, 16, 20, and 22 h at 30°C, respectively. This phenomenon can be explained by the sizes of the microbeads. It has already been discussed in the previous section that the smallest alginate beads (mean size of 150 μm) with the narrowest size distribution were produced using the SPG membrane emulsification technology, followed by the three homogenization treatments: 10,000, 7,000, and 5,000 rpm. Consequently, the results obtained are in agreement with other previous studies that have shown that lactic acid production increases as bead diameter decreases [1, 8, 27].

The pH change actually depends on the production of lactic acid by the selected lactic acid bacteria. However, in the case of entrapped lactic acid bacteria, the production rate of lactic acid was largely dependent on the temperature and size of the immobilized microbeads. Moreover, it is already known that control of bead size is one of the major concerns in many applications of immobilized living cells. Consequently, in order to overcome diffusion limitations, which is one of the major drawbacks of the microencapsulation techniques, a small bead is required. This is because smaller beads have the advantage of a higher surface-volume ratio, which facilitates good transport of essential nutrients and removal of metabolites into and out of the beads [1, 8, 27]. Conversely, diffusion limitations when employing larger beads may limit cellular metabolism owing to the lack of essential substances, such as oxygen, supplied to the interior of the beads [8, 9, 27].

Effect of Micro-Beading on the Kinetics of TTI Response in TA

As can be observed in Figs. 5A, 5B, and 5C, TA increased with increasing time, and the rate of change of TA was faster at higher temperature. In this study, the maximum TA production (which is equivalent to the production of lactic acid) was 0.085%, obtained at the lowest pH level of 3.9. However, the rate of TA change was highest for TTIs produced with SPG microbeads followed by TTIs made with microbeads formed through homogenization at 10,000, 7,000, and 5,000 rpm, respectively. Again, this phenomenon can be explained as being due to the size and size distribution of the beads.

The color change of a microbial TTI is due to the production of lactic acid by lactic acid bacteria. However, color measurement of TTI as an index of quality prediction is not always consistent owing to some difficulties, such as light interference. Therefore, in the present study, instead of using color as a variable directly, TA, which is an index that represents the total amount of acid present expressed as a percentage of the predominant acid, was employed. Although color is known to be represented by both TA and pH for estimating the acidity and hence the color changes, TA is considered to be a more reliable indicator than pH

Table 2. Comparison of the homogenization and SPG membrane emulsification techniques in terms of kinetic and Arrhenius parameters of the time-temperature indicators in titratable acidity.

Emulsification methods	Conditions	Temp. (°C)	$R^{2\,a}$
Homogenization	5,000 rpm	20	0.93
		25	0.96
		30	0.94
	7,000 rpm	20	0.94
		25	0.97
		30	0.96
	10,000 rpm	20	0.97
		25	0.97
		30	0.97
SPG membrane	10 μm	20	1.0
		25	0.99
		30	0.99

[a]Coefficient of determination.

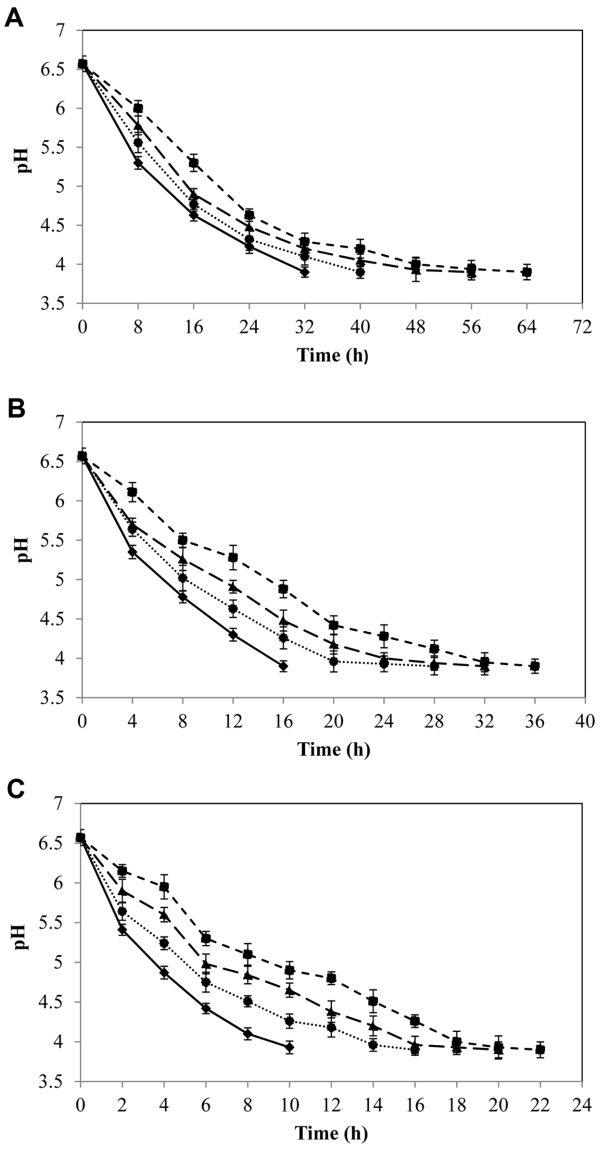

Fig. 4. Comparison of the changes of responses (pH) of the TTIs, with time with 2% alginate microbeads prepared using the homogenization and SPG membrane (10 μm) emulsification methods at (**A**) 20°C, (**B**) 25°C, and (**C**) 30°C.
■ 5,000 rpm, ▲ 7,000 rpm, ● 10,000 rpm, ◆ SPG membrane (10 μm).

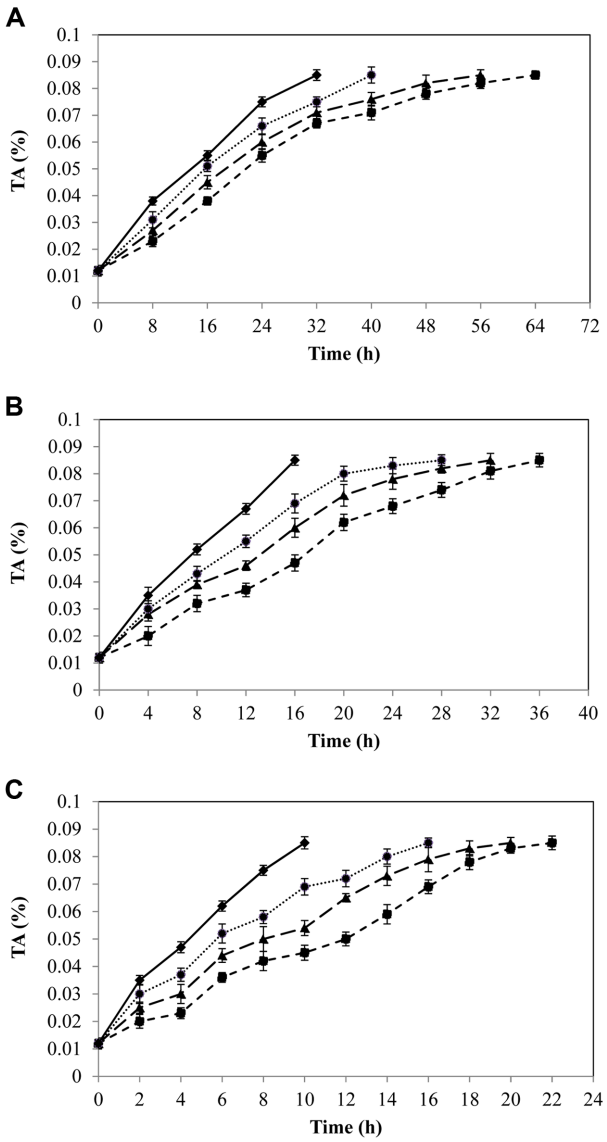

Fig. 5. Comparison of the changes of responses (titratable acidity) in percentages of the TTIs, with time with 2% alginate microbeads prepared by the homogenization and SPG membrane (10 μm) emulsification methods at 20°C, 25°C, and 30°C.
■ 5,000 rpm, ▲ 7,000 rpm, ● 10,000 rpm, ◆ SPG membrane (10 μm).

measurement owing to its greater sensitivity. Another reason for selecting TA instead of pH is that pH is defined as the negative log [H^+], and, consequently, pH change would not have been zero-order. It can be observed in Fig. 5 that the TA increased with time and followed a first-order reaction [17].

As can be observed in Table 2, the linearity (the higher the R^2 value, the better the accuracy of its predictions) of a TTI made with beads from the SPG membrane emulsification method was higher than TTIs produced by using homogenization methods. However, among the three TTIs produced with microbeads from homogenization techniques, poor linearities were observed for the 5,000 and 7,000 rpm treatments. Subsequently, microbeads generated by the SPG membrane technique and using homogenization at 10,000 rpm were selected for adjusting the E_a of the microbial TTI.

Adjustment of TTI Response Rates (pH and TA) by Varying the Quantity of the Microbeads Generated by the SPG Membrane Technique and Using Homogenization at 10,000 rpm

Figs. 6 and 7 illustrate that pH declined and TA increased with increasing microbead quantities (0.5 g → 1.0 g → 1.5 g) and temperatures (20°C → 25°C → 30°C). The time it took to reach the maximum TA production by TTIs containing 0.5, 1.0, and 1.5 g microbeads prepared with the SPG membrane method was found to be 36, 32, 28 h at 20°C; 20, 16, and 14 h at 25°C; and 14, 10, and 8 h at 30°C, respectively. Conversely, in the case of homogenization methods, the times were 48, 40, and 32 h at 20°C; 32, 28, and 20 h at 25°C; and 20, 16, and 10 h at 30°C, respectively. As mentioned above, TA is a more reliable indicator than pH measurement; consequently, the reaction rates in TA were estimated using the fit of the linear regression. They were found to be in proportional relationships with microbead quantities used in the TTI formulations for all temperatures. The results indicated that the endpoint of the microbial TTI using alginate microbead-entrapped microorganisms can be adjusted by changes in the quantity of the microbeads added to the microbial TTI.

The shelf-life adjustment of microbial TTIs is very difficult

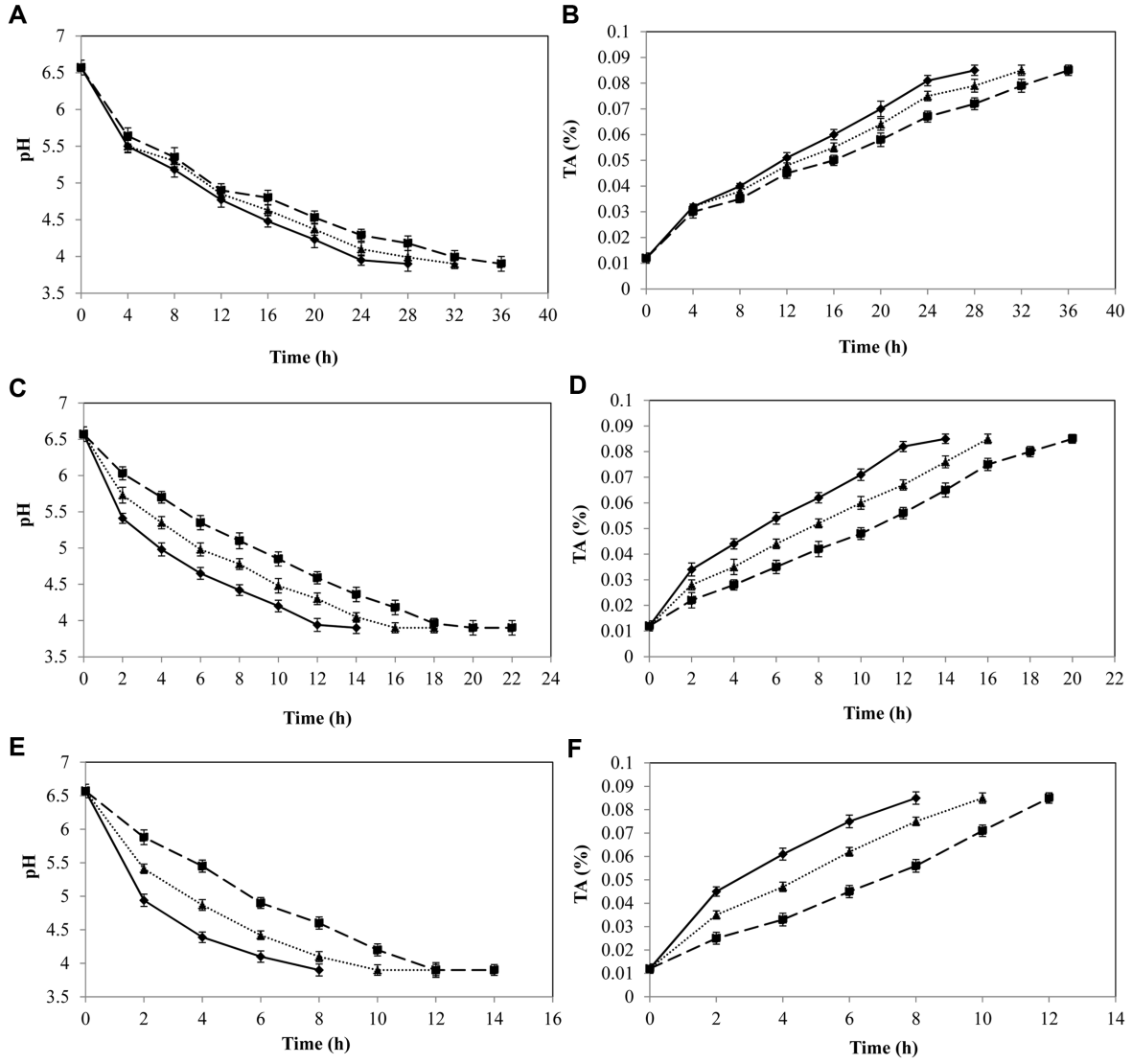

Fig. 6. Adjustment of TTI response rates (pH and titratable acidity) with different quantities of the microbeads prepared using the SPG membrane emulsification method at 20°C (**A** and **B**), 25°C (**C** and **D**), and 30°C (**E** and **F**).
■ 0.5 g/10 ml, ▲ 1.0 g/10 ml, ◆ 1.5 g/10 ml.

[38, 40], because although a given concentration of microorganisms is formulated in a TTI, their concentration changes as a result of growth. Traditionally, the adjustment of microbial TTI shelf-life has been attempted by varying the variety of carbon sources and the initial cell concentration. However, these treatments were not sufficient to cover the shelf-life of a variety of foods owing to limitations in altering the cell growth rates.

Consequently, the reaction rate of microbial TTIs was adjusted by changing the quantities of microbeads produced by employing SPG membrane emulsification technology [6]. It has previously been reported that the uniformity, size, and size distribution of microbeads may be key factors for controlling the reaction kinetics of microbial TTIs accurately and precisely. In the case of SPG membrane technology, a large variety of parameters, including membrane pore size, surfactants, pressure, and cross-flow rates, are known to influence the uniformity, bead size, and size distribution. However, most research has agreed that during the production of emulsions, the diameter of the droplets produced depends solely on the diameter of the pores of the membrane [12, 22]. A linear relationship between the sizes of the emulsion droplets and the membrane pores has previously been reported [21]. It is only possible to obtain uniform-sized particles when a marginal distribution of membrane-pore sizes is used [5].

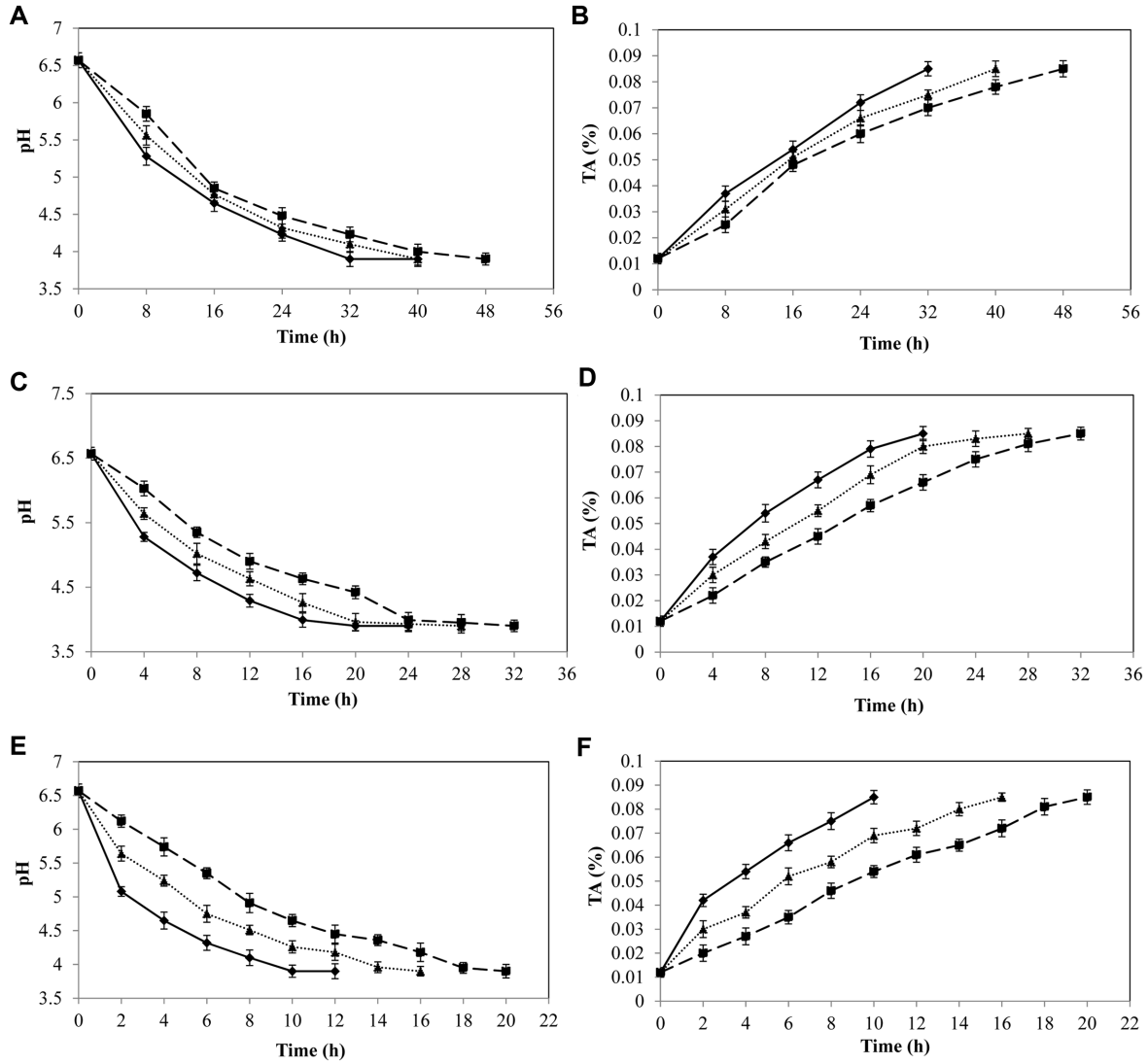

Fig. 7. Adjustment of TTI response rates (pH and titratable acidity) with different quantities of microbeads prepared using a homogenization speed of 10,000 rpm at 20ºC (**A** and **B**), 25ºC (**C** and **D**), and 30ºC (**E** and **F**).
■ 0.5 g/10 ml, ▲ 1.0 g/10 ml, ◆ 1.5 g/10 ml.

Table 3. Comparison of the kinetics and Arrhenius parameters of the TTIs (titratatable acidity) with different quantities of microbeads produced using the homogenization and SPG membrane emulsification techniques.

Emulsification methods	Microbead content of TTI (g/10 ml TTI base)	Temp. (°C)	k (TA/h)[a]	R^{2} [b]	E_a (kJ/mol)
SPG membrane	0.5	20	0.0018	0.99	86.0
		25	0.0037	0.99	
		30	0.006	0.99	
	1.0	20	0.002	0.99	83.5
		25	0.0041	0.99	
		30	0.0064	0.99	
	1.5	20	0.0023	0.99	76.6
		25	0.0044	0.99	
		30	0.0067	0.98	
Homogenization	0.5	20	0.0012	0.95	85.5
		25	0.0024	0.97	
		30	0.004	0.97	
	1.0	20	0.0018	0.97	73.5
		25	0.0034	0.97	
		30	0.005	0.97	
	1.5	20	0.0023	0.96	62.2
		25	0.0041	0.96	
		30	0.0054	0.97	

[a]TTI response rate.

[b]Coefficient of determination.

An exceedingly small pore size can generate a very small droplet but this may have a negative influence on the duration of particle formation time, whereas larger and less-uniform particles may be generated if a very large pore size is used [41]. Consequently, selection of the optimal pore size is very important for obtaining uniform-sized particles. Commercially, a wide range of tubular-shaped SPG membrane pore sizes, ranging from 0.1 to 20 μm, are available [5], whereas the diameter of the membrane pore used for developing the microbial TTI was 20 μm [6, 25], which may not be optimal. Moreover, investigation of temperature dependency, which is very important for higher accuracy because TTI-attached foods are known to experience different temperatures during storage or transportation, has not been performed. Consequently, among the four different pore sizes (1, 5, 10, and 20 μm) that are available in the laboratory, a membrane with a pore size of 10 μm was utilized in this study for making microbeads in order to develop a microbial TTI.

A regression analysis using the Arrhenius equation (E_a) was performed on the data of reaction rates versus temperatures (Table 3) to estimate activation energies. The linearity of TTIs with different quantities of 2% alginate beads made using SPG membrane technology was higher at different temperatures than the TTIs with microbeads made using a homogenization speed of 10,000 rpm. The Ea values of TTIs containing 0.5, 1.0, and 1.5 g microbeads prepared with the SPG membrane and the homogenization emulsification methods were estimated to be 86.3, 83.5, and 77.9 kJ/mol, and 85.9, 73.5, and 60.3 kJ/mol, respectively. Although both of the TTIs with microbeads prepared using the SPG membrane and the homogenization emulsification methods fulfilled the applicability requirements of TTIs (±25 kJ/mol), the SPG technology is much more efficient in controlling the temperature dependence and, consequently, the shelf-life of the microbial TTIs.

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