The gradient plate technique as a means of studying the recovery of heat-injured *Brochothrix thermosphacta*

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Summary Bacterial recovery from heat injury is influenced strongly by the nature of the recovery medium used to resuscitate cells. This study used agar gradient plates, in combination with image analysis, to study the synergistic effect of NaCl concentration (% w/v) and pH on the recovery of *Brochothrix thermosphacta* after heat treatment. Initially, exponential, early stationary and late stationary phase cultures of *B. thermosphacta* grown in all-purpose tryptone (APT) broth at 25 °C were heat-treated at 50 °C to measure thermal resistance. Late stationary phase cultures were found to be 2–3 times more heat-resistant than the other two, with a D-value of 14.8 min. Exponential and early stationary phase cultures were thermally treated and inoculated onto agar gradient plates (modified APT medium) and incubated at 25 °C. In the instance of the late stationary phase culture, there proved to be too low a cell concentration to obtain confluent growth. These plates had gradients of pH (4.0–7.4) and NaCl (1.5–8.1% (w/v)) running at right angles across them. After 48 h incubation, bacterial growth on these plates was measured by image analysis. In all bacterial cultures (heat-treated or control), optimal growth was found at pH 6.8 and 1.5% NaCl (w/v) concentration. The range of salt concentrations and pH values over which growth could be observed was shown to be reduced as a consequence of heat treatment. Overall, it is suggested that the gradient plate technique, in combination with image analysis, could be useful in determining combinations of different environmental factors which are effective in preventing the recovery of heat injured bacterial cells.

Keywords Combined effect of pH and NaCl, culture age, heat resistance, image analysis.

Introduction

A large number of pre-prepared food products, such as *sous vide* cooked meat products, depend on mild heat treatments for their microbiological safety. As a consequence of the low temperatures used, some concern has been expressed about the risks involved (Church & Parsons, 1993). To ameliorate such concerns, much work has been carried out to examine the thermal resistance of pathogenic bacteria. Examples of such studies can be seen in Roberts *et al.* (1996). The effect of differing types of media on the recovery of bacteria after heat treatment is also of great interest (Taormina *et al.*, 1998). The fact that small variations in media components can substantially influence the growth of damaged microorganisms was first reported by Wright (1917) in relation to the determination of phenol coefficients. Nelson (1943) pointed out that heat-injured bacteria are more exacting in their growth requirements than unheated organisms. Since then, numerous studies have demonstrated the increased sensitivity of heat-injured bacterial cells to changes in their environmental conditions, including salt concentration (Busta & Jezeski, 1963; Beuchat & Lechowich, 1968), incubation temperature (Ewald

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& Eie, 1992), media pH (Clavero & Beuchat, 1996) and the presence of oxygen (Bromberg *et al.*, 1998).

The use of the two-dimensional gradient plate to map the response of a bacterium to two different environmental conditions was first described by Caldwell & Hirsch (1973) and later developed by Wimpenny & Waters (1984). That study used a square agar plate with gradients of pH and NaCl concentration (% w/v) running across it to record the limits of growth of several different types of bacteria. McClure *et al.* (1989) compared data from gradient plates to that obtained from liquid cultures and concluded that they provide a good illustration of the conditions required to inhibit the growth of *Listeria monocytogenes*. In a subsequent study, image analysis was used to map bacterial growth by placing agar plates inoculated with *Salmonella typhimurium* on a lighting stage, and using a camera to convert the light transmitted to absorbency (Peters *et al.*, 1991). This proved to be a rapid and accurate means of recording growth. Furthermore, it allows simultaneous observation of the combined effect of different environmental variables. For example, Peters *et al.* (1991) showed that the minimum salt concentration required to inhibit the growth of *S. typhimurium* decreased with pH below 6.1.

The present study used gradient plates, with gradients of pH and NaCl concentration, to test the hypothesis that the recovery of heat-injured bacterial cells could be determined using image analysis to monitor growth. The food spoilage bacterium, *Brochothrix thermosphacta*, was used as a model bacterium for the purpose of demonstrating the application of this technique.

Materials and methods

Organisms and media

B. thermosphacta MR 165 (NCFB 2891) was maintained on Nutrient Agar (Oxoid, Basingstoke, UK) slopes at 50 °C. The working culture was grown at 25 °C for 18 h on brain heart infusion agar (Oxoid) and stored at 5 °C. The growth medium, all-purpose tryptone (APT), contained the following (g L^{-1} in distilled water): Tryptone (Oxoid) 10.0; yeast extract (Oxoid) 5.0; NaCl 5.0; K_2HPO_4 5.0; Na_3citrate $2H_20$ 4.0; $MgSO_4$ $7H_2O$ 0.8; $FeSO_4$ $7H_2O$ 0.04. The pH was adjusted to 7.0. Glucose solution was autoclaved separately and added to the growth medium to give a final concentration of 1% (w/v).

A vegetative inoculum was prepared by inoculating 100 mL APT broth with six loopfuls of bacteria from the working culture and shaking continuously at 200 r.p.m. for 18 h at 25 °C. From this inoculum, 10 mL was added to 90 mL fresh APT broth and incubated under the same conditions to produce the secondary inoculum. Different culture ages of secondary inoculum, i.e. exponential phase (5 h, 10^8 c.f.u. mL^{-1}), early stationary phase (18 h, 10^9 c.f.u. mL^{-1}) and late stationary phase (72 h, 10^6 c.f.u. mL^{-1}) were used to study bacterial heat resistance.

Thermal inactivation studies

One mL of culture was added to 50 mL of preheated APT broth contained in bottles immersed in a water bath at a temperature of 50 °C. The media temperature at the centre of the bottle was measured to an accuracy of ± 0.1 °C using a thermocouple. Samples of 1 mL were removed periodically and mixed with 1 mL 0.9% NaCl (w/v) (immersed in ice). Survivor cell numbers in the samples were determined by plating serial dilutions onto nutrient agar plates (pH 6.8, 0.5% NaCl (w/v)). Viable numbers were estimated from colony counts after an incubation time of 24–48 h at 25 °C. D-Values were calculated from the absolute value of the inverse slope of a regression line in the declining linear portion obtained from a plot of $\log_{10}$ of survival count against inactivation time. The lengths of the shoulder regions were calculated from the intercept of a horizontal line drawn from the initial value and the linear regression line. All experiments were performed in quadruplicate.

Heat-injury treatment

Two mL of exponential phase (5 h) or early stationary phase (18 h) culture were introduced into 25-cm-long capillary tubes. The tubes with culture were heated in a water bath at 50 °C for 5, 10 and 15 min for the 5-h culture and 2 and 7 min for the 18-h culture. After heat treatment, tubes were removed and immediately immersed in an ice

bath. The control experiments (no heat treatment) were conducted using the same procedure, i.e. introducing 2 mL of culture into capillary tubes and then placing these directly into an ice bath.

Gradient plate preparation

All plates were prepared in Sterilin wettable 10 × 10-cm square Petri dishes. The medium used contained the following (g L^{-1} in distilled water): tryptone 5.0; yeast extract 3.0; $MgSO_4$ 7-H_2O 0.8; $FeSO_4$ $7H_2O$ 0.04; agar 15. Each plate comprised four layers (see below) which were poured in the manner described by Wimpenny & Waters (1984).

Layer 1 (acid) 11.7 mL medium + 0.3 mL 1 M HCl.

Layer 2 (alkaline) 17.9 mL medium + 0.15 mL 1 M NaOH.

Layer 3 (salt) 13.4 mL salt medium (contained 21.6 g NaCl/100 mL medium) + 0.6 mL 25% glucose (w/v).

Layer 4 (plain) 15.4 mL medium + 0.6 mL 25% glucose (w/v).

Glucose was added to the medium used in layers 3 and 4 to give a final glucose concentration in the plate of 0.5% (w/v). The medium for each layer was prepared separately in McCartney bottles (20 mL). The media were heated to boiling point to melt the agar before autoclaving at 121 °C for 15 min. The glucose solution, HCl and NaOH were autoclaved separately. Once autoclaved, the media were maintained at 70 °C in a water bath until they were ready to be poured in order to avoid solidification of the agar.

The gradient plates were formed by vertical diffusion and subsequent equilibration of the NaCl and hydrogen ions after 24 h. Once formed, the gradients run horizontally across the plates at right-angle to each other. The pH gradients were determined with a flat-ended pH electrode (±0.01 pH unit) at intervals of 1 cm across the length of the plate (only the central, 9 cm × 9 cm, area was measured to reduce the edge effects).

The NaCl gradients were measured by taking samples of agar from the central 9 cm × 9 cm area on the plate and melting these in a known volume of distilled water and measuring the conductivity. A calibration curve was used to convert this to NaCl concentration (% w/v). The calibration curve was plotted using conductivity readings of samples containing known NaCl concentrations (% w/v). Gradients of both NaCl and pH were found to be approximately linear across the length of the plate with excellent regression coefficients of 0.99 and 0.98, respectively. The relationship between NaCl (% w/v) or pH as a function of distance (d, cm) along the plate were found to be as follows:

$$\text{NaCl (\%w/v)} = 0.77 \cdot d + 1.32$$

$$\text{pH} = 0.39 \cdot d + 3.92$$

The measured gradients were taken as average values from three plates. Growth on the gradient plate was measured every 0.5 × 0.5 cm, therefore the distance used in these calculations was at the centre of each area, i.e. 0.25, 0.75, 1.25,... cm. The NaCl gradient was found to vary between 1.5 and 8.0% w/v and that for pH between 4.0 and 7.4. These gradients cover the range of pH values and water activities of many food products. Gradients were found to be acceptably stable up to 48 h.

Inoculation of gradient plates

The experiments were performed in duplicate. Bacterial culture from the capillary tube (2 mL) was poured over the surface of the gradient plate, and excess fluid removed. The plates were allowed to stand for a few minutes, and then incubated at 25 °C for 48 h.

Mapping the growth and image analysis

A high-sensitivity CCD camera was attached to an image analyser (Photonics Science, East Sussex, UK). Images were captured and processed using Image-Pro Plus (Media Cybernetics, MD, USA) imaging software. As the distribution of light on the lighting stage was not uniform across the whole plate, each plate was analysed in four parts (each of 4.5 × 4.5 cm). A square was marked out in the centre of the lighting stage. The first quarter was placed in the light box on this square. The image was captured and the other quarters were processed in the same way. An image of an uninoculated plate obtained this way was used as background (blank). The bacterial culture was

inoculated onto this plate, which was then monitored at regular time intervals. The blank image was subtracted from the images captured during growth. The resulting grey scale values were converted to Microsoft Excel files for further processing. Using macros programmed in Excel, average greyscale readings from each 0.5×0.5 cm area were calculated and converted to optical density (OD). An area where there was no growth was selected from the image and the mean optical density determined. This value was used to re-scale the data, so that zero represented no growth. After careful examination of the data, it was concluded that evidence for bacterial growth could be reported for regions of the gradient plate with optical density values of 0.10 or higher.

Results and discussion

Thermal inactivation of bacteria

Survival curves and thermal resistance data of *B. thermosphacta* cultures of different ages after heating at 50 °C are shown in Fig. 1 and Table 1, respectively. The data presented are average values from four repeat experiments. The 5- and 18-h cultures showed a shoulder (lag period) followed by an exponential decrease. The lag time for the 18-h culture was much shorter than that for the 5-h culture and although the D-values are similar they were found to be significantly different ($P < 0.05$). The 72-h culture showed a different profile, with a continuous exponential decrease at an almost constant rate. This culture was found to be 2–3 times more heat-resistant than the other cultures, which is consistent with results reported in the literature for other bacteria. It is generally believed that cells which grow at a slower rate are more heat-resistant than those growing rapidly (Eliker & Frazier, 1938; Condon *et al.*, 1992). The increased heat resistance is believed to be due to the synthesis of proteins which provide protection against environmental stresses (Jenkins *et al.*, 1988).

The length of the shoulder region is longer for the 5-h culture than the 18-h culture. The 5-h culture thus appears more heat-resistant than the 18-h culture. The observation that exponential phase cells can be more heat-resistant than early stationary phase cultures has not, to the authors' knowledge, been reported previously. A number of different studies have identified cell ribosomes as the critical site for thermal injury (Iandolo & Ordal, 1966; Teixeira *et al.*, 1997). As exponential phase cells will be rich in ribosomes, it is possible that they may require a longer exposure to heat before sufficient damage is incurred such that they

Table 1 The thermal resistivity of cultures of *Brochothrix thermosphacta* of differing ages at 50 °C

Culture age (h)	Lag time (min) Mean ± SD	D-value (min) Mean ± SD	Time to obtain 4 log decrease (min) Mean ± SD
5	9.1 ± 1.7	5.7 ± 0.6	31.9 ± 2.2
18	1.3 ± 1.1	4.7 ± 0.3	20.9 ± 0.4
72	–	14.8 ± 1.6	62.7 ± 7.5

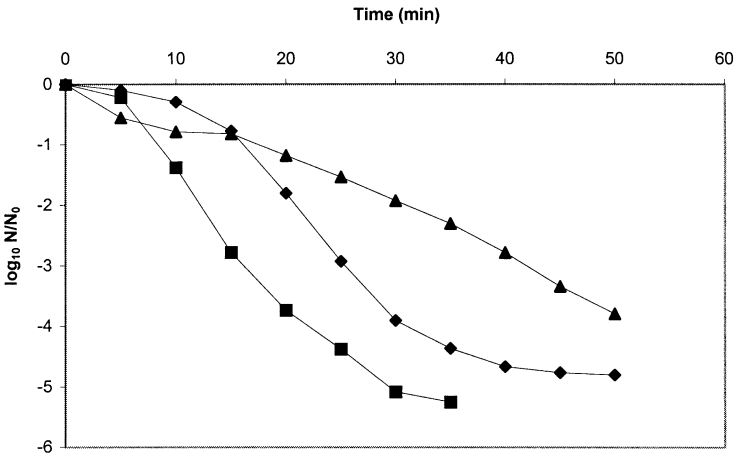

Figure 1 Survivor curves of different cultures (average from quadruplicate) of *Brochothrix thermosphacta* after heat treatment at 50 °C.

cannot repair themselves. To confirm this hypothesis, further experiments would be required.

The effect of NaCl and pH on the recovery of heat-injured cells

Experiments were performed in which cultures of *B. thermosphacta* of different ages were heated and then allowed to recover on agar gradient plates. Figures 2 and 3 show the representative contour maps of bacterial growth after 48 h incubation. Figure 2 illustrates results for the 5-h culture. Results for the unheated (control) culture are shown in Fig. 2a where it can be seen that growth occurred at salt concentrations up to 6.9% (w/v) and pH values as low as 4.8. This plate fails to fully illustrate the limits of growth for this bacterium. Brownlie (1966) reported that *B. thermosphacta* could grow at considerably higher salt concentrations. This was confirmed by our own liquid culture experiments (Rattanasomboon, 2000), where we found that growth could occur at salt concentrations of 9% (w/v) but not 10% (w/v) (under conditions of optimal pH and temperature, i.e. pH 6.8 and 25 °C). The growth limit value for pH reported here agrees well with liquid culture data. It has been pointed out by other workers (McClure *et al.*, 1989) that a limitation of the gradient plate technique is that experiments can only be performed over a relatively short time frame, owing to the diffusive breakdown of the gradients. Hence, if growth is very slow, it will not be detected.

The effect of heat treatment on the growth of these cultures can be seen in Fig. 2b–d. On all the plates tested, the highest ODs recorded were at pH 6.8 and the lowest salt concentration. As expected, the optimal conditions for recovery were observed under the best conditions for the growth of healthy cells. When exponential phase cells were subjected to 5 or 10 min of heat treatment (Fig. 1), there was very little reduction in cell numbers when enumerated by plate counts on nutrient agar (pH 6.8, salt concentration of 0.5% (w/v)). However, the diagrams (Fig. 2) clearly illustrate reduced OD values under optimal growth conditions, indicating the presence of a lag phase slowing the re-growth of heat-injured cells. The

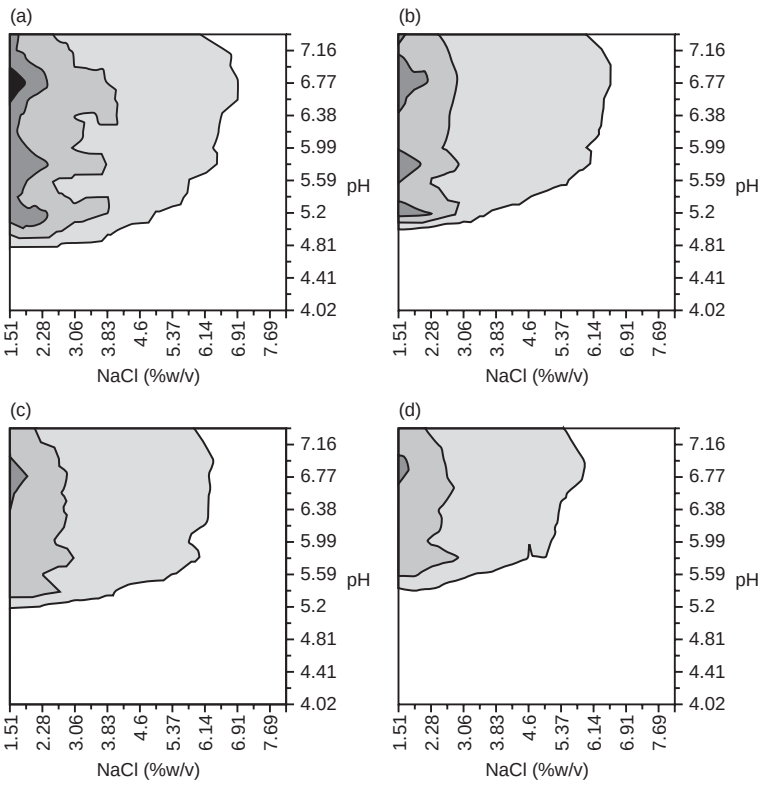

Figure 2 Contour map of the growth zone of 5 h (exponential phase) culture of *Brochothrix thermosphacta* after (a) 0, (b) 5, (c) 10 and (d) 15 minutes of heat treatment. □ 0.1 ≤ OD ≤ 0.2; ▒ 0.2 ≤ OD ≤ 0.3; ▓ 0.3 ≤ OD ≤ 0.4; ■ 0.4 ≤ OD ≤ 0.5.

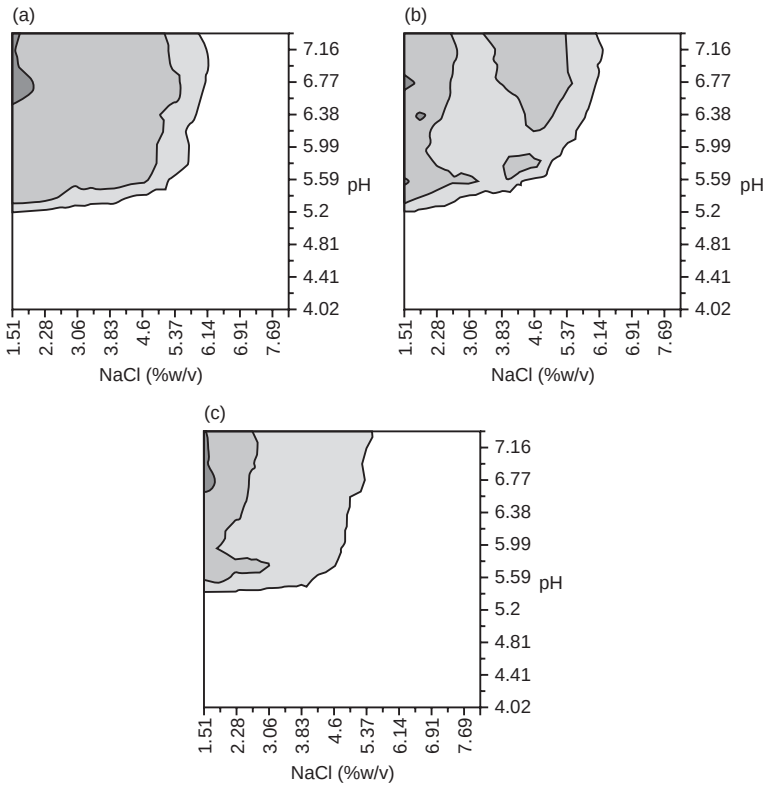

Figure 3 Contour map of the growth zone of 18 h (early stationary phase) culture of *Brochothrix thermosphacta* after (a) 0, (b) 2 and (c) 7 minutes of heat treatment. □ $0.1 \leq OD \leq 0.2$; ▒ $0.2 \leq OD \leq 0.3$; ■ $0.3 \leq OD \leq 0.4$.

highest salt concentrations and lowest pH values over which growth was observed to occur were also altered, to salt concentrations of 6.5% (w/v) and 6.1% (w/v) and pH values of 5.0 and 5.2, for 5- and 10-min heat treatments, respectively. Heat treatment of exponential phase cells for 15 min was sufficient to cause a 1 log reduction in cell numbers (Fig. 1). In spite of this, under conditions of neutral pH and low salt concentration, very similar OD values were reported to those observed for bacteria subjected to 5- or 10-min heat treatment. However, the range of salt concentrations and pH values over which growth occurred were further reduced, to a minimum pH value of 5.4 and maximum salt concentration of 5.8% (w/v). This illustrates that higher salt concentrations and lower pH values have a more pronounced effect on inhibiting the growth of heat-injured cells.

Figure 3 illustrates similar results for the 18-h culture, where 2 min heat treatment corresponds to little change in cell numbers and 7 min to approximately 1 log reduction. The range of conditions over which growth could be seen for the non-heat-treated 18-h culture was less than that found for the 5-h control culture. As the 5-h control culture comprised exponential phase cells, they would be expected to be more active and hence more growth would be seen over the limited time course of the experiment. The effect of heat treatment on the 18-h culture was qualitatively similar to that found for the 5-h culture, with a small reduction in the bacterial growth zone being observable. For both 5- and 18-h cultures, there was no evidence to indicate that the combination of a reduced pH and higher salt concentration has a particularly pronounced effect on bacterial recovery. This shows that the combined effect of low pH and high salt concentration alone may not be a good means of preservation of foods subjected to mild heat treatments.

Attempts were also made to grow the 72-h (late stationary phase) culture on an agar plate. However, the cell number density of this culture was found to be too low to observe a bacterial film growing on the gradient plate. We consider that cell concentrations of at least 10^7 c.f.u. mL^{-1} are required in order to be able to analyse bacterial growth using gradient plates in combination with

image analysis technology. This is a further limitation of the gradient plate technique. However, it might be possible to get the higher viable inoculum concentration by centrifugation and resuspension in a smaller volume.

The results demonstrate that heat-injured cells of *B. thermosphacta* exhibit growth at slightly lesser maximum salt concentrations and slightly higher minimum pH values than non-heat-treated cultures, as visualized using gradient plate technology. This is consistent with other published findings, where relatively low pH values or high salt concentrations are required to inhibit the recovery of heat-injured bacteria. Clavero & Beuchat (1996) demonstrated that lower pH values had a pronounced effect on the recovery of heat-injured *Escherichia coli* 0157:H7, but only when the pH was as low as 4.8. Gonzalez *et al.* (1996) found that when the pH of the recovery medium was reduced from neutral to 5.4, the ability of *Bacillus cereus* to survive heat treatment was not impaired in all strains. This caused the authors to question whether low pH foods treated by mild thermal processes have an additional safety margin provided by acidity. Beuchat & Lechowich (1968) demonstrated that high salt (NaCl) concentrations (6% (w/v) or higher) in the recovery medium were required to inhibit the recovery of heat injured *Streptococcus faecalis*.

Image analysis technology has found numerous applications in fundamental microbiological studies, including the effect of environmental factors on competition between different bacteria (Thomas & Wimpenny, 1993), mass transfer and growth kinetics of fungi (Lopez-Isunza *et al.*, 1997) and the ecology of marine bacteria (Pernthaler *et al.*, 1997). This is the first study to use image analysis, in combination with gradient plates, to study the recovery of heat-injured bacteria. Using *B. thermosphacta* as a model bacterium, some expectedly small effect could be seen on recovery as a consequence of reduced pH and increased salt concentration. However, it is a drawback of the gradient plate experiment that slow bacterial growth could not be detected on the plate within the time frame allowed due to the breakdown of gradients. As a result of this, the experiments could not be conducted at low, i.e. refrigeration temperatures (which is a major hurdle for the preservation of *sous vide* products). With respect to difficulties in detection of low cell concentrations, the details of growth over the unfavourable conditions could have been expanded by replicating the gradient plate after incubation onto a non-gradient, i.e. uniform plate (no added salt and neutral pH) and incubating at the same temperature. The growth zone from the replica plate would be greater than that of gradient plate. The additional growth would probably occur mainly on the growth boundary and would thus reveal the presence of viable cells which had not grown to a detectable optical density on the gradient plate. Overall, despite its limitation, the information of bacterial growth obtained from gradient plates might indicate some interesting combinations that could be examined in future liquid culture studies.

We have shown that the combination of the gradient plate technique and image analysis offers a way of screening growth conditions rapidly. There is the possibility of extending the present work to other food preservation techniques. For example, a number of naturally occurring compounds have been shown to have anti-microbial properties (Jay, 1996). It has already been demonstrated that the presence of nisin (Boziaris *et al.*, 1998) or sodium lactate (McMahon *et al.*, 1999) in the recovery medium has a significant effect on the recovery of sublethally injured pathogenic bacteria. These, or other potential food additives, can be introduced into gradient plates in combination with a salt or pH gradient. The application of this technology to study heat-injured food-borne bacteria could provide a wealth of information on the effect of combinations of differing environmental factors on bacterial recovery.

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