# Effects of ice-nucleation active bacteria on the freezing of some model food systems

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**Summary** Cells of ice-nucleation active (INA) bacteria, *Pseudomonas syringae* and *Erwinia herbicola*, were cultured at 18°C with media of nutrient broth and/or yeast extract and harvested at late log phase for maximum ice nucleation activity. These cells were able to nucleate water to freeze at temperatures as high as −2°C. They were incorporated into model food systems, including sugar, protein solutions and oil/water suspensions, representing all major components of foods, to investigate their effects on freezing. The nucleation temperatures of all the treated models were significantly raised by between 3.0 and 5.9°C compared with controls when the freezer temperature was set at −6 to −7°C. The application of the INA cells also caused freezing of certain model solutions at −6°C, such as sucrose solution (10%), which did not freeze at the same conditions without INA bacterial cells. Additions of INA cells also shortened the total freezing time of the model systems by between 20 and 38%. These results suggest that with the application of bacterial ice nucleation, some current food freezing processes may be modified to operate at higher subzero temperatures to provide guaranteed freezing, energy savings and improvement of efficiency and product quality.

**Keywords** Energy conservation, *Erwinia herbicola,* food preservation, freeze-facilitation, freezing temperature/time, *Pseudomonas syringae.*

## Introduction

Ice nucleation by species of the bacterial genera, *Pseudomonas*, *Erwinia* and *Xanthomonas*, is a major factor in frost injury to many frost-sensitive plants (Lindow, 1983). These plant pathogenic bacteria live on plant surfaces and initiate ice formation at temperatures as high as −1°C. The ice formed spreads quickly and mechanically disrupts the tissue, causing frost damage to the plants. Frost injury has been described as one of the main limiting factors to crop production in many locations in the temperate zone. Much attention has been paid to the alleviation and elimination of the frost injury due to bacterial ice nucleation (Lindow *et al*., 1978; Wolber, 1993). However, bacterial ice nucleation at higher temperatures may have important application in the freezing of foodstuffs (as reviewed by Watanabe & Arai, 1994; Li & Lee, 1995), where ice nucleation is usually limited in higher subzero temperatures (–2 to −5°C).

Arai & Watanabe (1986) studied the freeze texturing of proteins, such as egg white and soybean protein isolate, with the ice nucleation active (INA) cells of *Erwinia ananas*, and found that a flake-like texture was obtained. Ryder (1987), Ryder & Lee (1988) studied the effects of INA *P. syringae* on the freezing of salmon muscle. It was found that the nucleation temperature of fish fillet was elevated from −4.9 to −1.5°C, which means that the degree of supercooling (the temperature difference between the freezing-point temperature and the nucleation temperature of the sample, as illustrated in Fig. 1) was reduced by 3.4°C in this case. The total freezing time (defined as the time

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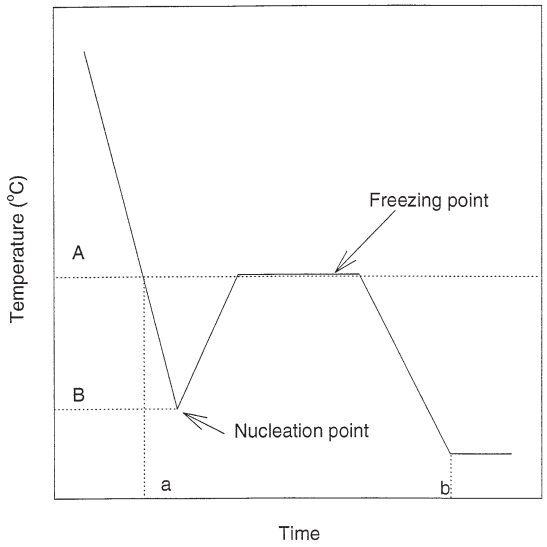


**Figure 1** A hypothetical freezing curve of water. Degree of supercooling is defined as the temperature difference between freezing point (A) and nucleation point (nucleation temperature) (B). Total freezing time is defined as the time difference between the time (a) when the temperature of water passes the freezing point and the time (b) when it reaches the freezer temperature (see text for more details).

elapsed from the instant the sample passed through its freezing-point temperature to the instant it reached the freezer temperature) for salmon muscle was reduced 33% by the addition of INA bacterial cells, when frozen with a freezer temperature of −5°C. Watanabe & Arai (1987) also attempted to apply the INA *E. ananas* cells to the freeze-drying of high salt-containing foods. It led to a shortening of their freezing time and efficient formation of powdered products. More recently, Clausse *et al.* (1991) found that the supercooling of emulsions in microsize droplets was reduced when *P. syringae* cells were added. The kinetics of heterogeneous nucleation by freeze-dried *P. syringae* and homogeneous nucleation of ice in aqueous systems including emulsions were investigated by Charoenrein & Reid (1989), and Ozilgen & Reid (1993) with the use of a Differential Scanning Calorimeter (DSC). They found that the reductions in heterogeneous nucleation temperature were linearly related to the solute-induced freezing point depression. These studies, however, were limited to samples with microdroplets.

*P. syringae* and *E. herbicola* were reported to be the most active ice nucleation bacteria (Lindow *et al.*, 1978; Maki *et al.*, 1974). Though it may be impractical to incorporate large quantities of bacterial cells into foods, their effects on the freezing of model food systems may provide valuable information for further application studies in the food industry. This work was undertaken to investigate the effects of INA cells on the nucleation temperatures, total freezing time and the freezing behaviour, etc., with model food systems of sucrose, egg white solutions and safflower oil/water suspensions/emulsions.

## Materials and methods

### Ice-nucleation active (INA) bacteria

*Pseudomonas syringae* pv. *pisi* (Cat. no. 11043) and *Erwinia herbicola* ssp. *ananas* (Cat. no. 11530) were both obtained from American Type Culture Collection (ATCC) (Rockville, MD). The bacteria were routinely cultured at 18°C in nutrient broth (supplemented with 2.5% (w/w) of glycerol) or in yeast extract medium, at a shaking speed of 200 r.p.m. until late log phase (Ryder, 1987; Obata *et al.*, 1990; Li & Lee, 1995; unpublished data). An absorbency of 1.0 at 660 nm was found to correspond to a cell concentration of $1.2 \times 10^9$ CFU $mL^{-1}$. The cells were then collected by centrifugation at 8000 g for 20 min. Aliquots of the collected cells were suspended in 0.85% NaCl at a population density of $10^8$ CFU $mL^{-1}$ in each. The cells were frozen at −70°C before use.

### Model food systems

Sucrose and egg white were purchased from Sigma Chemical Co., and dissolved with de-ionized distilled water at different concentrations (w/w). One millilitre of the bacterial suspension was added to 9 mL of each solution to give a cell density of $10^7$ CFU $mL^{-1}$ and mixed well on a mixer. Safflower oil (food grade) was obtained from a local supermarket, and diluted with distilled water and 1 mL of the bacterial suspension, giving ratios of 10 to 60% oil/water (v/v) and a cell density of $10^7$ CFU $mL^{-1}$, and homogenized (16 000 r.p.m.) in a Servall Omni-Mixer (Norwalk, CT) for 1 min. When the control sam-

 

ple was mixed with 1 mL of inactivated INA cells (by heating to 100°C for 5 min), we found that there was no difference in nucleation temperatures and freezing times for the controls with inactivated INA cells or without INA cells at all. Therefore, the controls reported were tested in the absence of INA cells. Ten millilitres of each solution/suspension in a Falcon polypropylene test tube (16 mm 75 mm, 15-mL capacity) (Becton Dickinson and Company, Franklin Lakes, NJ) were subjected to a freezing test. Four separate tubes were tested for each sample.

### Ice nucleation activity test

Ice nucleation activity of the bacterial suspension was measured at constant supercooling temperatures with a droplet method modified from Vali (1971). The bacterial suspension was first diluted with a buffer of 20 mM Tris (pH 7.6) containing 10 mM $MgCl_2$. Thirty drops, each of 10 μL, were produced with an automatic pipette on an aluminium weighing dish, which were then held at a given supercooling temperature for 3 min. The frozen drop number was counted and recorded. The ice nucleation activity unit per mL was calculated with the following formula,

$$\text{INA}\ (T) = \text{D}\ f/\text{V}$$

where, INA ($T$) is the ice nucleation activity at a given supercooling temperature, $T$°C, units $mL^{-1}$; D is the dilution factor from the original suspension; $f$ is the frozen fraction of the total drops, that is the frozen drop number divided by the total drop number, let $f < 40\%$; and V is the volume of an individual droplet (mL).

The ice nucleation activity was expressed as activity units/$OD_{660}$ at a specific supercooling temperature, that is, the total amount of ice nucleation activity units per unit volume are divided by the $OD_{660}$ value per unit volume.

### Freezing study

The samples were subjected to freezing in a custom-designed ScienTemp Lo-Cold Model Freezer (ScienTemp Co., Adrian, Michigan) at −6°C unless otherwise indicated. The temperature of each sample was monitored with a Type T Copper-Constantan Thermocouple (Omega Engineering Inc., Stamford, CT), placed in the geometric centre of the test tube and connected to a WB-AAI data acquisition system (Omega Engineering Inc., Stamford, CT), which was interfaced with an IBM 386 compatible computer through the QuickLog PC Data Acquisition and Control Software (Strawberry Tree, Sunnyvale, CA). Four separate measurements were tested for each sample.

### Nucleation temperature and total freezing time

The ice nucleation temperature was defined as the minimum temperature attained by the sample before freezing occurred (Reid, 1983). On the freezing curve (Fig. 1) it is the temperature point at which the sample temperature starts to return to its equilibrium temperature or freezing point. The total freezing time was defined as the time period elapsed from the instant the sample temperature passed through its freezing-point temperature to the instant it reached the freezer temperature after freezing.

## Results and discussion

The ice nucleation activities of *P. syringae* and *E. herbicola* were evaluated at constant supercooling

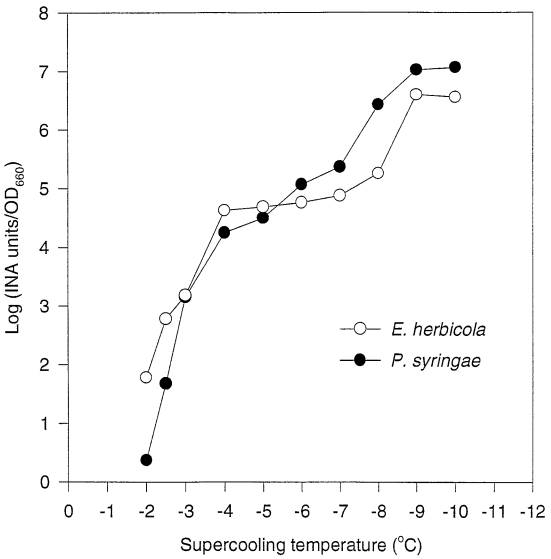


**Figure 2** Ice nucleation spectra of the INA cells of *P. syringae* and *E. herbicola.* The cells were suspended in 20 mM Tris buffer (10 mM $MgCl_2$) and diluted serially for each supercooling temperature for the activity tests as described in Materials and methods.

temperatures from −2 to −10°C. The ice nucleation spectra are shown in Fig. 2. It is apparent that the ice nucleation activity (INA) increases dramatically as the supercooling temperature decreases. This means that at a lower temperature, more and more bacterial cells are available to serve as active ice nucleators. At −3°C, for example, the INA of *E. herbicola* is $10^3$ units/$OD_{660}$ cells; at −6°C, however, the INA increases to $10^5$ units/$OD_{660}$ cell. This indicates that bacterial cells have a stronger ice nucleation activity at lower supercooling temperatures which confirms previous reports (Maki *et al.*, 1974; Lindow *et al.*, 1978).

The effect of cell concentration on the nucleation temperature of water was also investigated. A concentrated cell suspension (~$10^{10}$ CFU $mL^{-1}$) was diluted serially by a factor of 10 to give a cell concentration of 10, $10^2$, ... $10^8$, CFU $mL^{-1}$, respectively. Ten millilitres of each suspension were subjected to freezing at −6.0 ± 0.5°C. The nucleation temperature was found from each time–temperature curve. The results are presented in Fig. 3. It is apparent that an increase in cell concentration results in nucleation temperatures close to 0°C, the melting point of ice. However, the relationship between cell concentration and nucleation temperature is not linear (Fig. 3). For a significant increase of nucleation temperature of water, it requires the presence of $10^7$–$10^8$ CFU $mL^{-1}$. With a bacterial suspension of $10^7$ CFU $mL^{-1}$, we have roughly $10^2$–$10^5$ INA units $mL^{-1}$ at above −5°C, which was demonstrated to be required for the freezing of model food systems (Ryder, 1987; Izquierdo, 1988). Watanabe & Arai (1987) also reported that a $10^6$–$10^7$ cell number of *E. ananas* in the unit volume (2 mL) gave a significant difference in the degrees of supercooling for the bulk water.

The effect of INA bacterial cells on the freezing curves of water is shown in Fig. 4. When the temperature of the freezer is set at −6°C, the temperatures of both the control and sample descend very quickly to 0°C. There is no difference at this stage (i.e. the prefreezing stage). The temperature of the control keeps descending to the freezer temperature, −6°C, and does not change thereafter during the whole process (the minor change

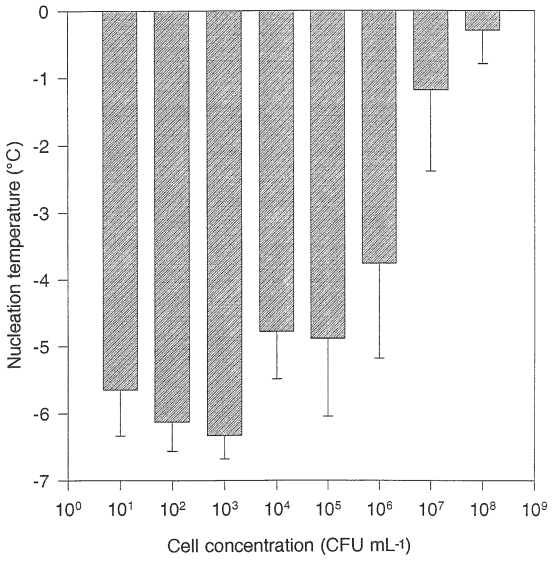


**Figure 3** Effect of cell concentration on nucleation temperatures of water. Ten millilitres of each cell suspension was subject to freezing at −6.0 ± 0.5°C. The nucleation temperature was found in each freezing curve (see Fig. 4). Results are an average of three separate measurements.

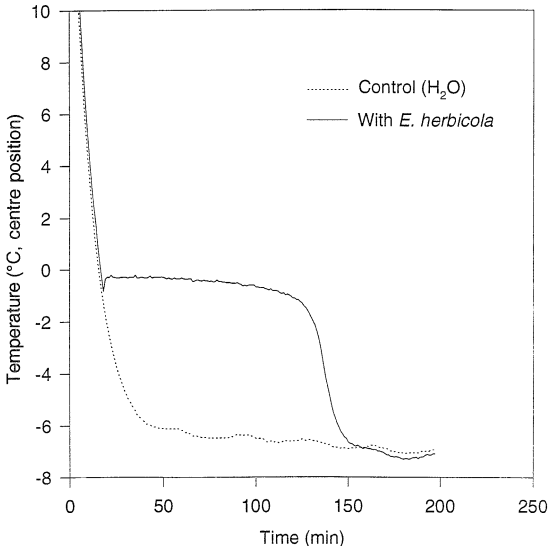


**Figure 4** Typical freezing curves of distilled water in the absence and presence of INA cells (*E. herbicola*, $10^7$ CFU $mL^{-1}$). The freezer temperature was set at −6.0 ± 0.5°C and temperatures of samples, each of 10 mL in a polypropylene tube (15 mL capacity) were continuously monitored over the time with a QuickLog PC Data Acquisition and Control Software (Strawberry Tree, Sunnyvale, CA). As shown in this figure, the control without INA cells stayed as supercooled liquid through the whole process and the sample nucleated at around −0.82°C and was completely frozen in about 140 min.

was due to the freezer temperature fluctuation). In other words, the control could not be frozen at these conditions. There was a significant difference, however, in the treated sample, with the presence of INA *P. syringae* cells. Its temperature descended a little below its freezing point to −0.82°C and then came back to 0°C, the freezing stage, for ≈100 min, and the liquid water was converted to ice completely. After this stage, the temperature of the ice descended to −6°C, the freezer temperature (Fig. 4). The total time was around 140 min, calculated from the time when the temperature of the sample passed its freezing point and the time when it reached the freezer temperature (as defined in Fig. 1). This experiment has two important implications: (1) it is possible to add INA bacterial cells to certain model food systems which are difficult to freeze at relatively higher subzero temperatures, such as −6°C, and make them freeze. In other words, the nucleation temperatures of some model food systems may be significantly elevated by the addition of the INA bacterial cells; (2) The total freezing time for different model food systems may be shortened. The following experiments were conducted to demonstrate these ideas.

As food materials have three major components other than water: carbohydrate, protein and lipid, three typical model food systems were chosen to test the effects of INA bacterial cells on their freezing: sucrose solutions (5 to 35%, w/w), egg white solutions (1 to 20%, w/w) and safflower oil/water suspensions (10 to 60%). Bacterial cell suspensions were prepared, diluted and added to corresponding model systems to give $10^7$ CFU $mL^{-1}$ in the final samples for all the tests.

The effect of the INA cells on the nucleation temperatures of sucrose solutions is presented in Table 1. It is interesting to note that with even 5% of untreated sucrose solution, the nucleation temperature (–4.6°C) is lower than that of the treated samples (–0.9°C and −0.7°C). Sucrose solutions of concentrations higher than 10% could not be frozen at −6°C, however, the addition of INA cells, either *P. syringae* or *E. herbicola*, allowed these solutions to freeze very easily at or above the freezer temperature. The difference in nucleation temperatures as affected by these two strains of bacteria for all of the sucrose solutions was not significant (lower than 0.6°C). There was a general trend that the higher the concentration of the sucrose solution, the lower the nucleation temperature.

**Table 1** Nucleation temperatures (°C) of sucrose solutions frozen at −6 ± 0.5°C in the absence and presence of INA cells[a,b]

| Sucrose solution (w/w) | Control (without INA cells) | With *P. syringae* ($10^7$ CFU $mL^{-1}$) | With *E. herbicola* ($10^7$ CFU $mL^{-1}$) |
|---|---|---|---|
| 5% | −4.6 ± 1.8 | −0.9 ± 0.3 | −0.7 ± 0.3 |
| 10% | DNF[c] | −1.8 ± 0.4 | −1.3 ± 0.2 |
| 20% | DNF | −3.0 ± 0.2 | −3.1 ± 0.2 |
| 35% | DNF | −6.0 ± 0.5 | −5.3 ± 0.1 |

[a]Four separate samples were each tested at a volume of 10 mL in a polypropylene tube (16 mm × 75 mm), and a thermocouple (Type T) tip was centred in the tube.
[b]One control out of four nucleated at −6°C.
[c]DNF means 'Did Not Freeze'.

The addition of either *P. syringae* cells or *E. herbicola* cells to the egg white solutions also resulted in a significant increase in their nucleation temperatures at −6°C (Table 2). In the absence of INA cells, egg white solutions of 1 to 9% usually nucleated at −4.3 to −5.1°C. The addition of *P. syringae* cells ($10^7$ CFU $mL^{-1}$) elevated the nucleation temperatures to −0.2 to −0.6°C; a difference of 3.7 to 4.5°C compared to the control. The effect on the elevation of nucleation temperatures by *E. herbicola* cells was less marked when compared to *P. syringae* cells (Table 2). The reason is not clear. When the concentration of egg white was higher than 20% (w/w), the control did not freeze at −6°C, but both of the treated samples froze easily. This

**Table 2** Nucleation temperatures (°C) of egg white solutions in the absence and presence of INA cells[a]

| Egg white solution[b] (w/w) | Control (without INA cells) | With *P. syringae* ($10^7$ CFU $mL^{-1}$) | With *E. herbicola* ($10^7$ CFU $mL^{-1}$) |
|---|---|---|---|
| 1% | −4.3 ± 0.3 | −0.2 ± 0.2 | −0.8 ± 0.2 |
| 5% | −4.1 ± 1.7 | −0.5 ± 0.2 | −1.2 ± 0.1 |
| 9% | −5.1 ± 0.7 | −0.6 ± 0.2 | −1.9 ± 0.1 |
| 20% | DNF[c] | −1.7 ± 0.2 | −2.2 ± 0.3 |

[a]The freezer temperature was set at −6°C ± 0.5°C.
[b]Each sample was tested for four individual times at a volume of 10 mL in a polypropylene tube (16 mm × 75 mm).
[c]DNF means 'Did Not Freeze'.

means that in the presence of INA *P. syringae* or *E. herbicola* cells, the freezing behaviour of the egg white solution was determined by the nucleation abilities of the bacterial cells. This is an advantage for freezing processes, because the freezer temperature may not need to be set as low, resulting in energy-savings.

Safflower oil, a polyunsaturated oil (77.5% linoleic acid), was tested for ice nucleation in a combination with water using *P. syringae* and *E. herbicola*. Safflower oil was prepared at ratios of 10% to 60% (v/v, oil/water) in the presence or absence of $10^7$ CFU $mL^{-1}$. These suspensions after homogenization were stable within 30–45 min, and nucleation also occurred during that time period. When the freezer temperature was set at −7.5°C, these suspensions nucleated at temperatures ranging from −0.5 to −3.0°C in the presence of *P. syringae* cells (Table 3). Untreated samples presented lower nucleation temperatures, i.e. −5.4 to −6.9°C. Due to the instability of the mixture, ice nucleation of oil/water suspensions with ratios of more than 50% did not occur consistently without the bacteria cells.

Generally speaking, the solute, either an organic or an inorganic compound in a solution, will cause the freezing point to drop. That is to say, it inhibits the freezing of its solution or its suspension. The presence of INA bacterial cells, however, catalyses the freezing of the solution or suspension, although it may also decrease the freezing point at the same time. The bacterial cells serve as templates for water binding, with structures favouring the growth of ice in the liquid phase at a relatively higher subzero temperature (Wolber, 1993). Ice nucleation is the critical point for bulk water to start to freeze. The ice nucleation point is the result of the counteraction of the two contradictory forces caused by the solute and the INA cells. By using the DSC and emulsion techniques, it was demonstrated that the freezing point depression induced by added solute was linearly related to the lowering of both homogeneous and heterogeneous nucleation temperature (Charoenrein *et al.*, 1991). It was also confirmed that the type of nucleation template was important in determining the temperature effects of the solutes on the heterogeneous nucleation rates (Ozilgen & Reid, 1993).

**Table 3** Nucleation temperatures (°C) of safflower oil/water suspensions in the absence and presence of INA cells[a]

| Safflower oil/water[b] (v/v) | Control (without INA cells) | With *P. syringae* ($10^7$ CFU $mL^{-1}$) | With *E. herbicola* ($10^7$ CFU $mL^{-1}$) |
|---|---|---|---|
| 10% | −5.9 ± 0.5 | −0.5 ± 0.1 | −1.1 ± 0.1 |
| 20% | −6.1 ± 0.6 | −0.8 ± 0.4 | −1.3 ± 0.1 |
| 30% | −6.9 ± 0.1 | −1.0 ± 0.3 | −1.7 ± 0.3 |
| 40% | −5.4 ± 2.6 | −1.5 ± 0.6 | ND[c] |
| 50% | (1) | −1.5 ± 0.7 | ND |
| 60% | (2) | −3.0 ± 1.5 | ND |

[a]The freezer temperature was set at −7.5°C (±0.5°C) except for the tests added with *E. herbicola* cells for which the temperature was set at −6.0°C (±0.5°C).
[b]Each sample was tested for four individual times at a volume of 10 mL in a polypropylene tube (16 mm × 75 mm).
[c]ND means 'not determined'.
(1) Three controls nucleated at −5.8 ± 0.6°C out of four samples.
(2) Three controls nucleated at −6.2 ± 0.7°C out of four samples.

The highest nucleation temperatures of INA cells (–2.0°C for *P. syringae*; −2.5°C for *E. herbicola*) tested with the droplet method were different from the nucleation temperatures obtained from freezing curves as shown in Fig. 4 (–0.82°C for *E. herbicola*). This was due to the effect of sample volumes (10 μL in droplet methods and 10 mL in the freezing test). Clausse *et al.* (1991) also reported that ice nucleation activity by iodide and bacteria in microsize droplets dispersed within emulsions is lower than that observed for bulk samples. However, the mechanism by which the expansion of the total volume for the freezing test could elevate the nucleation temperature remains unclear. One possible explanation is that as the sample only freezes once, only the most active ice nucleator is recorded in bulk systems. The larger the sample the more likely a bacterial cell with activity in the range 0 to −2°C will be present to nucleate the sample.

In addition increasing the nucleation temperatures of various solutions/suspensions, the presence of INA bacterial cells also reduced the total freezing time compared to that of controls (Table 4). The most apparent difference between controls and samples is that, with the addition of INA cells, sucrose of more than 10% solution, egg white of more than 20% solution and oil/water

 

**Table 4** Reduction of total freezing time of model food systems with the presence of INA cells[a]

| Samples[b] | Control (min) | With *P. syringae* (min) | Freezing Time Saving (%) |
|---|---|---|---|
| **Sucrose solution (w/w)** | | | |
| 1% | 177.6 ± 11.0 | 142.4 ± 3.4 | 20% |
| 5% | 174.9 ± 10.4 | 140.6 ± 11.5 | 20% |
| 10% | DNF[c] | 163.9 ± 5.2 | ++ |
| **Egg white solution (w/w)** | | | |
| 1% | 165.3 ± 14.7 | 141.3 ± 11.4 | 15% |
| 9% | 167.0 ± 15.1 | 135.8 ± 8.4 | 19% |
| 20% | DNF | 188.0 ± 3.0 | ++ |
| **Safflower oil/water (v/v)** | | | |
| 10% | 177.6 ± 4.5 | 136.2 ± 6.1 | 23% |
| 40% | 202.6 ± 7.2 | 126.0 ± 10.1 | 38% |
| 60% | (1) | 143.5 ± 9.5 | ++ |

[a]The freezer temperature was set at −6.0 ± 0.5°C for all the tests.
[b]Each sample was tested for four individual times at a volume of 10 mL in a polypropylene tube (16 mm × 75 mm).
[c]DNF means 'Did Not Freeze'.
(1) Three controls froze in 156.2 ± 8.9 min out of four samples.
++indicates that the comparison is impossible as the control does not freeze at the same conditions.

suspension of more than 50% can be frozen at relatively higher subzero temperatures (–6 to −7°C), while controls could not be frozen at the same conditions.This may serve as a basis for an alternative design for some freezing processes in the industry. The total freezing time savings were difficult to compare in this case. For the representative solutions/suspensions, a range of total freezing time savings from 15 to 38% were observed in our experiments when both control and treated samples could be frozen at the same conditions.

Freezing experiments were also conducted at different freezer temperatures, such as −10, −15, and −20°C. It was found that at a lower freezer temperature, the effect of INA cells on the freezing was less apparent (data not shown). When the freezer temperature was set at −20°C, the difference between the presence and the absence of INA cells during freezing was insignificant (data not shown). This temperature-dependent function is probably due to more and more unknown foreign particles contained in the solutions/suspensions able to serve as ice nucleators at temperatures lower than −15°C (Yankofsky *et al.*, 1981). This property of bacterial ice nucleation is both an advantage and a limitation for its application. Efficient freezing is a key step to many industrial processes, such as frozen storage, freeze-drying, freeze-concentration and freeze-texturing. At a higher subzero temperature, the energy used for the freezing may be reduced, productivity may be improved and the quality of certain products can be modified on purpose. Watanabe *et al.* (1989) applied INA cells of *E. ananas* in the freeze concentration of raw egg white, milk and fruit juice for various purposes at −5°C. Therefore, some new procedures were proposed for the special products, non-heated jam and milk jelly.

While it may not be practical to add large doses ($10^7$ CFU $mL^{-1}$) of bacterial cells to foods, the results presented here will focus the attention of investigators on understanding the mechanisms of bacterial ice nucleation. To alleviate the safety concern of this aspect of application and to have it approved by FDA, ideally, only purified and biochemically defined ice nucleators should be used in food systems for freezing. This has proved difficult because fragmentation of the intact bacterial cells resulted in significant loss of ice nucleation activity (Yankofsky *et al.*, 1981; Govindarajan & Lindow, 1988; Collette, 1989). INA sites may also contain lipids and saccharides and are membrane bound (Kozloff *et al.*, 1991; Turner *et al.*, 1991). Extracellular ice nucleators from *Erwinia herbicola* (Phelps *et al.*, 1986; Zheng & Lee, 1990), *Pseudomonas fluorescens* and *Erwinia uredovra* (Obata *et al.*, 1993) may be one of the solutions for the safety issue. Another method is to isolate food-grade microorganisms with ice nucleation activity such as *Xanthomonas*, kill them by pressurization, and then use them for freezing (Watanabe *et al.*, 1991). A third way is to clone the *ina*$^+$ gene (Wolber, 1993) into generally recognized as safe (GRAS) microorganisms, such as yeast and/or *Lactobacillus*.

Further research with real foods in the presence of ice nucleation active bacterial cells and/or purified ice nucleators is still needed to confirm their effects on the nucleation temperature elevation and freezing time reduction. We are now working on the application of purified extracellular ice nucleators from *E. herbicola* in the freezing of some real food systems.

## Conclusions

The application of bacterial ice nucleation in food processing is a new and promising field. It requires the addition of about $10^6$–$10^7$ CFU $mL^{-1}$ to water to have a significant increase in the nucleation temperature of water. In the presence of INA cells, the bulk water in model food systems, including sucrose and egg white solutions and oil/water suspensions, freezes at a relatively higher subzero temperature. The nucleation temperatures have been elevated by between 1.2 and 5.9°C. More interesting, it could cause some model systems with higher concentrations to freeze easily at −6 and −7°C, which normally does not happen. In other words, the guaranteed freezing of some foods at higher subzero temperatures may be achieved by the use of INA cells in the systems. The total freezing time with the addition of INA bacterial cells was also shortened by between 15 and 38% compared to controls. The INA bacterial cells, most active only at above −10°C, may not be suitable for the application with the current food freezing industry, which usually operates at very low temperatures (–35°C for mechanical freezing). However, the findings in this study imply definite potential for further modifications of the present food freezing processes in the industry to save energy and to improve efficiency and product quality where, operating at a higher subzero temperature, a completely frozen state could be achieved by the application of bacterial ice nucleation.

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