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Ice crystallization induced by silver iodide and bacteria in microsize droplets dispersed within emulsions

D. Clausse*, D. Bouabdillah, N. Cochet, M.P. Luquet and S. Pulvin

Départements de Génie Chimique et Génie Biologique de l'Université de Technologie de Compiègne, BP. 649 - 60206 Compiègne Cédex, France

Abstract - Water dispersed within a suitable oil medium shows 40°C undercooling. Specific ice nucleating agents such as silver iodide or less known bacteria such as *Pseudomonas syringae*, have been found to reduce undercooling to a value depending on different parameters relevant to the emulsification process or to the pretreatment of the aqueous phase to be dispersed. Furthermore, it appears that when there is activity, it is lower than that observed for bulk samples. Sonication, filtration and ultrafiltration tests performed on bulk water bacterial samples show that the size of the active fragments ranges 0.006 μm to 0.2μm.

INTRODUCTION

Numerous experimental works have investigated the ice crystallization induced by an INA (Ice Nucleating Agent) introduced into droplets whose diameters are generally more than 1 mm. The best known INA are silver iodide and less active lead iodide. For example, ice nucleation temperatures are found to be around-4°C when silver iodide is used as INA (ref. 1).

Ice nucleation induced by silver iodide, within much smaller droplets is difficult to study for two main reasons. The first one is that it is not easy to be sure that the droplets are actually seeded. The other one is that a rather large number of droplets must be studied for statistical analysis of the ice nucleation temperatures, which are scattered around a mean temperature, even if the droplets are identical (ref. 2).

Previous works have shown that these difficulties can be overcome by studying water droplets seeded by silver iodide and dispersed within a suitable oil medium (refs. 3 and 4). Silver iodide particles within the dispersed water droplets have been obtained by coalescing droplets from two W/O emulsions whose dispersed phases are dilute aqueous solutions of KI and $AgNO_3$ respectively. The formation of solid silver iodide particles within the droplets is the result of the chemical reaction between $AgNO_3$ and KI, potassium and nitrate ions remaining in the water droplets. The inseminated droplets mostly freeze around -22°C, though some still freeze around the ice nucleation threshold observed without INA. For bulk water samples inseminated by silver iodide obtained by precipitation from two aqueous solutions of KI and $AgNO_3$, the ice nucleation threshold is around -4°C.

To further investigate this difference between the activities of silver iodide for bulk microsized samples, experiments with a completely different kind of INA have been undertaken. The ability of certain bacteria to catalyze ice nucleation at relatively warm temperatures is a recent observation, and the study of this phenomenon attracts considerable attention (ref. 5). Among the Gram- negative bacteria studied, *Pseudomonas syringae* is well known to plant pathologists as an INA (ref. 6).

In this article, we describe our latest results concerning the ice nucleation in dispersed aqueous suspensions of bacteria submitted to various treatments before being dispersed. This study has two objectives, namely to examine the activity of INA within microsize droplets, as mentioned above, and to obtain more information about the mechanism of ice nucleation with bacteria. Little is known about the molecular mechanism involved in this phenomenon, but some recent works have established that the cloned ice nucleation genes from *Pseudomonas syringae* encode a 180 kD protein (ref. 5). Moreover the ice nuclei appear to be located in the outer membrane of the bacteria (ref. 7).

The method for detecting ice nucleation within emulsions is also described and the results obtained for pure water are given as a reference.

ICE NUCLEATION IN PURE WATER DROPLETS DISPERSED WITHIN EMULSIONS

The crystallization of pure water droplets dispersed within a suitable oil medium has been studied thoroughly. The main characteristics of this process are given here. More details can be found in refs. 8 and 9.

Water emulsification is obtained by using a high speed blender Ultra-Turax 8,00-13,500 r.p.m. Various oil media have been used : 1) liquid paraffin ; 2) methylcyclohexane ; 3) silicone, viscosities : 100-500 or 1,000 SI.

To reduce the instability of the emulsions, different amounts of various surfactants have been added : a) lanolin 5%-25 % (Wt/Wt) ; b)-c) span 65, 4 %-10 %. The value of P, the weight fraction of dispersed water, has been taken as 25 % (Wt/Wt) from stability tests. Electron or optical microscopy of the emulsions has shown droplets whose diameters can be as small as 0.5 μm and are always less than 10 μm.

Crystallization is detected by calorimetry of the emulsion subjected to steady cooling. Analysis (ref. 10) of the freezing thermogram has shown distribution of the crystallization temperatures around a mean temperature T*, referred to the most probable crystallization temperature. No delay is observed for the melting of the frozen droplets that occurs at 0°C for the whole droplet. For pure microsize droplets, T* is -39°C i.e 39°C undercooling.

ICE NUCLEATION IN AQUEOUS SUSPENSIONS OF BACTERIA DISPERSED WITHIN EMULSIONS

Bacterial suspension

The strain was routinely cultured on trypto-casein-soja agar (Institut Pasteur Production) and the slants were incubated for 48 hours at 25°C.

Cultures were carried out in a liquid medium containing in $g.l^{-1}$: glucose : 5.0 ; peptone : 5.0 ; and yeast extract : 5.0.

After inoculation, 500 ml flasks containing 100 ml of medium were incubated at 20°C on an oscillatory shaker until the culture reached the stationary phase.

Activity as INA

The activity of the bacteria was first checked on bulk samples, as described below. As there is undercooling, the freezing temperatures are scattered around a mean temperature and several samples must be studied to obtain a significant result.

The ice crystallization temperature of a sample can be obtained from the heat released and detected by a calorimeter. Although this technique is not the most suitable as only one sample is studied, we used it for our initial assays. An apparatus providing the freezing temperatures of several samples during a single experiment is being built.

The liquid is placed in a closed aluminium cell whose volume is a few mm^3.This is placed in a calorimeter (DSC 111 Setaram) and steadily cooled at $2°C.min^{-1}$. After complete solidification, the sample is heated until complete melting at T_f. During cooling, the sample crystallizes, at a temperature T_c which corresponds to the onset of the freezing peak (Fig. 1). The same experiment has been performed on several identical samples.

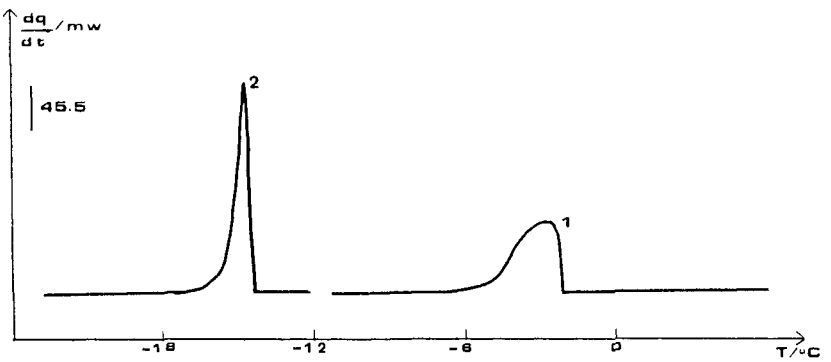

Fig. 1. Freezing thermogram of a bulk sample 1 : suspension bacteria ; 2 : culture medium

The results obtained on pure water, culture medium and culture medium inoculated with bacteria and at the stationary phase are reported on Table 1, where n = the number of samples.

TABLE 1. Freezing and melting temperatures and undercooling for pure water, culture medium and stationary bacteria suspension

sample	n	T_{min} (°C)	T_{max} (°C)	T_c (°C)	T_f (°C)	ΔT (°C)
pure water	14	-10.0	-17.5	-13.0	0.0	13.0
medium culture	20	-10.0	-19.0	-14.5	-0.8	13.7
bacteria suspension	45	-4.4	-1.7	-2.8	-1.0	1.8

All samples melted without delay at their T_f. The freezing temperatures are scattered between T_{min} and T_{max}, with a non uniform density. A mean value T_c can be defined and hence a mean undercooling $\Delta T = T_f - T_c$.

It is clear that bacteria are INA, as ΔT is reduced from 13.7°C to 1.8°C.

The influence of different treatments on this activity was also studied.

First, we found that preheating of the suspension to 120°C for 10 minutes eliminates the activity, ΔT being around 14°C.

To determine the active point, we subjected the suspension to sonication, followed by filtration and microfiltration.

A sonifier cell disrupter (model Branson B12) was used to break the concentrated cell suspension at 50 W for 10 x 6 minutes. Every six minutes, the sample was allowed to cool at room temperature. The sonicated suspension was then passed through a 0.22 µm pore filter (Minisart - N.M.L. - Sartorius). The absence of full bacteria in the filtrate was determined by plating on trypto-casein-soja agar, as no contamination was observed. Lastly, the filtrate was passed through a 0.006 µm pore filter (300,000 MW - diaflo ultrafilter Amicon system - model 8050).

The activity tests on these sonicated and filtrated suspensions gave the results in Table 2. Activity is related to the undercooling ΔT (°C) (ΔT = 14°C, nil activity ; ΔT = 2°C, full activity).

TABLE 2. Activity of sonicated and filtrated suspensions

sample	bacterial suspension	sonicated suspension	filtrate Φ = 0.22 µm	microfiltrate Φ = 0.006 µm
ΔT (°C)	1.5	4.9	7.2	13

These results show that sonication does not seem to destroy the freezing nuclei. However, the activity is reduced as ΔT is increased from 1.5°C (full activity) to 4.9°C. Filtration reduces the activity (ΔT = 7.2°C), whereas microfiltration destroys it (ΔT = 13°C).

From these results, it appears that the size of the active bacterial fractions induced by sonication is between 0.006 and 0.22 µm.

Dispersed bacterial suspension

Suspensions were next dispersed within the suitable emulsions described in part one and the activity of the dispersed phase was assessed through the undercooling ΔT as for the pure water emulsion.

Influence of emulsification

This was determined on bacterial suspensions with maximum activity. The suspension was dispersed at different speeds in different oil media, the proportion of water phase being always : 25 % (Wt/Wt). The thermograms are shown in Figure 2. For comparison, we also studied ice nucleation to dispersed medium culture with no activity (see Table 1) and in the water phase obtained by demulsification with hexane.

Illustrative thermograms are given in Figure 2.

From Fig. 2 (a2) it can be seen that medium culture droplets freeze at around -40°C, which is lower than the mean temperature observed for pure water (see B) as solutes are present in the medium. When a bacterial suspension is dispersed in the same conditions, nearly the same thermogram (Fig. 2. a1) is obtained and it can be deduced that the suspension has lost its INA activity. To check that emulsification does not alter the activity, we demulsified the emulsion with hexane and tested the activity of the aqueous phase withdrawn as described in C.2. The undercooling ΔT was around 2°C, showing that the lack of activity when the suspension is dispersed is not the result of emulsification. When the amount of surfactant in the oil phase is drastically reduced from 25 % (Wt/Wt) to 5 % (Wt/Wt), the results are different (Fig. 2.b1). The medium culture crystallizes around two main temperatures, -33.2°C and -40.2°C, most droplets freezing around -33.2°C. This can be attributed to the fact that the droplets are bigger, the smaller the amount of surfactant. Consequently the undercooling is reduced. When the bacterial suspension is dispersed, thermogram Fig. 2.b2 is obtained. An INA activity is still observed as the droplets freeze around -20°C for the most part, a few still freezing around -40.5°C and then showing no activity. This result is emphasized when other oil media known to give more unstable emulsions are used. This is clearly

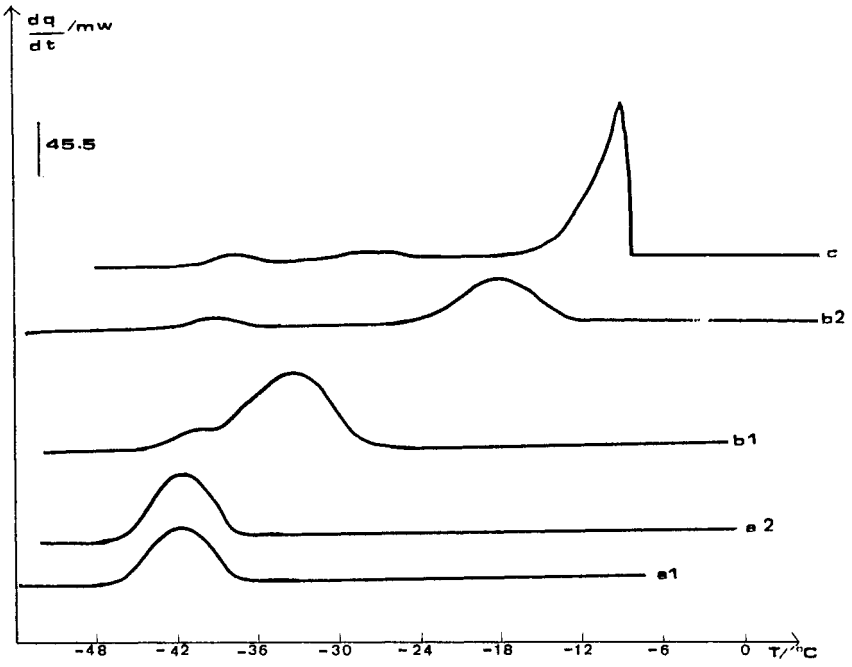

Fig.2. Freezing thermograms of :
a1 : suspension bacteria emulsion (lanolin 25 %)
a2 : medium culture emulsion (lanolin 25 %)
b1 : medium culture emulsion (lanolin 5 %)
b2 : suspension bacteria emulsion (lanolin 5 %)
c : suspension bacteria emulsion (silicone and span)

shown by the thermograms Fig. 2.c obtained when the mixture silicone oil + span 65 was used. Some droplets still freeze not only around -39°C (no activity) and -27°C, but also at about -9°C.

It can thus be deduced that an INA activity can be observed on dispersed bacterial suspensions. This activity is nearly nil for the smallest droplets and always lower than that observed in the bulk samples.

In the next part, we report our initial results obtained when a sonicated suspension is dispersed.

Dispersed bacterial suspension submitted to sonication

Few experiments have as yet been performed on these kinds of suspensions. Dispersed sonicated suspensions show less activity, as has been found for bulk sonicated samples (see C.2).

CONCLUSIONS

Previous works had pointed out that silver iodide is less active as an INA when it is introduced in microsized water doplets dispersed within an oil medium. This result has been confirmed with a completely different kind of INA, namely bacteria *Pseudomonas syringae*. When INA activity is observed, it is always weaker than that obvserved on bulk samples. Futhermore, studies on bacteria have shown that fragments whose sizes are between 0.006 µm and 0.2 µm are still active. Further studies will be devoted to the activity of these fragments when they are introduced within microsized water droplets.

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