

Bacterial ice nucleation: Prospects and perspectives

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In this new emerging era of biotechnology, ice nucleating proteins find varied applications in different fields like artificial snow making, freeze texturization of foods, freeze drying, freeze concentration, detection of bacterial pathogens and also in the control of frost damage to plants. However, the commercial exploitation of this novel biobased and energy saving technology is still under consideration.

BIOLOGICAL systems to initiate a physical process are not new to man. Perhaps the best characterized biological indicators are bacterial ice nuclei, which trigger crystallization of ice in super-cooled water. Detailed characterization of ice nuclei has been carried out in certain bacteria of plant epiphytic communities like *Pseudomonas syringae*, *P. fluorescens*, *P. viridiflava*, *P. coronafasciens*, *Erwinia herbicola*, *E. ananas*, *E. ure-dovora*, *E. corotovora*, *Xanthomonas campestris* and *X. translucens*. In all the strains investigated so far, the phenotype is encoded by a single gene¹⁻⁵. The product of this gene expression is a membrane-bound protein. This protein, alone or in combination with membrane phospholipids, mimics the structure of an ice nucleus and thus, acts as a template for ice formation which is responsible for imparting ice nucleating activity to the bacterium. Such bacteria are referred to as ice-plus (ice⁺) or ice nucleation active (INA) bacteria.

Ice used in daily life is created either due to ice nucleators or due to secondary nucleation. Ice and water can co-exist in equilibrium at 0°C provided heat is neither added nor removed. Removal of heat causes water to convert to ice without need of nucleation. The system becomes totally solid, maintaining a temperature of 0°C. But, when ice is initially absent in the system, solidification follows a different path. When primary nucleation proceeds to crystal growth upon cooling, the temperature of the system falls below freezing point, called super cooling, before nucleation. Nucleation is a necessary step in order to surmount an energy barrier (activation energy) before water converts to ice. Thus, nucleation can be defined as a process that generates within the supercooled state of water (metastable mother phase), the initial fragment of a more stable phases. These fragments then spontaneously develop into gross fragments and the phase change is complete.

Types of ice nucleation

Nucleation is of two different types – homogeneous nucleation (that occurs in 100% pure water) and heterogeneous nucleation (that occurs in impure water). Heterogeneous ice nucleation is common in nature, where water aggregates in a crystalline arrangement and suspended foreign bodies, surface film or wall of the container serve as nucleating agent⁶. A decreased degree of super cooling is required for the heterogeneous ice nucleation. Many organic and inorganic substances like silver iodide⁷, phloroglucinol dihydrate, alpha phenazine, metal aldehyde, kaolinite, covellite, magnetite⁸, cholesterol⁹, amino acids¹⁰, lectins and proteins¹¹ are well known ice nucleators. However, biological ice nucleators are also well established. Among all these, INA bacteria are the most active.

Most of the INA bacteria are inhabitants of plant surfaces and some of them are also plant pathogens¹². In general, they are gram-negative nonspore forming rod-shaped bacteria. Some of them are known to produce plant toxins (syringomycin from *P. syringae*) but are non-toxic to humans. These organisms have faster growth rate¹³ and spread through aerosols¹⁴.

Molecular biology of ice nucleation

INA protein, conferring ice-nucleating property to a bacterium is encoded by a single gene located on a small stretch of chromosomal DNA whose size ranges from 3.4 kb to 7.5 kb. The *Ina* genes that have been sequenced completely are from genus *Erwinia* (*InaA*, *InaU*, *IceE*), *Pseudomonas* (*InaZ*, *InaW*) and *Xanthomonas* (*InaX*). The *Ina* gene contains one open reading frame (ORF) of ~3600 bp, of which 80% consists of a series of imperfectly repeated DNA sequence with lengths of 24, 48, 144 bp. The DNA sequences of the ORF are highly homologous, whereas the sequences lying outside the ORF are usually non-homologous and

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the translation product is comparatively more conserved than the DNA sequence¹⁵. INA proteins produced by different INA⁺ bacteria, immunologically cross react with each other indicating their similar antigenic structure. The translation product of INA gene is predicted to contain 1200 amino acids and has a molecular weight of around 120 kDa, comprising of contiguous repeats of a consensus octapeptide (Ala-Gly-Tyr-Gly-Ser-Thr-Leu-Thr) (ref. 1).

INA protein consists of three distinguishable domain structures – a unique N-terminal domain (~15% of total sequence), which is relatively hydrophobic and contains the membrane anchor for ice nucleation protein; a unique C-terminal domain (~4%) which is rich in basic residues and very hydrophilic; and a central repeating domain (81%), which is hydrophilic and rich in alanine, glycine, tyrosine, threonine, serine and leucine. This central domain can be sub-divided into three regions. The first two of these repeats consist of 48 peptide units of high fidelity; each 48-peptide unit can be further divided into three 16-peptide repeat units of medium fidelity; again, each 16-peptide unit can be further divided into two 8-peptide repeat units of low fidelity in which only two of the eight peptides are strongly conserved. The third region of the repeating domain contains only an 8-peptide repeat sequence (Figure 1).

The repeating domain is naturally suited to formation of a repeating template structure. This domain is rich in amino acid residues with neutral hydrophilic side chains capable of forming hydrogen bonds with water molecules^{2,16}. Removal of several 8-amino acid repeating blocks lowers the nucleation-threshold temperature but does not abolish the nucleation activity¹⁷. The symmetry of this sequence is similar to that of certain crystal planes of ice^{2,18}. Highly active bacteria contain only a few thousand copies of protein in each cell¹⁹⁻²¹. The repeating domains of this INA surface protein are capable

of organizing water into an ice-like structure in three dimensions. The secondary structure of the protein is thought to be a beta-pleated sheet, punctuated by 5 to 6 turns per 48 amino acid sequences²².

Factors affecting ice nucleation

Several chemical compounds known to disrupt membranes have been shown to decrease or abolish bacterial ice nucleation activity. Loss of membrane lipids causes loss of ice nucleation activity^{23,24}. Treatment of bacterial cell wall with lipases, lipid extracting agents, quaternary ammonium compounds, detergents and dyes, etc., decreases the ice nucleation activity. The treatment of bacterial cell membrane with proteases and protein denaturants²⁵ affects functionality of INA protein. The change in temperature also causes disaggregation of INA protein²¹, antibodies²⁶ and antifreeze glycopeptides²⁷, which physically blocks the water-binding surface of INA protein. Nucleation threshold temperature and nucleation frequency of INA⁺ bacteria depend upon the dynamic assembly of multiple, membrane-bound aggregates of ice-nucleation protein. Though in most cases, INA protein is observed in cell membrane, *E. herbicola*, on the other hand, shed ice nuclei into medium. These are released as membrane vesicles of size 50–200 nm in diameter, which are active at –2 to –10°C and show similar freezing spectrum like that of intact nuclei²⁸. These vesicles can also be stored at 0 to 4°C for weeks with no loss of activity. But, heating of these cell-free nuclei at 30°C causes rapid and irreversible loss of ice-nucleation activity.

Nucleation capability mainly depends upon the cell concentration. If the cell number/population is high, the temperature required for nucleation will also be higher. The dead cells also retain INA⁺ activity, if there is no lysis. Approximately, 10⁶ cells are required at –2°C to initiate nucleation, but at very high cell concentration (> 10⁷ cells/ml), nucleation activity is independent of cell number/population. The other factors that contributed to the nucleation efficiency of INA protein are its lattice match and symmetry, water solubility, size of nucleation site, polarization ability, hydrophobicity, surface charge, number and strength of surface sites capable of absorbing water molecules²⁹.

Assay for ice nucleation activity

The best and rapid method for measurement of bacterial ice nucleation activity is droplet freezing assay. In this process, INA⁺ bacteria are diluted and about 10 µl sample mix is placed on hydrophobic surface which remains floating on the surface of a refrigerated bath. The temperature is lowered step by step and at each point, the number of droplets frozen is counted to estimate the

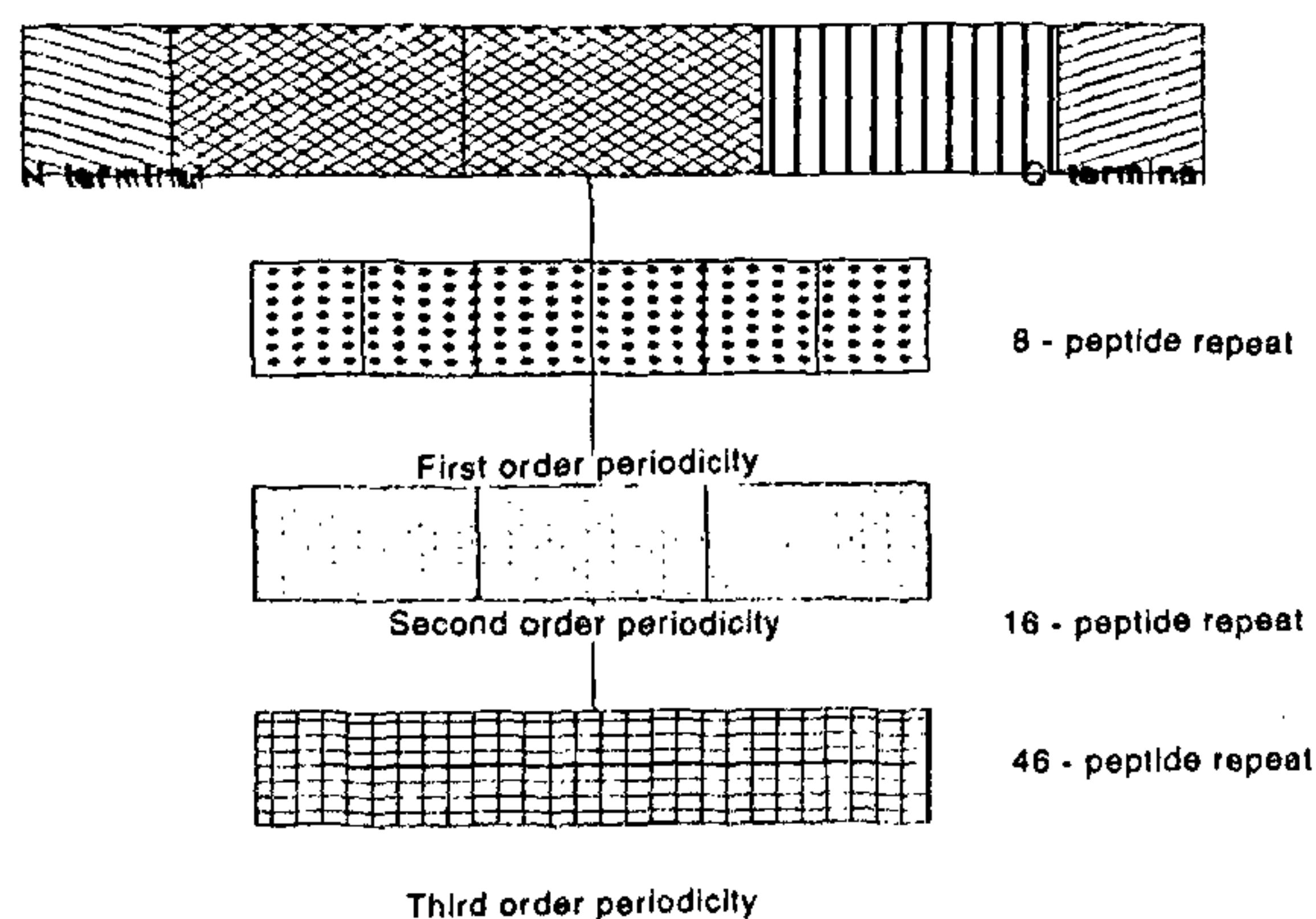


Figure 1. Diagrammatic representation of INA protein. (Adapted from ref. 52).

nucleation efficiency^{30,31}. The common factors which probably affect bacterial ice nucleation assay are temperature, pH and volume of the drop, heat transfer capacity of cooling surface, concentration of cells, the ratio of ice nuclei per cell, time period and the temperature at which droplets are held.

Contrary to INA proteins, antifreeze glycopeptides seen in some fish, spiders, mites, plants, fungi (*Pleurotus ostreatus*, *Flamulina velupites*), bacteria (*Micrococcus cryophilus*, *Rhodococcus erythropolis*, *P. putida*)³² interact directly with ice crystals and stop the increase in size of ice crystals. As it binds to surface of ice crystal embryo, it physically blocks part of the water-binding surface of INA protein. This results in neutralization of the action of INA protein and hence causes freezing point depression by adsorption inhibition²⁷. At times, the presence of antifreeze proteins in food causes problems in freeze texturization³³.

With the advancements of recombinant DNA technology, the *InaA* gene from *E. herbicola* has been successfully cloned and expressed in *E. coli*³⁴. Not only *InaA*, some other genes like *InaZ* of *P. syringae*, etc. associated with ice nucleation have also been cloned successfully in *E. coli*²⁰ and other hosts, thereby, leaving ample scope to explore their commercial value and applications in myriads of areas.

Applications of bacterial ice nucleation

Artificial snow making

Snow is a loose ice mass entrapping air inside, which is highly resistant to temperature. In general, snow is manufactured by spray freezing operation using a snow-gun, which handles between 10 and 100 gallons of water per minute along with several hundred cubic feet of compressed air and the process is very energy intensive. Snow is commonly used for recreational skiing, skating and ice hockey grounds. Recently, Eastman Kodak in collaboration with Advanced Genetic Sciences, Inc. (now a part of DNA Plant Technology Corp.) has patented a product called SNOMAX which is nothing but a freeze dried, radiation sterilized powder of bacteria *P. syringae* PS 31. Each gram of freeze dried powder contains about 10¹¹ ice nuclei with nucleation threshold of -5°C or warmer. The main advantages imparted by Snomax include reduction in energy consumed by snow-making machines for each unit of snow produced and an improvement in the recreational quality of artificial snow³⁵. This product has not yet been marketed in India and as it is prepared by following common fermentation technology, its cost will come around the same as already exists for freeze dried preparations of *Bacillus huringiensis* used all over the world as a bioinsecticide. Use of this product reduces the time and temperature

required, therefore, considerably improving the economics of snow making.

Freeze-texturing using INA⁺ bacteria

INA⁺ *E. ananas* cells can be used for freeze texturing of foods. Addition of INA⁺ bacterial cells to isotropic aqueous dispersion of hydrogels composed of proteins and polysaccharides converts bulk of water into directional ice crystals at subzero temperature, not lower than -5°C and forms an anisotropically textured product. Slicing rightangle to the lamellae of flakes formed produces fibrous textured food. INA⁺ bacteria inhibit supercooling of the bulk water in aqueous dispersions and hydrogels of food proteins and polysaccharides. This effect changes the ice crystallization process which improves the texture of food. This method is commonly applied to heat-sensitive foods which need texturing. INA⁺ bacterial texturization has been successfully experimented in raw egg white, bovine blood, soybean curd, milk curd, corn starch paste, agar hydrogel, soybean protein slurries, hydrogels of glucomannan and calcium bridge glucomannan gel, etc³⁶. Some of the advantages imparted by INA⁺ bacterial texturization of food include preserving foods with high quality, saving the refrigeration cost, shortening the freezing time, improvement in quality of food especially flavour and taste, reduction in degree of super cooling, reduced initial freezing temperature, improved efficiency of production, increased freezing rate, formation of less damaging ice crystals and above all, saving of energy.

High pressure sterilized cells of INA⁺ *X. campestris* INXC-1 have been permitted for food use in Japan. A Japanese company QPCo, Tokyo has already started commercial production of *X. campestris*. The food grade INA⁺ bacteria must be environmentally safe, non-toxic, non-pathogenic and palatable to human beings. Generally, the use of live INA⁺ cells in food systems is discouraged and hence, these should be killed before use by high pressure treatment or radiation³⁷. But, this has a promising future as the ice nucleation gene can be cloned to edible food grade organisms like *Lactobacillus* or yeast which can be consumed directly.

Freezing foods using INA⁺ bacteria

INA⁺ bacteria have successfully been used for freezing of salmon fish muscle, whole fish, surmi, ice cream, agar gel, sucrose solution, fruit juices, egg white, coconut oil, sunflower oil, milk and dairy products. The application of INA⁺ bacteria in freezing provides quick freezing, increase in nucleation temperature and energy saving. It has been reported that the INA⁺ *P. syringae* cells can cause a whole fish to freeze consistently at -5°C within few hours, whereas up to 33% of the un-

treated fish do not freeze at the same temperature³⁸. The total energy saving is due to the savings made during the freezing process as a result of the total freezing time being shortened and the saving made during the storage of frozen foods. For example, a frozen food plant may be able to operate at -30°C rather than at -40°C , because the use of INA⁺ bacteria will ensure that the foods freeze at a relatively higher temperature²⁴. If this plant operates at 5000 kV, the difference of electrical load between these two temperatures will be around 313 kW. Hence, the use of INA⁺ bacteria will save a lot of energy.

Freeze drying using INA⁺ bacteria

The cells of *E. ananas* were applied for the freeze drying of food items such as soy sauce and soybean paste, which are difficult to freeze under normal conditions. Such high salt containing food items are usually diluted for effective freezing³⁹. The addition of INA⁺ cells has made it possible to shorten the freezing times of these foods and to obtain powdered freeze-dried product with greater efficiency.

Freeze concentration using INA⁺ cells

The cells of *E. ananas* entrapped in calcium alginate are also used for freeze concentration purpose. Generally, INA gel is placed in a desired position in liquid to be concentrated and gradually the temperature is lowered. Ice crystals formed at INA gel site along with INA gel are removed to get a concentrated product⁴⁰. *E. ananas* IN-10 and *X. campestris* INXC-1 can be used for freeze concentration. Fresh milk, lemon juices, strawberry paste, raw egg white have been successfully concentrated using this technique⁴⁰.

BIND assay

Bacterial ice nucleation diagnostic (BIND) assay was developed by DNA Plant Technology Corporation, USA for detection of *Salmonella* in food⁴¹. This method uses the recognition specificity of a bacteriophage P22 and the sensitivity of the gene (*InaZ*) encoding ice nucleation protein for detection of pathogens in foods. To explain it at molecular level, *Ina* gene introduced to a bacteriophage is kept under the control of a strong promoter by recombinant DNA technology, the phage is allowed to transduce the target bacteria and the test sample is cooled to -9.5°C in presence of a freezing dye which shows fluorescent green colour at super-cooling. This indicates absence of target organisms and shows non-fluorescent orange colour at freezing, indicating the presence of target organisms, i.e. *Salmonella*. The fre-

quency of freezing of test samples can indicate the degree of contamination. This method has been employed successfully in raw egg, milk, meat, gelatin, etc. to detect *S. typhimurium*, *S. dublin*, *S. paratyphi B*, *S. enteritidis*, *S. gallinarum* and *S. typhi*. The method is quick and shows high specificity and sensitivity and can detect even < 10 cells/ml of sample⁴². In BIND assay, non-target organisms do not interfere and it can also detect injured cells as only 100–200 INA protein molecules are required for detection. But, it has certain disadvantages, i.e. it cannot detect phage-resistant cells and also fails to detect dead cells. Moreover, as this method fully depends upon specificity of phages, it cannot be employed for detection of certain bacteria for which bacteriophage is not known.

Control of frost damage to plants

As most of the INA⁺ bacteria are the common inhabitants of phyllosphere, they cause great damage to crop plants like wheat, oat, barley, mustard, etc. by inducing ice formation on plant surfaces⁴³. The economic impact of frost damage in US has been estimated to be over a billion dollars per year⁴⁴. Several methods are now in vogue to control frost damage to plants like genetic improvement, use of protective covers, use of wind machines and heaters, application of water to plants, application of chemicals, use of antifreeze proteins, etc. of which ice-minus bacteria are gaining importance day by day.

Ice-minus bacteria are nothing but the INA⁺ bacteria engineered genetically (*Ina* gene removed) to lose its ice-nucleating power^{45,46}. Generally, ice-minus bacteria are sprayed on plant surfaces before ice-plus population develop and grow to a sufficient number to prevent the latter from colonizing. This method has given excellent results as 70–80% reduction of ice accumulation has been observed on strawberry plant⁴⁷. But, till date Environmental Protection Agency and government have not permitted release of these organisms to nature. However, these ice-minus bacteria do not express any competitive advantage over the wild strains of ice-positive bacteria, therefore, it is difficult to control INA⁺ population on plant surfaces in uncontrolled natural environment.

Other possible uses

Bacterial ice nucleation finds possible applications in replacement of silver iodide in artificial rain (cloud seeding and cloud precipitation); in air-conditioning for improvement of its efficiency; in refrigeration system and ice-flacking machine; processing of antifreeze protein containing foods; identification of pathogens and reduction of cold tolerance of insects in frozen grain

storage^{48,49}. Certain US patents concerning the use of INA protein and its related products for fluorescent immuno assay kit, treatment of hazardous waste, purification of organic chemicals have also been developed. Use of BIND assay can be extended to study the sensitivity of the bacteria to bacteriocidal agents. The initial discovery that plant surfaces shed bacterial ice nuclei in the environment was prompted by the general interest by meteorologists in ice nucleation⁵⁰. Since then, a lot of intensive investigations have been continuing by different groups on the incidence and importance of bacterial ice nucleation in the atmosphere⁵¹. But till today, the meteorological significance of bacterial ice nuclei remains unproven.

Conclusion

Ice nucleating bacteria can play a significant role not only in affecting our environment but also influencing our day to day life. Although most of the possible applications involving these ice nucleation proteins are at their embryonic stages at the moment, their application and scope are bound to increase in the near future as more basic information on these wonder proteins pour in.

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