Freeze Drying of Lactic Acid Bacteria (Probiotic) with Maximum Activity and Stability

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Introduction

Foods or supplements containing probiotics do not necessarily meet a particular level or even state how much of the culture is present in a viable form within the product. This unregulated "state of affairs" combined with increased scrutiny of supplement and food claims by regulatory authorities and the scientific community has led to a desire to ensure that probiotic products can provide a high enough concentration of live probiotic cells to actually a benefit to a individual.

Freeze-drying has been used in a number of applications for many years, most commonly in the food and pharmaceutical industries. Freeze-drying involves the removal of water or other solvent from a frozen product by a process called sublimation. Sublimation occurs when a frozen liquid goes directly to the gasseous state without passing through the liquid phase. In contrast, drying at ambient temperatures from the liquid phase usually result in changes in the product, and may be suitable for some materials. The advantages of freeze-drying are obvious. It is convenient method for the preservation and long-term storage of a wide variety of microorgansisms. However, special precautions are needed for the preservation of microorganisms sensitive to dessication, light, oxygen, osmotic pressure, surface tension and other factors. To produce final products in powderised form containing live cells for commercialization at reduced cost, cheaper protective agent and stabilizer must be used in the formulation during freeze-drying.

Reseach was focussed on the development of freeze drying method for the preparation and formulation of product containing live probiotic bacteria for commercial applications. Research activities includes; (1) optimisation of freeze-drying conditions on minimising dehydration inactivation, (2) effect of dehydration conditions on leakage of essential cell components through the membrane, (3) feasibility of using cheap and locally available protective carbohydrate or stabilizer to maintain the activity and stability of the freeze-dried bacterial cells, (4) interaction between stabilizers and cell physiological and membrane damage using microscopic examination, and (5) storage study of the powderised products containing live cells.

Materials and Methods

The probiotic cultures, *Lactobacillus* spp, were grown in appropriate media until late logarithmic phase of growth was achieved. A thick cell suspension (at least 10^8 cells/mL), harvested from culture broth using centrifugation technique, was prepared in various protective solutions to be investigated. The cell suspensions were kept in an ice bath prior to filling in the container containing the stabilizer.

Freeze drying experiments of product containing probiotic cultures were carried out using Virtis lab scale freeze dryer Christ pilot scale freeze dryer. Freeze-drying involves the removal of water from frozen cell suspension by sublimation under reduced pressure. The out line of the freeze-drying procedure and the major steps involved; (i) pre-freezing, (ii) primary drying, and (iii) secondary drying. In prefreezing step, products to be freeze dried consist primarily of water (*solvent*) and materials dissolved or suspended in water (*solute*); and are *euthetics* (a mixture of substances that freeze at lower temperature than surrounding water). When aqueous suspension is cooled, changes occur in solute concentration of product matrix. As cooling proceeds, water separated from solutes as it changes to ice, creating more concentrated areas of solutes. These pockets of concentrated materials have lower temperatures than surrounding water. Mixture of various concentrations of solutes with solvent constitutes the eutectic of suspension. Only when all of eutectic mixture is frozen at the eutectic temperature, suspension is properly frozen. It is important to prefreeze product to below eutectic temperature prior to freeze drying process. During primary drying, conditions must be established in which ice can be removed from frozen product (obtained from prefreezing stage) via sublimation, resulting a dry and structurally intact product. These require very careful control of two parameters, temperature and pressure. Rate of sublimation of ice from frozen product depends upon the difference in vapour pressure of product compared to vapour pressure of ice collector. After primary drying, although all ice has sublimed, bound moisture is still present in the product with residual moisture content of about 7-8%. Secondary drying is necessary at warmer temperature to reduce residual moisture content to optimal values. This process is called isothermal desorption as the bound water is desorbed from the product.

For the estimation of cell viability, the total plate count was done using serial dilutions of the freeze dried products in liquid media. The number of colonies are counted from the plates and average colony forming units per sample were calculated. The revived cultures were also observed for mutation and change in colony morphology. The samples were also examined under the Scanning Electron Microscope (SEM) to observe the ultrastructure. The freeze dried products were attached on double

stick adhesive tape on SEM stubs and were the sputter-coated. The specimens were kept in the dessicator until placement in the SEM chamber.

Results and Discussion

Research activities includes; (1) optimisation of drying condition on minimising dehydration inactivation, (2) effect of dehydration conditions on leakage of essential cell components through the membrane, (3) feasibility of using cheap and locally available protective carbohydrate or stabilizer to maintain the activity and stability of the freeze-dried bacterial cells, (4) interaction between stabilizers and cell physiological and membrane damage using microscopic examination, and (5) storage study.

From this study, several freeze-dried products, formulated using locally produced lactic acid bacteria and locally available stabilizers, for various commercial uses (human, aquaculture and chicken) have been developed. The morphological changes that occurred on the lactic acid bacteria powder after being freeze-dried, which was examined under scanning electron microscope, with different cryoprotectants and stabilizing agents have been carried out.

The feasibility of protective agent and stabilizer to maintain the activity and stability of the freeze-dried bacterial cells has been carried out. The differences in microstuctures of different cryoprotectants and stabilizing agents, reflects the physical properties of the dried-powders. Hence, the suitable cryoprotectants and stabilising agents to be used in freeze drying of different lactic acid bacteria can be chosen. The use of sago starch as stabilizer was capable to maintain the stability of the live probiotic bacteria in powderised form up to 42% after six months storage at 30°C. On the other hand, when non-fat dry milk and cellulose was used as the stabilizer, the stability of the products was about 63% and 59% after storage at the same time and conditions, respectively.

The protective agent was also found very important to reduce cell damage during freeze drying. This agent acts to minimize leakage of essential cell components through the membrane during dehydration. When the cells were freeze dried without protective agent, the % decrease in viable count of *L. rhamnosus* during storage for 20 days at 25°C was 99.5%. However, when ascorbic acid and monosodium glutamate was added to the cells and used as protective agent, the % decrease in viable cell count was only at a range of 10% to 20%.

Result from SEM analyses were also be used to optimise the formulation and process variables. The products developed can be stored at room temperature with only 40% loss in viability after 6 months. In addition, in freeze-drying, the material does not go through the liquid phase. Furthermore, freeze dried probiotic cultures was not only an efficient barrier against chemical entities such as moisture, oxygen and acids but also a good protector against short exposure to high temperature. Thus, it allows the preparation of a stable product that is easy to use and aesthetic in appearance.

Conclusions

From this study, several freeze-dried products, formulated using locally produced lactic acid bacteria and locally available stabilizers, for various commercial uses (human, aquaculture and chicken) have been developed. The morphological changes that occured on the lactic acid bacteria powder after being freeze-dried, which was examined under scanning electron microscope, with different cryoprotectants and stabilizing agents have been carried out. The differences in microstuctures of different cryoprotectants and stabilizing agents, reflects the physical properties of the dried-powders. Hence, the suitable cryoprotectants and stabilising agents to be used in freeze drying of different lactic acid bacteria can be chosen. Result from SEM analyses can be also be used to optimise the formulation and process variables. The products developed can be stored at room temperature with only 10% loss in viability after 6 months. In addition, in freeze-drying, the material does not go through the liquid phase. Thus, it allows the preparation of a stable product that is easy to use and aesthetic in appearance. Although the cost of the specialized equipment required for freeze drying can be substantial, the process may appear to be an expensive undertaking. However, savings realized by stabilizing an otherwise unstable product at ambient temperatures, thus eliminating the need for refrigeration, more than compensate for the investment in freeze-drying equipment. Thus, it has great potential to be used for preparation of stable commercial products containing live cells with maximum activity and viability.

Benefits from the study

Formulation and Freeze-drying method for preparation of commercial products containing live and active lactic acid bacteria (probiotic) for various applications (human, aquaculture and chicken). Probiotic bacteria in the form of powder that can be stored at room temperature with minimal loss in viability and stability for long time. The same technology could also be applied to other products containing live cells for commercial applications such as microbial inoculant, starter cultures and biopesticides. The use of local materials such as zeolite, rice husk, sago starch as stabilizer for freeze drying of live bacteria for commercial use is novel. Some results related to the findings on the mechanism of cell damage during dehydration and the role of stabiliser to protect cell membrane from damage during dehydration is also novel. The technology has great potential to be commercialised. Most of live and active microbial cultures used in chemical and agriculture industries in Malayisa such as probiotic microorganisms for human, aquaculture and animal feeds; and microbial inoculant; biopesticide (*B. thurigiensis*) are imported. Locally produced products may less expensive and more suitable for local applications.

Patent(s), if applicable:

Nil

Stage of Commercialization, if applicable:

Nil

Project Publications in Refereed Journals:
Nil

Project Publications in Conference Proceedings:
Nil

Graduate Research

Name of Graduate	Research Topic	Field of Expertise	Degree Awarded	Graduation Year
Lim Chin Ming	Mass production of probiotic microorganisms for chicken	Bioprocess Engineering	PhD	Expected to graduate in 2004

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