A novel preservation and delivery technology for live probiotics, enzymes and vitamins.

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INTRODUCTION

Biological materials, such as proteins, vitamins, bacteria and viruses, are generally unstable when stored at ambient temperature (approximately 25°C). For example, the thermal reduction rate of probiotic bacteria such as *Lactobacillus acidophilus* at 25°C, is over six times higher than that at 4°C (Tsen et al., 2007). Typical methods for preserving sensitive biological materials include freeze-drying, cryopreservation, and spray-drying. These drying techniques involve the use of extreme temperature excursions which may initiate structural damages to the cell membranes, protein denaturation, and/or DNA damage and can lead to a decrease in cell viability (Leslie et al., 1995). A common strategy to protect and stabilize the biological materials during the freeze drying is to add low molecular weight compounds such as sugars, polyols, amino acids and/or methyl amines to the aqueous suspensions prior to the freeze drying process (Hubalek, 2003). Studies have shown that the addition of non-reducing disaccharides such as sucrose and trehalose, could stabilize the structure and function of intracellular proteins by forming hydrogen bonds with the dehydrated proteins (Crowe et al., 1998). Another common strategy to minimize the freezing and drying damage is to immobilize the biological material in a protective matrix of cross-linked polysaccharides such as calcium-alginate (Kailasapathy, 2002), *k*-carrageenan (Tsen et al., 2007) or gelatin (Annan et al., 2007). However, polysaccharide-based immobilization has limited applications in the probiotic industry, due to high bacterial loss during the encapsulation and drying processes, poor shelf life stability of the probiotic product, and difficulties in industrial scale-up (Champagne et al., 2001). Here, we report on a novel formulation and drying process technology (MicroMatrix™), which provides a superior stability to several types of biological materials during manufacturing, drying, ambient storage, and gastric excursion. The technology utilizes only food grade, non-toxic and solvent-free ingredients, and requires very mild exposure to temperature and pH.

MATERIAL AND METHODS

The MicroMatrix™ formulation is composed of a proprietary mixture of polysaccharides, sugars and proteins. Dry microparticles containing probiotic bacteria, enzymes, or vitamins were formed using a controlled desiccation protocol (liquid drying process) under low temperature and vacuum exposure. A specified size range of the particles was obtained after milling and sieving through a series of mesh screens (Figure 1). Cultures of probiotic bacteria were obtained from various commercial sources. The bacteria were fermented with a standard MRS media and glucose as a source of energy. The bacteria was harvested after 22 hours and concentrated into a paste by a centrifugation (7000g x 20 min. at 4°C). The concentrated bacterial paste was added with 10% (w/w) of trehalose solution and kept frozen at –80°C.

Storage stability of the probiotic bacteria was determined under accelerated conditions (40°C and 33% RH). Simulated gastric juice was prepared according to the protocol of US Pharmacopoeia (2000) & National Formulatory (USP 24NF19, Rockville, MD.) and adjusted to pH = 1.2. Free or

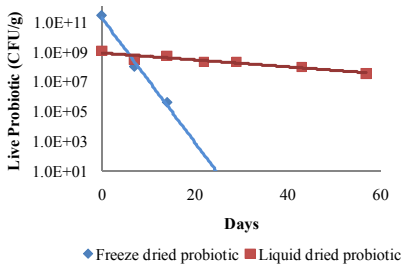
stabilized bacteria and enzyme were incubated in the gastric juice on a shaker (150 RPM) at 37°C for 2 hours.

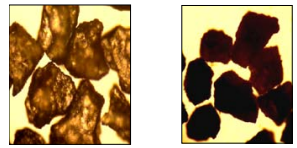
Free or stabilized oil were exposed to 100°C for up to 6 hours and oxidation was measured by the appearance of malonyl dialdehyde (MDA). Free or stabilized enzyme (Savinase®, Novozyme) was incubated in simulated gastric juice for 2 hours or heated at 90°C for 20 minutes and the protease activity was determined with PDQ Protease Assay™ kit (Athena E.S., Baltimore, MD. USA)

Figure 1. Liquid drying process and formation of microparticles loaded with probiotic bacteria.

RESULTS AND DISCUSSION

Dehydration of probiotic bacteria is commonly performed by freeze-drying or spray drying. This harsh process can cause extensive damage to cell membranes even if the drying media contains sufficient cryo-protectants. Figure 2 shows the eight weeks stability at 40°C and 33% RH of freeze-dried or MicroMatrix stabilized and liquid dried *L. rhamnosus* GG (LGG). The stabilized and liquid dried probiotic lost approximately two logs under the high temperature and humidity conditions; while the commonly freeze dried probiotic saw an almost total loss of viability within the first 3 weeks of testing. Figure 2 also shows the appearance under a light microscope of particles obtained from the liquid or freeze drying process. Particles obtained through the controlled liquid drying process were transparent and hard, while freeze-dried particles appeared agglomerated and easily disintegrated under a finger pressure.





Light microscopy showing the appearance of the microparticles after a liquid (Left) or freeze (Right) drying process (particle size range - 125-250µM)

Figure 2. Effects of a common freeze drying process or a controlled liquid drying process of MicroMatrix™ formulation on the survival of *L. rhamnosus* GG at 40°C and 33% RH.

The storage stability of a biological material is probably the most important economic factor in determining market feasibility. Among the several factors affecting storage stability; temperature and humidity are responsible for the majority of degradation. Figure 3 illustrates the relationship between temperature and humidity on the degradation rates of *L. paracasei*. The Arrhenius plots of the stabilized bacteria, obtained under accelerated temperature conditions, were linear for each relative humidity, indicating that excessive denaturing stress or bacterial damage was avoided over a wide range of temperature and relative humidity conditions; up to 50°C and 33%RH. The independent variable in the equation represents the activation energy value, or the energy barrier, that must be exceeded for the thermal degradation reaction to occur (Geoffrey and Milida, 1991). Based on the projected *k* value for ambient conditions (25°C and 23% RH) the stabilized probiotic will lose no more than one log of potency over 6 month storage period.

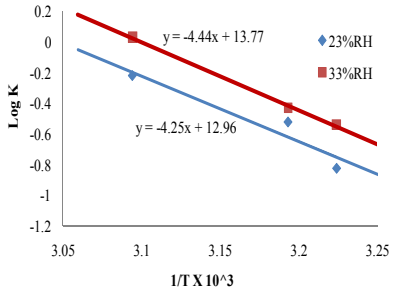


Figure 3. Arrhenius plots of the thermal degradation of *L. papracasei* at 23% and 33% relative humidity. The probiotic bacteria was mixed with the MicroMatrix™ ingredients and dehydrated under a controlled liquid drying process.

One of the most significant losses of probiotic viability is encountered during passage of the material through the stomach. Figure 4 shows that stabilizing various probiotic bacteria using the MicroMatrix significantly improved their survival in simulated gastric juice as compared to the survival of non-protected cells. This protection allows for a 100 to 1000-fold increase in the number of viable bacteria reaching their preferred site of action in the intestinal tract, thereby either improving their efficacy or allowing a manufacturer to provide the same result with a lower initial dose of the probiotic in the consumed food.

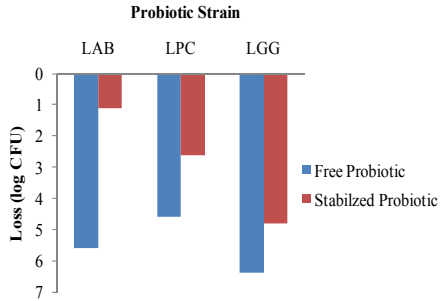


Figure 4. Survival of free and MicroMatrix™ stabilized probiotic bacteria *L. acidophilus* (LAB), *L. paracasei* (LPC), and *L. rhamnosus* (LGG) after 2 hours incubation in simulated gastric juice at 37°C and pH=1.2.

Additional applications of the MicroMatrix stabilization technology have been demonstrated with several other biological materials such as enzymes, vitamins and vaccines. Figure 5 demonstrates

the impact of the MicroMatrix on the stabilization and preservation of the quality and the activity of fish oil and an enzyme (Savinase®, Novozyme).

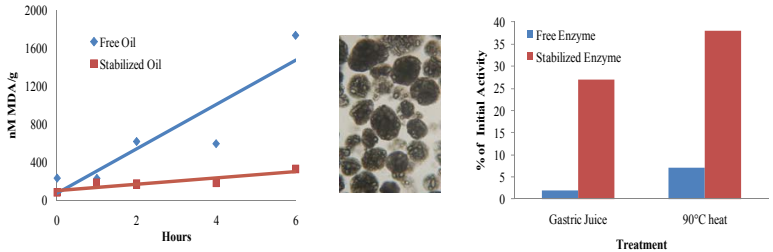


Figure 5. Stabilization of fish oil (left figure) and enzyme (right figure). The picture in the middle shows the appearance under light microscopy of particles loaded with oil (50-200μm).

CONCLUSIONS

The MicroMatrix™ stabilization and delivery technology is a unique approach to providing enhanced stability for a variety of biological materials at ambient storage conditions and protection during passage through the stomach. The technology involves a controlled desiccation process (liquid drying) of a viscous formulation of natural polymers surrounding the biological material to be protected. As a result, the protected material retained its activity for a long period of time under challenging manufacturing, storage, and gastric conditions. This technology enables the food industry to offer a wide range of new products containing sensitive biological materials outside the cold chain of distribution.

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