فصلنامه علمی زیست‌شناسی میکروارگانیسم‌ها
سال یازدهم، شماره ۴۴، زمستان ۱۴۰۱ ، صفحه ۹۴-۷۷
تاریخ دریافت: ۱۴۰۰/۱۲/۲۵ – تاریخ پذیرش: ۱۴۰۱/۴/۶
(مقاله پژوهشی)

مقایسهٔ انواع روش‌های حفظ باکتری‌های اسید لاکتیک برای استفاده به‌صورت کشت آغازگر

نفیسه سادات نقوی: استادیار گروه میکروبیولوژی، دانشکدهٔ علوم زیستی، واحد فلاورجان، دانشگاه آزاد اسلامی، اصفهان، ایران، nafiseh_naghavy@yahoo.com

گلنوش رضائی زاده: دانشجوی کارشناسی ارشد گروه میکروبیولوژی، دانشکدهٔ علوم زیستی، واحد فلاورجان، دانشگاه آزاد اسلامی، اصفهان، ایران، golnooshrezaiezade23101369@gmail.com

فهیمه صرامی: دانشجوی کارشناسی ارشد گروه میکروبیولوژی، دانشکدهٔ علوم زیستی، واحد فلاورجان، دانشگاه آزاد اسلامی، اصفهان، ایران، sarmifahime1996@gmail.com

منصوره سادات خیام نکویی: دانشجوی کارشناسی ارشد گروه میکروبیولوژی، دانشکدهٔ علوم زیستی، واحد فلاورجان، دانشگاه آزاد اسلامی، اصفهان، ایران، _manoos.kh@gmail.com

کیانوش خسروی دارانی*: استاد گروه تحقیقات صنایع غذایی، انستیتو تحقیقات تغذیه‌ای کشور، دانشگاه علوم پزشکی شهید بهشتی، تهران، ایران، kiankh@yahoo.com

چکیده

مقدمه: در صنایع غذایی برای تولید انواع مختلف محصولات تخمیری از تک‌سویه‌ها یا مخلوط مزوفیل و ترموفیل باکتری‌های اسید لاکتیک به‌عنوان کشت‌های آغازگر استفاده می‌شود. نگهداری این کشت‌های آغازگر مرحله مهمی است که طی آن باید زنده‌مانی و پایداری ژنتیکی کشت آغازگر در بالاترین حد حفظ شود. روش‌های سنتی نگهداری کشت‌های آغازگر، بیشتر براساس روش‌های انجماد بود. امروزه خشک‌کردن روش معمول نگهداری کشت‌های آغازگر شده که عمدتاً شامل خشک‌کردن پاششی و انجمادی است.

* نویسندهٔ مسئول مکاتبات

مواد و روش‌ها: در این بررسی مروری روایی، انواع روش‌های نگهداری کشت‌های آغازگر با کمک جستجو در پایگاه‌های داده‌ای مانند Google Scholar، Scopus، PubMed و سایر پایگاه‌های اطلاعاتی معتبر مرور شده‌اند. پس از نگاه کلی به انواع روش‌های نگهداری، مزایا و معایب آنها مقایسه و راه‌حل‌هایی برای غلبه بر مشکل بقا معرفی شده‌اند

بحث و نتیجه‌گیری: در این مطالعه، مقایسه روش‌های خشک‌کردن از نقطه‌نظرهای مختلف می‌تواند به طراحی پروژه‌های تحقیقاتی مؤثر یا انجام فرایندهای تولید در این حوزه کمک کند.

واژه‌های کلیدی: باکتری‌های اسید لاکتیک، کشت آغازگر، انجماد، خشک‌کردن پاششی، خشک‌کردن انجمادی

Biological Journal of Microorganism
Year 11, Vol.11, No.44, Winter 2023
Received: 2022-03-16 - Accepted:Page: 2022-06-27
P:77-94

A Comparison between Different Methods for the Preservation of Lactic Acid Bacteria for Usage as a Starter Culture

Nafiseh Sadat Naghavy
Department of Microbiology, Faculty of Biological Sciences, Falavarjan Branch, Islamic Azad University, Isfahan, Iran, nafiseh_naghavy@yahoo.com
Golnoosh Rezaeizadeh
Department of Microbiology, Faculty of Biological Sciences, Falavarjan Branch, Islamic Azad University, Isfahan, Iran, golnooshrezaiezade23101369@gmail.com
Fahimeh Sarami
Department of Microbiology, Faculty of Biological Sciences, Falavarjan Branch, Islamic Azad University, Isfahan, Iran, sarmifahime1996@gmail.com
Mansoureh Sadat Khayam-Nekooii
Department of Microbiology, Faculty of Biological Sciences, Falavarjan Branch, Islamic Azad University, Isfahan, Iran, manoos.kh@gmail.com
Kianoush Khosravi-Darani*
National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran
kiankh@yahoo.com

Abstract

Introduction: Single or multiple strains of mesophilic and thermophilic lactic acid bacteria are used as starter cultures in food industries to produce different types of fermented products. Preservation of these starter cultures is a crucial step in which the highest viability and genetic stability of the starter culture should be retained. Traditional methods for the preservation of starter cultures were mainly based on the freezing method. Nowadays, the usual method for the preservation of starter cultures is drying which mostly included spray drying and freeze-drying.

Materials and Methods: In this narrative review of studies, a comprehensive review is conducted on all methods for the preservation of lactic acid bacteria through searches in databases such as Google Scholar, Scopus, Pubmed, and other valid databases. After a glance at the types of preservation methods, advantages and disadvantages are compared and solutions to overcome the problem of survival have been introduced.

Discussion and Conclusion: Comparisons of the preservation methods from different points of view in the present overview may help to design more effective research projects or production processes in this area.

Key words: Lactic Acid Bacteria, Starter Cultures, Freezing, Spray Drying, Freeze-drying

*Corresponding Author

10.22108/BJM.2022.133007.1459

Introduction:

Fermented foods are known as conventional civil products and have been important ingredients of the human diet since time immemorial. Starter cultures are the basis of fermented foods production. Lactic acid starter cultures are the most fundamental type of bacterial starter cultures. In-plant sub-culturing is eliminated by the use of frozen or freeze-dried starter cultures, reducing the cost of preparing the bulk cultures and the risk of bacteriophage infection as well as improving their resistance to antimicrobial compounds and bile salts, and increasing their viability in low pH values and acidification ability. Thus, today, indigenous flora and liquid cultures need to be displaced with commercial condensed cultures (1,2).

Commercial starter cultures were first offered in liquid form, before the initiation of the use of condensed starter cultures. Manufacturers of fermented foods are required to set up intermediate and bulk starters for fermentation processes. Later, by progress in biomass manufacture like neutralization and centrifugation methods (3), direct inoculation of condensed starter cultures to the food matrix was used in frozen form or as freeze-dried products. These cultures, as a direct-to-vat set culture, have many benefits that have made them very favored. They delete in-plant sub-culturing, lower the cost of preparing for mass cultivation, and inhibit the growth of bacteriophages. Thus, today, dairy indigenous flora and starter cultures in liquid form have largely been replaced by the commercial intense starter cultures offered by starter culture producers. The dairy industry currently uses about US$ 1.5 billion in commercial starter cultures worldwide to produce fermented milk products (1,4,5).

Bacterial Starter Cultures: Concentrated microbial cultures individually or in the form of mixed cultures are used to promote fermentation in food products and are known as starter cultures or starters. Bacteria, especially lactic acid bacteria (LAB) and coagulase-negative staphylococci (CNS), along with some yeast and molds, can be used as starters, which increase the safety and shorten the time of ripening for fermented foods. On the other hand, starter cultures, allowed as GRAS (generally recognized as safe) according to the US Food and Drug Administration (FDA), can eliminate the growth of unwanted microbes including pathogenic and deterioration-causing microorganisms (6,7,8). The substrates, strain(s) characteristics, food safety needs, and quality properties should be considered as selection criteria for starter cultures (7). Starter cultures contain different species and strains of microorganisms to limit the growth of inappropriate microorganisms and suppress the growth of spoilage-causing microorganisms (9,10).

Currently, starter cultures used in the production of fermented products have received special interest. Traditional products are manufactured in different countries around the world. The use of these cultures is a crucial and sustainable way to preserve some food products with known technological benefits (11-15).

Dairy Starter Cultures: Raw milk for processing is usually obtained from cows, but sometimes it comes from other mammalians such as sheep, water buffalo, and goats. Milk is then converted into various dairy products. Many dairy products such as yogurt, cheese, butter, cream, and kefir have been produced and consumed around the world for thousands of years (16). Dairy products are energy-rich food products that are produced from milk (17). The plant-based diets are varied with the inclusion of dairy products. However, both in the scientific and traditional literature, the role of milk and dairy products in human nutrition has been discussed in recent years (16). As a result,

the number and variety of dairy products produced in the world are increasing (Figure 1).

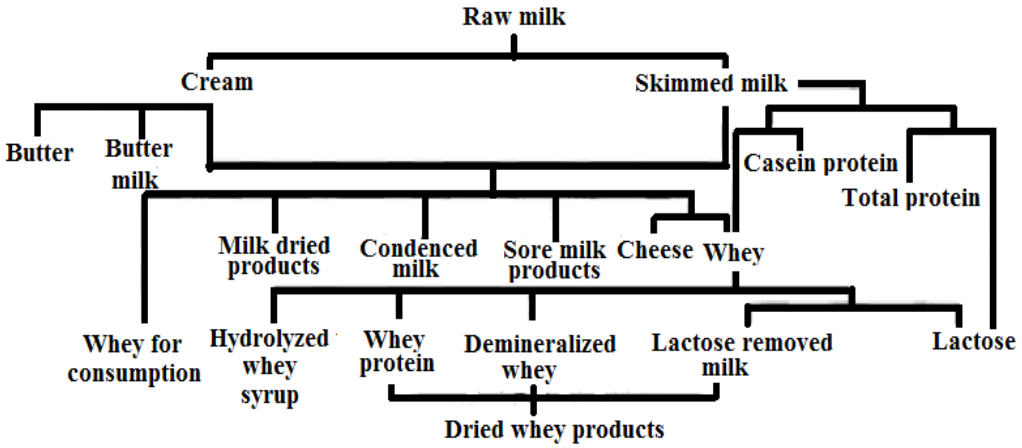

Fig. 1- Milk Processing Procedures

Fermented milk products are made from milk, whole, mostly, skimmed, concentrated, or substituted; pasteurized or sterilized, by using special microorganisms (18). These products have also been considered carriers for alive probiotic microorganisms. Many types of fermented milk and fermented milk products are produced using starter cultures with known properties in dairy industries (16). Milk products have favorable taste and odor and also act as a crucial vehicle for transmitting probiotic bacteria. Probiotics had associated with dairy products for a long time (19).

The starter cultures are like the active bacteria of harmless food-grade microorganisms, which grow deliberately in milk or whey, or other formulation environments to create the desired and predictable taste and texture in milk products. The microorganisms used in fermented milk that produce various types of fermented milk products are cultures of individual strains or mixed strains of LABs (20,24). They have also different beneficial effects on human health in accordance with the treatment of various digestive diseases (25,26), chronic kidney disease (27), immune system disorders (28), and high blood cholesterol (29).

Different lactic starter cultures are applied in the production of industrial fermented milk products around the world. They can be classified into two cultures, mesophilic and thermophilic (Table 1) (16).

Table 1- Taxonomical View of Bacterial Dairy Starter Cultures

Starter culture type	Old name	New name	Major function	Usage
Mesophilic	*Streptococcus lactis*	*Lactococcus lactis* subsp. *lactis*	Acid production	Sour cream, buttermilk, cheese
	Streptococcus cremoris	*Lactococcus lactis* subsp. *cremoris*	Acid production	Sore cream, buttermilk, cheese
	Streptococcus diacetylactis	*Lactococcus lactis* subsp. *Lactis* biovar *diacetylactis*	Flavor, acid production	Sore cream, buttermilk, ripened butter, cheese
	Leuconostoc cremoris	*Leuconostoc mesenteroides* subsp. *cermoris*	Flavor	Sore cream, buttermilk, ripened butter, cottage cheese
	Leuconostoc lactis	Unchanged	Flavor	Sore cream, buttermilk, ripened butter, cottage cheese
Thermophilic	*Streptococcus thermophilus*	Unchanged	Acid production, flavor	Yogurt, fermented milk types, Italian cheese, Emmental cheese

	Lactobacillus bulgaricus	*Lactobacillus d*elbruekii subsp. *bulgaricus*	Acid production, flavor	Yogurt, fermented milk types, Italian cheese, Emmental cheese
	Lactobacillus lactis	*Lactobacillus d*elbruekii subsp. *lactis*	Acid production, flavor	Yogurt, fermented milk types, Italian cheese, Emmental cheese
	Lactobacillus helveticus	Unchanged	Acid production, flavor	Yogurt, fermented milk types, Italian cheese, Emmental cheese

Mesophyll Starter Culture: Mesophyll starter cultures are detected by the best grow at 25-30 ℃. Mesophilic cultures are widely used in the milk fermentation industry to produce products such as 'feta, 'ymer' (in Denmark), 'filmjo¨lk', and 'lactofil' (in Sweden) (20,21). Mesophilic starters are mainly contained *Lactococcus lactis* subsp. *cremoris*, but this bacterium is rarely used alone. Combined starters containing *Lactococcus lactis* subsp. *cremoris* with *Leuconostoc* species are used in Norway, Sweden, and Finland for the production of buttermilk, 'la˚ngfil', and 'viili'. Lactobacilli also are used as cheese starter culture and have been isolated from natural cheese such as Poosti cheese from ewe's milk (22) and Siahmezgi cheese which have been traditionally produced in Iran (23). Sour cream, cultured buttermilk, and kefir are other fermented dairy products made with mesophilic starters (20). Additionally, mesophilic cultures are used to produce stirred types of yogurts.

Thermophilic Starter Cultures: Thermophilic starters which are active at higher temperatures (37-45 ℃) are used for manufacturing yogurt, Bulgarian buttermilk, and different other products made with intestinal bacteria, primarily lactobacilli, and bifidobacteria. Today, thermophilic starter cultures are used to produce different types of fermented milk products like many kinds of yogurt (30) and some cheese products (20). Also, thermophilic starter cultures especially lactobacilli have well-known effects on biological decontamination in fermented foods (31,32).

Meat Starter Cultures: Fermented meat as one of the "functional foods", is a new approach to achieving a healthier status that reduces the risk of diseases such as type 2 diabetes, cardiovascular disorders, and colorectal cancer (33). LABs play a crucial role as starters in meat fermentation products by preventing corruption as well as creating the desired taste and texture (34). The most important meat fermenting bacteria are *Lactobacillus sakei*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus carvatus,* and *Lactobacillus xylosus*. Other bacteria such as Gram-positive, coagulase-negative *Staphylococcus*, and *Kocuria* spp. are recommitted as a basic technology in meat fermentation (35,36). *Lactobacillus sakei* is one of the most remarkable lactic acid bacteria that is used as a starter culture in fermented meat. The ability to adapt to the meat conditions and components is a unique feature of these bacteria (37). It is proposed that the phenotypic changes in the morphology of colonies and cell shape during *Lactobacillus sakei* colonization in the gastrointestinal tract are advantages leading to selective growth in meat (38). *Lactobacillus sakei* also produces sakacin, which is a member of class II bacteriocins with a strong antimicrobial effect on a wide range of foodborne pathogenic microorganisms, particularly members of *Enterobacteriaceae* (39). *Lactobacillus plantarum* is another important bacterium in fermented meat. Different strains of *Lactobacillus plantarum* can ferment sugars into L and D isomeric forms of lactic acid .Besides, this bacterium has shown high antibacterial activity on pathogenic bacteria such as *Escherichia*

coli, *Salmonella*, and *Listeria monocytogenes* (9,40).

Common Methods for Bacterial Cell Preservation: To protect microorganisms, many conservation methods have been used. Continuous growth, dehydration, and frozen storage are the used techniques that can be divided into three categories. These categories can be further subdivided. Maintaining the viability and genetic stability of the culture by decreasing the level of metabolism and thus increasing the period between subcultures is one of the tools for the improvement of conservation methods (41).

Regular Subculture: Microbial culture can be preserved by periodic transfer to a sterile, fresh environment. The culture stored in this way is preserved by intermittent cycles of exponential growth and preservative periods resulting in sets of subcultures. A well-known technique to all involved microbiologists is a subculture that requires basic skills of aseptic culturing with no special equipment. The number of transfer times depends on the type of the organism. For instance, *Escherichia coli* culture should be transferred at monthly intervals. The slants can be preserved for 20-30 days at low temperature, after growth for 24 hours at 37 ºC. To maintain the cultures alive, it is required to use a suitable culture medium and the right storage temperature. Culturing in a low-nutrient environment reduces cell metabolisms, and as a result, the number of subcultures can be reduced. Numerous factors are considered in the storage of microbial cultures by the sub-culturing method (41). Solid media should be preferred to broth media because the contamination of broth media is more likely to happen. Slope cultures are used most of the time for preservation, but microaerophilic and facultative anaerobes sometimes benefit from stab culture; therefore, the tubes should be sealed sufficiently after the sub-culturing of these bacteria. Cotton wool plugged tubes are not suitable, because the media dries quickly and the bacteria will be killed. The sub-culturing has many drawbacks, mostly changing in characteristics. Changes may be often occurring between strains when short transmission intervals are considered. When a large number of cultures are required and different persons do the transferring steps, contamination occurs frequently. Another drawback is mislabeling. Cultures may be labeled with false numbers or names, leading to distortion and unrecognizing. Loss of cultures also happens occasionally and is maybe more usual in delicate organisms. Fluctuations in temperature in incubators or refrigeration units may influence the possibility of loss (41).

Paraffin Method: This is a simple and low-cost way to keep the bacterial and fungal cultures at room temperature for a longer time. Sterile liquid paraffin, in this way, is poured on the slant culture of microorganisms and stored directly at room temperature. The paraffin layer prevents medium dehydration. The metabolic activity slows down in this method, by reducing growth via reducing oxygen tension. It is also possible to preserve the culture by a layer of sterile mineral oil that covers the agar slants about half an inch above the slant surface. The oil should not expose the tip of the slanted area. Cultures covered with mineral oil are kept at room temperature or, if it is possible, at 0-5 ºC. Some of the microorganisms have been satisfactorily preserved by this method for 15-20 years or more. There is something to keep in mind when preserving cultures in oil. Unless the oil is far above the highest surface of the medium, the medium may dry up, separate from the tube wall, and floats to the surface of the wall, where living organisms may be found dead. The purity of the oil is so important, because

any spoilage or toxic materials may be harmful to the cultured organisms. Preferably the oil is sterilized in a hot air oven at 150 to 170 ºC for one hour; because during autoclaving, the mixture of moisture with the oil gives it a milky appearance (41,43).

Preservation in Soil: Different fungi like *Aspergillus*, *Fusarium*, *Alternaria*, *Penicillium*, and *Rhizopus* have been successfully stored in sterile soil. Storage in soil includes inoculation of spore suspension in soil (twice sterilized in autoclave) and incubation for 5-10 days at room temperature. This period of early growth leads to the usage of moisture by the fungus and gradually enters dormancy. The containers are then preserved in the refrigerator (41). It has been shown that the soil type and the type of stored microorganisms influence the viability of the microorganism. For instance, the microbial community in arable land soils has been more persistent than that in forest soils. On the other hand, fungal communities have been more stable than bacterial communities in soil (44).

Preservation in Silica Gel: It is possible to store bacteria or yeast in silica gel powder for 1-2 years at low temperatures. In this way, heated and cooled silica powder is mixed with a concentrated suspension from the cells and kept at a low temperature. The main principle of this method is rapid drawing at a low temperature, which results in cell survival for a long time (45). Sol-gel processing is used for the preparation of silica gel in which the reaction of soluble silica precursors resulted in the formation of amorphous networks composed of siloxane bonds. Among bacteria, Gram-positive bacteria have been preserved better in silica gel (46).

Preservation at Refrigerator or Cold: When the temperature is lowered to 4 ºC, live microorganisms in a culture medium can be kept alive in refrigerators or cold rooms. In this temperature range, the metabolism of microbes is falling a lot but does not stop. For this reason, bacterial metabolism will fall down and only fewer quantity of nutrients will be used. It is not possible to use this method for very long times because toxic products may be accumulated and microbes may die. This method of storage is used only for the short-term storage of cultures. It has been shown that among bacteria, *Bacillus* and *Acinetobacter while* among yeast, *Candida* and Saccharomyces were the most genera stored by this method (47). Some factors can influence the long-term survival of lactic acid bacteria, including sufficient oxygen content in accordance with the bacterial metabolism characteristics, sufficient water activity and acidity, and low osmotic stress (48).

Freezing Methods: The usual process for the storage of bacteria is freezing. Thereby, concentrated bacterial suspensions can be used at a temperature of -30 ºC. The microbial metabolism decreases with decreasing temperature and in the state of severe preservation in liquid nitrogen at -196 ºC, the metabolism drops to zero. A method for the actual lysis of cells is freezing and thawing. In addition, by conversion of water during freezing to ice, the concentration of electrolytes in unfrozen water can also be harmful, because the osmotic pressure of the cells outside will highly be varied from inside which leads to osmotic stress. Cultures can be so effective if freezing is done in the presence of a cryoprotectant, which diminishes the damage caused by ice crystals. Glycerol or dimethylsulphoxide (DMSO) are usually utilized as cryo-protectants. Generally, 15% (v/v) glycerol is added to the culture and then the culture is stored at -20 or -80 ºC in a freezer is the easiest way to preserve a culture. Cultures can be kept in glycerol at a temperature of -40 ºC in a freezer for many years. Glycerol

solution with the approximate volume of 2 ml is added to the agar slant culture in this method. Culture can emulsify by shaking. Next, the emulsion is transferred to ampoules and each ampoule contains 5 ml of the culture and is quickly frozen at -70 ºC. Ampoules are then taken and placed directly in a deep freeze at -40 ºC to be used for stock cultures. Before using for cultivation on the agar plates, the ampoules are placed in a water bath at 45 ºC for a few seconds (49).

Utilizing ultra-low temperature in cryogenic storage method gained by freezing in liquid nitrogen at -196 ºC has shown to be an easy standardized method for preserving a broad range of microorganisms and mammalian cells. Also, availability as a live suspension, slight loss of viability, quick resuscitation, and speed of preparation are advantages of liquid nitrogen storage. The high cost of the apparatus and regular usage of liquid nitrogen which has the risk of explosion when injected at room temperature, the loss of a large number of cultures if the level of liquid nitrogen is not closely monitored and probable contamination of the liquid nitrogen in the storage tank, if an ampoule is broken, are some of the disadvantages of liquid nitrogen storage (49). Some other approaches also have been proposed for reach to low temperatures such as a cryogenic apparatus based on high electric fields for producing a high density of Ultra-Cold Neutrons (50).

The high viable maintenance during storage is a main important feature of LAB starter cultures which are directly inoculated to food matrices to produce products with probiotic characteristics. Keeping at a low storage temperature such as −80°C and rapid thawing have been proposed for reaching the high viability of the frozen cells (51). Processing conditions affect the survival rates of lactic acid bacteria during freezing and frozen storage. Fonseca et al. (2000) evaluated the resistance of *Streptococcus thermophiles* and *Lactobacillus delbrueckii* subsp. *bulgaricus* during freezing and frozen storage by determination of the decrease in acidification activity. The effects of 13 factors were assessed by using a Plackett and Burman experimental design. All factors except the thawing temperature showed significant effects on the acidification activity. Cryo-protection temperature and its duration influenced acidification activity. The higher activity was obtained in lower temperatures and shorter durations. Also, fermentation conditions including pH control by the use of NaOH instead of NH_4OH, the addition of Tween 80 in the culture medium, and faster cooling resulted in better cryo-tolerance. The high freezing rate and low storage temperature improved the resistance to frozen storage (52).

Preservation of Microorganisms by Drying: Some sensitive strains to freeze-drying can be stored by drying from the liquid state without the initial freezing. Some methods have also been developed for drying bacterial suspensions for protective purposes and are used in laboratories that cannot provide expensive apparatus used for very low temperatures storage or frozen drying, or where culture storage is rarely performed. (42).

Drying on a Disk: Bacteria in the form of a concentrated suspension is poured on sterile discs of high-absorbing paper and then dried by using a vacuum drier in the flow of phosphorus pentoxide ($P_2O_{5)}$ (53). A concentrated microbial suspension can be also added to nutrient gelatin. Bacterial suspension that drops in gelatin is put on a sterile waxed paper or a plastic Petri dish and after that dried off over P_2O_5 under vacuum (42). Different kinds of bacterial species, e.g., *Enterobacteriaceae*, *Branhamella*, *Haemophilus*, *Neisseria*,

Flavobacterium, *Streptococcus*, *Gemella*, *Pseudomonas*, and *Bacteroides* species, were successfully kept for 1 to 5 years via the method of drying on gelatin disks. The activity of beta-lactamase in some penicillinase-producing bacteria such as *Neisseria gonorrhoeae* was maintained by this method for more than 3 years. Airmailing of many strains of *N. gonorrhoeae* surrounded in gelatin disks from Japan to the US also has been successful. The organisms of *Neisseria*, *Gemella*, *Branhamella*, and *Haemophilus* have been suspended in the solution utilized for preparing the gelatin disks and stored for 6 to 12 months at -20 ºC after freezing the cell cultures. Plus, changing the gelatin disk storage method made feasible the safe preservation and short-distance transport of clinical strains (54). By another method, thick concentrations of bacteria are made in a desiccator over P_2O_5 in a vacuum before drying and then drops on sterile cellophane or starch, peptone, or dextran pre-dried plugs (42).

Liquid Drying: Liquid cultures of microorganisms, especially vesicular-arbuscular mycorrhizal fungi, are directly dried using a vacuum pump and in small ampoules. A water bath is used to control the temperature of the liquid drying. Using this method, the suspension of the organisms is dried in the vacuum from a liquid state without pre-freezing. In this method, cell suspensions are dropped on activated charcoal thin discs containing skim milk and a sufficient protective substance and then dried in mild vacuum conditions. The new method has been successfully used to preserve a collection of microorganisms sensitive and will be damaged by freezing or freeze-drying (55). There are some recommendations for dry liquid:

1. Using a small volume of suspension distributed over a large surface area, before freezing the material is dried by high-rate evaporation.

2. It is possible to prevent freezing by decreasing the water vapor flow from the dried material, or by placing cotton plugs into the ampoules, by a vacuum controlled by using a valve.

3. To prevent the suspension from freezing under a vacuum, immersing the ampoules in a water bath can obtain sufficient heat input in the culture medium (42).

Spray Drying: A method of making a dry powder from a liquid or slurry mixture by rapid drying using hot gas flow is called spray drying (Figure 2). The usual method for drying numerous thermally sensitive materials like foods and pharmaceutical is spray drying. The constant distribution of particles is the advantage of spray drying many industrial products including catalysts. The air hits the drying suspension; however, in the case of flammable suspensions such as ethanol or when the product is sensitive to oxygen, nitrogen gas is used. The spray-drying process for bacteria is done on a larger production scale with lower energy costs than current freeze-drying processes, which is more sustainable. Microencapsulation of bacteria in different protective matrices is also a promising way to maintain their better viability in digestive pressures during preservation and processing (42).

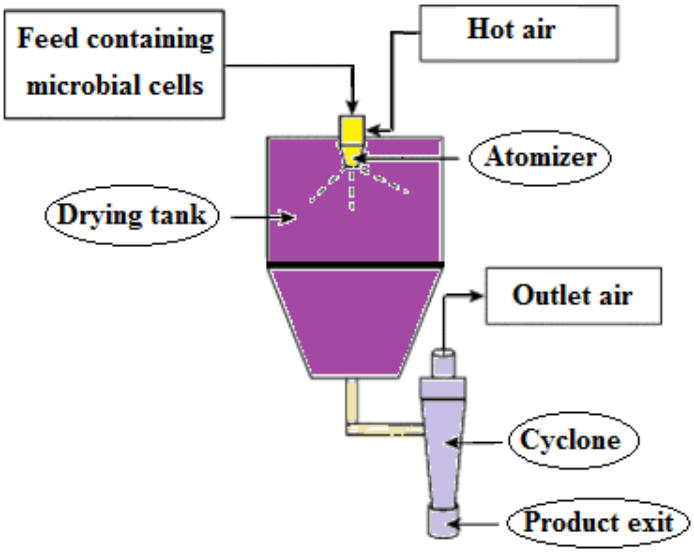

Fig. 2- The Schematic View of the Components of a Spray Drier Apparatus

Freeze Drying: Freeze-drying is the most confident technique to preserve bacterial cultures. Lyophilization is another name for freeze-drying in which bacterial cultures or virus liquids are dried and stored in the dry state under appropriate conditions. A high concentration of salt is produced in the later stages of drying which causes damage to protein, organisms' death, and breakdown of serum. In the freeze-drying technique, the culture or serum is rapidly dried in a vacuum from the frozen state. The material is properly frozen by an applicable method and then dried via sublimation of the ice. The technique is a multi-step procedure in which, by freezing, the metabolic activity is temporarily stopped at the beginning, then the removal of water with no thawing (sublimation) has happened, and a dry product is obtained (Figure 3). The yield product is sealed either by vacuum or by inert gas and can be kept at room temperature because of the inhibition of metabolic activity and no need for water or nutrients. Freezing should be very fast, and the temperature should drop below 0 ºC (-20 to -80ºC), because slow freezing causes exposure to the denaturing of cell macromolecules. The liquid must be frozen in a thin layer with a large surface to be evaporated (56-58). Freeze-drying also can be used for the preservation of beverage starter cultures. In this area, Jafari et al. (2020) investigated the drying of kombucha starter culture and its effect on invertase enzyme and antioxidant activities in the manufactured tea. No significant difference was shown between the fresh and dried starter cultures in both activities (59).

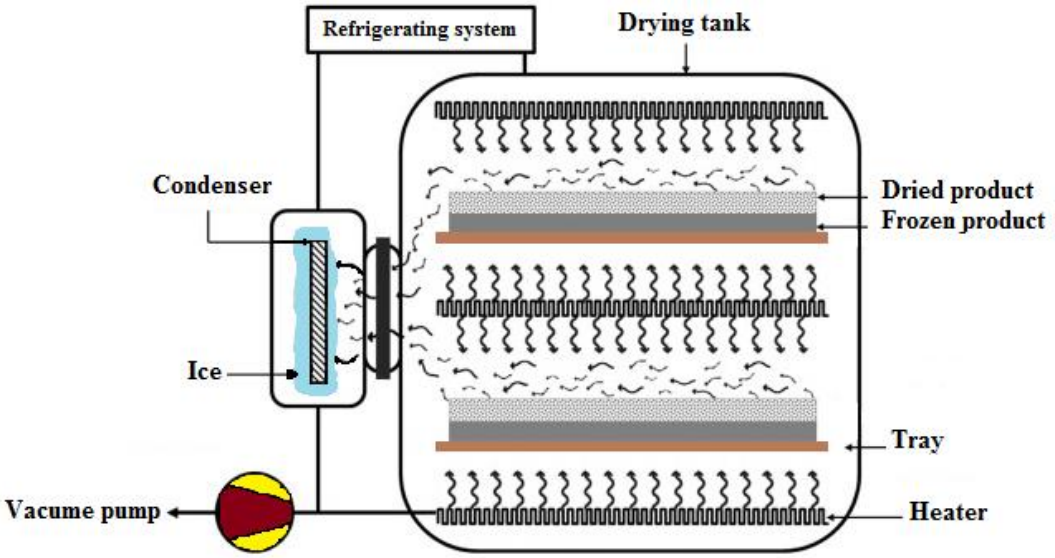

Fig. 3- The Schematic View of the Components of a Freeze Drier Apparatus

Freeze-drying contains several steps are included:

a) Pre-drying of cultures: A vital criterion in this stage for some microbes is the type of culture media used for freeze-drying. The pre-drying culture and the freeze-dried culture may be the same or different. The pre-drying environment must have a high concentration of microbes. Another important criterion is the age of the culture because the cultures that have reached the desired stage of development will be viable better than the cultures that are still in the stage of development.

b) Preparation of the ampoules: Ampoules that are used to preserve the culture must manufacture from neutral glass and be sufficiently sterilized before use. After tying the cotton wool, the ampules should be properly disinfected.

c) Cultures harvesting: After incubation, cultures grown on agar slants must harvest for 3-5 days. Suspending fluids such as horse serum, glucose, and nutrient broth must use to harvest the culture. The cultures should be immediately transferred to the ampoules.

d) Primary drying: Specified centrifuges are utilized for this step. The initial drying process is done between 2.5 to 4 hours, during which about 90% of free water is lost. Then the slow entrance of the air into the vacuum chamber is allowed. Finally, the centrifuge head is removed from the machine and the ampoules are sealed with cotton wools.

e) Secondary drying: The secondary dryer contains high-grade P_2O_5, in which the ampoules are transferred, and then a vacuum pump is attached to it. The ampoules remain on the dryer for 18-20 hours which results in the reduction of moisture content to 1%. The ampoules are inspected for vacuum persistence and then closed with a flame. The obtained powdery culture in the ampoules can be kept at 4 ºC (42).

The centrifugal and shelf are two types of commercial freeze-dryer that are commonly used. In initial freezing, evaporation happens when the vacuum is used, and the cell cultures are centrifuged during this step to boost the surface medium and protect frothing. The centrifugal method for progressed culture collections has the advantage of minimizing the possibility of contamination as the ampoules may be closed at the end of the secondary drying step after filling and closed under a vacuum. According to the

manufacturer's directions, lyophilized cultures should be rehydrated and survive via repeated transfers or freezing steps. One of the main advantages of freeze-drying is that the ampoules are especially reasonable as a tool for culture distribution, as the integrity and viability of the ampoules remained unchanged in conditional changes during airmail services. The relatively high-cost equipment is a disadvantage of freeze-drying (42).

Freeze Drying Conditions Affecting Rehydration Process: Dried starter cultures are required to be prepared in suitable conditions in which the microorganisms maintain the maximum survival rate during rehydration (60-63). The protective medium used in the process of freeze-drying is an important factor affecting the viability of microorganisms after rehydration. Some studied protectants include monosaccharides such as glucose, sucrose, fructose, lactose, trehalose, sorbitol, mannitol, and maltose; amino acids such as sodium glutamate; polyols; and yeast extract (64). However, the used protectant usually varied based on the species of starter culture microorganism (65). Sucrose can act as a protective agent for bacteria. It is safe for consumption and can boost sweetness (66). The addition of sucrose as a cryo-protectant prevents the destruction of proteins in microbial cells (67). The usual use of coating material (cryo-protectant) comes from encapsulants, like carbohydrates, gum, and proteins to cover the main ingredients (bacteria) with certain goals, such as protection as opposed to environmental influences, masking bad taste and odor, preventing evaporation, and increase the stability. Utilizing protein as a cryo-protectant can sustain bacterial resistance while using carbohydrates can maintain the bacterial resistance and improve microcapsule texture (63). The initial cell concentration is the other factor affecting cell viability during rehydration. It has been shown that the highest surviving values are achieved when the freeze-drying process has been initiated with the cell concentrations between 10^9 and 10^{10} CFU/ml (61). Freezing temperature before drying is also a crucial factor for maintaining the survival of freeze-dried starter cultures. It has been shown that the highest survival levels for yeasts reached at -20 and -80°C, although these levels were obtained at -196 °C for lactic acid bacteria (68). These results may have been affected by the type of the used cryo-protectant compounds and the bacterial strain. In this regard, Wang et al. (2020) detected the survival rates of freeze-dried *Lactobacillus plantarum* strains at 4 different pre-freezing temperatures in the presence of different protectants. Pre-freezing at the temperatures of −196°C, −40°C, and −20°C led to the highest survival rates for 3 strains AR113, AR307, and WCFS1, respectively, by using phosphate-buffered saline solution and sorbitol protectants. By using trehalose, the best survival results were obtained at −20°C for the strains AR113 pre-freezing ensured, and −60°C for the strains AR307 and WCFS1 (69).

Although freeze-drying is considered a more effective method for drying starter cultures, some improvements are required to this method to decrease its destructive effects on the integrity and fluidity of cell membrane (70,71), and the integrity of the protein’s structures (72). Sugars and skim milk are the most used protectants for LABs viability maintenance during passage through freeze drying and storage (73,74).

Oxygen is another factor that negatively affects the viability of LABs during the production and rehydration of freeze-dried starters because of its oxidative stress induction. Therefore, oxygen-free gas flow may enhance the survival of LABs during these processes (75).

Microwave Vacuum Drying: Compared to ordinary vacuum drying, this method has

benefits related to shortening the drying time, while retaining product features especially for drying biologically sensitive materials. According to the volumetric microwave input, it is possible to reduce the time down to 90%. When drying viscous liquids, the remaining foamed structure is stable drying which has some advantages because the third drying stage is progressed with the porous structure. Because foams not only have to be thermally resistant within the microwave vacuum method, but also have to withstand the vacuum. So, a special method for foam drying by microwaves under low-pressure states was created. The foam formation and stabilization will be gained via utilizing a synergistic combination of carbohydrates and proteins. Studying the surface activity and foaming conditions when drying *Lactobacillus paracasei* showed an important positive sign. *Lactobacillus paracasei* was shown to be adsorbed directly at the air-water interface. In addition, the structure of the liquid layers was assumed. Plus, the drying time was decreased to at least 50% in comparison to the drying in the microwave vacuum which was lack of foaming. It was shown that the slight decrease in the viability of the cells in this method is mainly because of the relatively high moisture content and high vacuum levels at the initiation steps of the procedure. The use of continuous drying foam suggests a proficient and energy-saving option to the present techniques for adopting sensitive material. When sensitive materials such as protein involving drugs is dried, this process can be used to preserve the starter cultures and probiotics as well as in pharmaceuticals manufacturing. This process is particularly reasonable for freezing-sensitive and thermo-labile materials. So, factors such as foaming, vacuum use, and final drying don’t affect bacterial cell viability. Therefore, by combining lactic acid bacteria in foam structures, drying can be done in a fraction of the time, and produce more results in high-product quality (76).

A comparison of different methods of the preservation of lactic acid starter cultures is presented in Table 2. The advantages and disadvantages of different methods for drying microbial starter cultures may come from several factors including the acid produced during storage, the oxygen level in the products and the package permeability to oxygen, and the production of antimicrobial agents by the cultures during drying and storage process (77).

Table 2- Major Advantages and Disadvantages of Different Preservation Methods for Lactic Acid Starter Cultures

Preservation method	Advantages	Disadvantages	Additives	References
Regular subculture	Without the risk of cell death during preparation; ready to use	Alteration of genetic content because of a high number of passages; by the preparation of the cells growth curve in the preservative culture, the approximate subculture times can be calculated	No additives; medium containing minimal nutrition lowers the metabolism of the organism	41
Paraffin	Simple; cost-effective	It is better to keep the cultures at 0-5 °C; drying up the medium is probable which causes cell death	A layer of mineral oil above the paraffin layer can inhibit the drying of the culture	41,43
Soil	Simple; cost-effective; genetic stability	Only suitable for spore suspension; more applicable for fungal communities than bacteria	No additives; arable land soils are more suitable than forest soils	41,44
Silica gel	Simple; cost-effective	Low quality; usable for a limited range of microorganisms	No additives	46
Cold	Simple; cost-effective	Alteration of genetic content; high risk of cell death	No additives; medium containing minimal nutrition lowers the metabolism of the organism	47,48
Freezing	Available; cost-effective	High loss of viability in temperatures upper than -196 °C; Costly (especially when liquid nitrogen is used)	Glycerol (5-30%), sugars such as saccharose, and DMSO (5-10%)	49-52
Drying on a disk	Simple; cost-effective; suitable for short-distance transport of cultures	Low quality; usable for a limited range of microorganisms	No additives	42,53,54
Liquid drying	Lower cell destruction in comparison to freeze-drying;	Lower quality than freeze-drying	No additives	55
Spray drying	Cost-effective; rapid; stability during storage	Thermal destruction of cultures; Requirement for pre-processing methods such as microencapsulation	Skimmed milk, polydextrose-based prebiotic substances	77,78
Freeze-drying	High-quality and high-value dehydrated cultures; storage stability; easy handling and transportation	Slow dehydration process; expensive; protectant requirement; strain dependent	Skimmed milk, Glycerol (5-30%), dimethylsulfoxide (DMSO, 5-10%), non-permeable additives like polysaccharides, prebiotics like inulin and oligofructose, plant oils, $CaCl_2$ (0.5-2%), fatty acids such as oleic acid, tween 80, sugars such as saccharose	79,80,81
Microwave vacuum drying	Rapid; retaining the fine quality of the product	High energy consumption	No additives	80,82

Conclusions

Starters are considered the heart of fermented products and are the most important component in producing high-quality fermented foods. The comparison of drying methods shows that selection of the method can strongly depend on condition and scale. Spray drying is a preferred method for thermally-sensitive materials such as starter cultures which can undergo heated air exposure. Spray drying is a large-scale method of production of starters, and is more cost-effective and sustainable than freeze-drying. Freeze-drying is a more widely used technique in which the metabolism of the cells is first stopped by rapid freezing and then the culture is dried by the ice sublimation process. In this process, the damages caused by the accumulation of salts in the medium are also avoided. The maximum survival and stability rates after rehydration can be achieved when the cultured are prepared in suitable conditions before freeze-drying, and by using proper protectants based on the species of the dried microorganisms. The protective medium used in the process of freeze-drying is an important factor affecting the viability of microorganisms after rehydration. The sufficient initial cell concentration (10^9-10^{10} CFU/ml) may also affect the cell

viability during rehydration. Future studies can be done on the progression of the available methods and propose alternative drying processes. The disadvantage of frozen starter cultures is that they require very low transport or storage temperatures preferably -20 to -40 °C. In addition to the risk of thawing, the high cost of transportation may restrict the marketing of frozen starter cultures economically in remote regions or countries. So, many efforts have been made to breed lower-cost alternative drying processes.

Acknowledgment

The authors thank the plant improvement and seed production research center, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan, Iran, for technical support, and Shahid Beheshti University of Medical Sciences for financial support.

ORCID

Kianoush Khosravi-Darani 0000-0002-0269-6385

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