MINIREVIEW

# Practice and prospects of microbial preservation

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## Abstract

A growing interest in culturable diversity has required microbiologists to think seriously about microbial preservation. In addition to the isolation and cultivation of pure strains, adequate preservation without changes in morphological, physiological and genetic traits is necessary. This review consolidates different methods used for preservation of microorganisms with an emphasis on cryopreservation and lyophilization. The critical points of cryopreservation and lyophilization are highlighted to explain how several extrinsic and intrinsic factors affect the cell survival and recovery during the process of long-term preservation. Factors responsible for alteration in genotypic and phenotypic integrity of cultures during preservation and methods used for their evaluation have been incorporated. We emphasize the importance of depositories and highlight their current funding status. Future areas for preservation research, including cell dormancy, ecosystem and community level preservation and the effects of the viable but non-culturable state on post-preservation recovery of the cells are also discussed.

## Introduction

Extensive research in microbiology has revealed that almost all ecosystem services, including biogeochemical cycling of materials, degradation of pollutants, wastewater treatment and production of oxygen, are mediated by microbial metabolism (Hayat *et al.*, 2010; Senni *et al.*, 2011). Microbes are the backbone of modern biotech industries and are utilized for generation of bioenergy and biofuels (Stahl & Wagner, 2006; Bhardwaj & Garg, 2012). They are a natural source of novel therapeutics, used as bio-pesticides and bio-fertilizers, and for maintaining the sustainability of the environment (Zaidi *et al.*, 2009; Hayat *et al.*, 2010). Despite providing valuable ecosystem services, microbes also act as causative agents of diseases and affect human health and hygiene (Arrigo, 2005; Lal *et al.*, 2010; Singh *et al.*, 2010; Kostka *et al.*, 2011).

Increasing awareness about culturable diversity and the development of modern cultivation approaches is constantly increasing the numbers of new and previously uncultivated taxa of microorganisms in culture collections (Zengler *et al.*, 2002; Leadbetter, 2003; Giovannoni & Stingl, 2007; Alain & Querellou, 2009; Pace, 2009). Cultivation and characterization of microorganisms alone is not adequate without preservation techniques that do not alter the morphology, physiology or genetics of pure strains. Careful preservation is imperative for future research, teaching and industrial applications (Ward *et al.*, 2001, Emerson & Wilson, 2009).

The current review discusses the crucial points of microbial preservation, highlights the importance of microbial depositories, and outlines future areas of preservation research. In addition to providing the current status of preservation research, the aim of this article is to assist the reader in designing better preservation protocols, including selection of the right cryoprotectants and appropriate culture conditions to ensure long-term preservation of microorganisms.

## Microbial resource centers and preservation research

Microbial resource centers (MRCs), or microbial depositories, are generally considered knowledge hubs for the life sciences and underpin biotech-industries (OECD, 2001; Cypess, 2003; Stern, 2004; Janssens *et al.*, 2010). Despite the immense importance of MRCs in research, many of these depositories are underfunded. Of the 476 culture collections registered under the World Federation of Culture Collections, only a few are fully funded,

whereas others are sustained by generation of their own funds or face shutting down (Smith, 2003; Stackebrandt, 2011). MRCs, along with other organizations such as the American Society for Microbiology, Society for General Microbiology and Federation of European Microbiological Societies should promote the concept of microbial diversity and highlight the role of MRCs by devoting special session in meetings, and seminars and publishing special issues on microbial preservation. These activities will create an awareness of the importance of microbial culture collections among researchers, private industry and government agencies, and will encourage more funding to support MRCs. Stackebrandt (2011) also raised the point that underfunding and insecure carrier prospects of depositories demotivates existing staff and fails to attract young scientists to the area of cultivation and preservation research.

Many depositories focus mainly on their service components and do not support research on microbial cultivation, new media formulation, and preservation. As mentioned above, sufficient funding and/or the appropriate scientific staff may not be available at these facilities. Furthermore, major stakeholders of MRCs, including universities, institutions, hospitals and biotech industries, do not fully utilize the research capabilities possible with microbial collections. Therefore, it is advisable that the curators of the centers maintain their service as well as research components, and develop plans to expand collaborations with other stakeholders.

MRCs should also encourage researchers to deposit their strains in depositories with complete information on taxonomic status, pathogenicity, method of preservation, and stability of traits after revival. In many cases, even after isolation of the novel taxa, researchers do not submit isolates to the depositories. As a result, information on the optimization of cultivation and preservation for specific strains is not transferred, and consequently valuable diversity may be lost. Similarly, a researcher may immediately submit the strains to culture collections without checking the long-term viability and consistency of observed traits. It is often assumed that the expert staff at MRCs will maintain the strains, but there is a lack of resources for optimization and cultures may become nonviable or extinct before a preservation protocol is established. It is advisable for researchers, particularly after isolation of novel organisms to optimize culture conditions and test preservation methods. Furthermore, the viability and stability of the traits should be confirmed for up to 1 year before submitting the strains to culture collections. In addition to focusing on taxonomy, MRCs may secure more funding and become self-sustainable by expanding expertise in the areas of kit development for rapid diagnosis, formulation of low-cost media for industrial scale cultivation, and development and formulation of bio-fertilizers and bio-pesticides. Bio-prospecting of existing strains for industrial exploitation may also provide funding opportunities.

## Methods of microbial preservation

Several methods have been successfully used for the preservation of microorganisms: repeated sub-culturing, preservation on agar beads (Winters & Winn, 2010), oil overlay of slant-grown cultures (Nakasone *et al.*, 2004), use of silica gel and other sterile supports (Liao & Sholenberger, 2003; Pérez-García *et al.*, 2006; Smith *et al.*, 2008), cryopreservation (Gorman & Adley, 2004; Smith *et al.*, 2008) and lyophilization (Berner & Viernstein, 2006; Morgan *et al.*, 2006). Among these, cryopreservation and lyophilization are highly utilized for culture collections and industry, and a discussion of technical aspects and the pros and cons of both the methods is warranted.

A large body of information regarding the practice and protocols of microbial preservation is now available on the website of the Organization for Economic Co-operation and Development (OECD; http://www.oecd.org/dataoecd/7/13/38777417.pdf), as well as some of the largest culture collections: American Type Culture Collection, Deutsche Sammlung von Mikroorganismen und Zellkulturen, and 'The Common Access to Biological Resources and Information' (http://www.cabri.org). Access to this information will unveil the lacunas of the field and create possibilities for future research and innovations. Several articles including novel methods, use of cryoprotectants, effect of cooling rate and media composition on post-preservation viability, and scale-up of lyophilization have been published recently (Mukamolova, *et al.*, 2006; Krumnow *et al.*, 2009; Kuppardt *et al.*, 2009; Patel & Pikal, 2011; Heylen *et al.*, 2012; Hoefman *et al.*, 2012).

## Role of cryoprotectants in long-term preservation

In cryopreservation and lyophilization, cells are subjected to cryogenic temperatures which promote ice crystal formation in the suspension medium and within the cell interior. The resulting osmotic imbalance induces biophysical and biochemical changes (e.g. disruption of organelles and loss of membrane integrity) and causes cryo-injuries and cell death (Lovelock, 1954; Fuller, 2004; Mazur, 2004; Woke, 2007). Cryoprotectants protect the cells from cryo-injuries during cryopreservation. Several excellent reviews on cryoprotectants have been published elsewhere (Israeli *et al.*, 1993; Leslie *et al.*, 1995; Fuller,

2004; Mazur, 2004; Jackson *et al.*, 2006; Chian, 2010; Heylen *et al.*, 2012), and a detailed description is beyond the scope of this review. Cryoprotectants can be broadly classified as penetrating or non-penetrating, and cell-penetrating cryoprotectants are generally considered ideal. They protect the cell by lowering the freezing point of water, promoting hydrogen bond formation and vitrification of solvents, and preventing ice crystal formation inside the cells (Fuller, 2004; Chian, 2010). Glycerol (10–15%) and dimethyl sulfoxide (5%) are frequently used in cryopreservation of microorganisms, and both have cell-penetrating capacity. At physiological temperatures, glycerol works best, but at lower temperatures it does not penetrate well inside the cell and consequently provides less protection. DMSO has a better penetrating ability than glycerol but its use is limited due to toxic effects at higher concentrations. There are other types of compounds employed for cryopreservation apart from glycerol and DMSO but they are not in common use. These compounds are also produced by small animals and bacteria in response to cold temperatures to protect against cryo-injuries. An ideal cryoprotectant should meet all of the following criteria: be highly water soluble, penetrate inside the cell, have a low toxicity, be non-reactive, and not precipitate at high concentrations.

## Cryopreservation

The term cryopreservation refers to the preservation of biological materials at cryogenic temperatures, generally −80 °C, (dry ice) or −196 °C, (liquid nitrogen). Low temperature protects proteins and DNA from denaturation and damage and slows the movement of cellular water. Consequently, biochemical and physiological activities of the cells are essentially halted and cells are protected for long periods of time. Preservation of cells at −20 °C is not recommended for long-term preservation. Preservation at −80 °C is adequate, but −196 °C is considered ideal because the chances of DNA mutations are almost zero at that temperature. During cryopreservation, cryovials can be stored immersed in liquid nitrogen (at −196 °C) or in its vapour phase (−135 to −150 °C). Storage in vapor phase is considered better because it prevents the entry of liquid phase nitrogen into the cryovials, protecting against bursting and viral contamination (Smith *et al.*, 2008).

Most microbiologists prefer preservation of microorganisms at cryogenic temperatures (−80 °C or −196 °C) with 10–15% glycerol and/or 5–10% DMSO, and there are few studies using other cryoprotectants (Crespo *et al.*, 2000; Gorman & Adley, 2004; Nagai *et al.*, 2005; Bryukhanov & Netrusov, 2006; Cody *et al.*, 2008). Although the methods of cryopreservation and lyophilization are established and give good response in terms of viability and genotypic integrity with most of the microbes, more attention needs to be paid to the optimization of cryoprotectants and other conditions using diverse group of microorganisms (Nagai *et al.*, 2005; Berner & Viernstein, 2006; Cody *et al.*, 2008; Stielow *et al.*, 2012).

The rate of cooling and thawing is another crucial point for preservation and resuscitation of cells during cryopreservation. A controlled cooling rate (−1 to −5 °C $min^{-1}$) and rapid thawing (37 °C water bath) are reported to be optimum for cell viability (Smith *et al.*, 2008). The effect of cooling rate on survival of different types of cells (yeast, bacteria and eukaryotic cell) was studied by Dumont *et al.* (2004). The authors reported high cell recovery at low and high cooling rates, whereas intermediate cooling rates were detrimental to cell viability. They also concluded that the response of cells to cooling is not only dependent on cooling rate but also on cell size, water permeability, and the presence of a cell wall (Dumont *et al.*, 2004). Thus, storage temperature and a controlled rate of cooling, in addition to selection of the right type of cryoprotectants, are critical components for successful cryopreservation of microorganisms (Morgan *et al.*, 2006; Smith *et al.*, 2008).

## Freeze-drying or lyophilization

Lyophilization is the preferred long-term preservation method in most MRCs due to the low cost of maintenance and ease of transportation of lyophilized cultures. Lyophilization gives satisfactory results for the preservation of many bacteria, yeast and sporulating fungi, but does not adequately preserve non-sporulating fungi (vegetative hyphae), some species of yeast (*Lipomyces*, *Leucosporidium*, *Brettanomyces*, *Dekkera*, *Bulleera*, *Sporobolomyces*) and certain bacteria [*Aquaspirillum serpens*, *Clostridium botulinum*, *Helicobacter pylori*; Smith *et al.*, 2008)]. Lyophilization exerts stress on the cells during vacuum desiccation, and cells raised under stress may respond better to lyophilization (Morgan *et al.*, 2006; Smith *et al.*, 2008). For instance, a culture at stationary phase and low pH condition survived better during lyophilization than did cells in log phase grown at circumneutral pH (Palmfeldt & Hahn-Hagerdal, 2000; Corcoran *et al.*, 2004). However, the above-mentioned generalization is not true for all groups of bacteria (Boumahdi *et al.*, 1999). In general, a suspension medium with $1 \times 10^8$ cell $mL^{-1}$ or more gives a better recovery, whereas glass-forming cryoprotectants are preferred over a eutectic crystallization salt (palmfeldt *et al.*, 2003; Morgan *et al.*, 2006). Although techniques of lyophilization are well established, optimization of lyoprotectants and suspension media are still necessary for certain microorganisms (Crowe *et al.*, 1998; Miyamoto-Shinohara

*et al.*, 2000; Carvalho *et al.*, 2003; Gomez Zavaglia *et al.*, 2003; Streeter, 2003; Berner & Viernstein, 2006; Tindall, 2007; Siaterlis *et al.*, 2009). An ideal suspension medium for lyophilization should contain lyoprotectants and matrix materials or excipients (http://www.opsdiagnostics.com/notes/ranpri/rpbacteriafdprotocol.htm). The use of stationary phase cultures, borosilicate ampules, a 1–2% final moisture content of the lyophilized specimen, and storage at 4 °C in the dark are recommended for higher cell viability and longer stability with lyophilization (Morgan *et al.*, 2006; Smith *et al.*, 2008).

Both cryopreservation and lyophilization have advantages and disadvantages, and the response of preservation varies by species. Even different strains of the same species may respond differently to the same preservation method. The viability and longevity of microorganisms under preservation depends on some critical factors: (1) composition of the suspension and rehydration medium, (2) type of cryoprotectant used, (3) rate of cooling and thawing, (4) growth stage of the culture, (5) cell size and type, lipid content, water content, and initial density of cells (De Kievit *et al.*, 2001; Hubalek, 2003; Ren *et al.*, 2004; Morgan *et al.*, 2006; Smith *et al.*, 2008; Chian, 2010).

## Monitoring and management of genotypic and phenotypic stability of preserved cultures

The aim of microbial culture collections is not only to achieve long-term viability but also to maintain the genotypic and phenotypic stability of its preserved cultures, as the genotypic and phenotypic integrity is absolutely essential for the authentication of previous findings. Researchers often claim that the culture collection provides a mutant version of the expected wild-type strain. This indicates that culture collections may be dealing with mutants rather than original wild microorganisms. Therefore, a comprehensive characterization of cultures on morphological, anatomical, physiological, immunological and molecular grounds is a must before and after preservation (Muller *et al.*, 2007; Broughton *et al.*, 2012; Smith & Ryan, 2012). Generally, sub-culturing of active cultures induces more mutations. Cultures preserved using lyophilization and cryopreservation showed more genotypic and phenotypic stability but still need optimization for better result (Simione, 1992; Lang & Malik, 1996; Muller *et al.*, 2007). Evaluation of the post-preservation genomic integrity in *Trichoderma* spp. by Broughton *et al.* (2012) showed that a robust method like cryopreservation can also, at times, induce genetic changes. Similarly, stability of biodegradation potential in bacteria was evaluated by Lang & Malik (1996) and they concluded that 'use of the right preservation method and cryoprotectants are essential to avoid the genetic alteration'.

Exact mechanisms leading to induction of genotypic and phenotypic changes in microbial cells during preservation and sub-culturing are not yet clear, and demand more investigation. It is presumed that several factors including temperature shock, oxidative stress, toxicity of cryoprotectants, cryo-injuries, intracellular ice formation, pressure of vacuum in freeze- and liquid-drying, and formation of free-radicals may induce different kinds of genotypic and phenotypic changes in the cultures (Fleck, 1998; Day *et al.*, 2000; Fleck *et al.*, 2000; Benson & Bremner, 2004). Apart from the harsh preservation conditions, fluctuations in temperature during freezing and thawing, or even transfer of cultures from one freezer to another, may be viewed as factors inducing these changes in microorganisms.

A nice note about validation of preservation success has been provided by Smith & Ryan (2012). In brief, several typing methods including amplified fragment length polymorphism, multilocus sequence typing, pulsed-field gel electrophoresis, PCR binary typing (P-BIT), real-time PCR and whole genome comparison can be used to monitor the genetic drift in bacteria, fungi and microalgae (Muller *et al.*, 2007; Ragimbeau *et al.*, 2008; Cornelius *et al.*, 2010; Angela *et al.*, 2010). Extensive pre- and post-preservation monitoring of physiological and genotypic traits is recommended for authentication of the preserved cultures, at least for the strains of ecological, medical and biotechnological importance to begin with. Accurate inventory with backup facility, validation of storage temperature and quality, good training for specimen handling, minimum sub-culturing, availability of sufficient distribution stocks and preservation using freeze-drying, liquid-drying and cryopreservation at liquid nitrogen temperature are recommended for maintaining the phenotypic and genotypic integrity of the cultures during preservation (Simione, 1992; Lang & Malik, 1996; Muller *et al.*, 2007; Smith & Ryan, 2012).

Research towards maintaining the genotypic integrity and validation of preserved stocks is not very encouraging. The focus is mostly restricted towards plasmid stability in genetically modified bacteria and organisms of medical importance (Koenig, 2003; Kim *et al.*, 2005; Marston *et al.*, 2005). Even the major culture collections are unable to devote efforts to understanding the factors behind the genomic stability of the preserved cultures. Consequently, the process of authentication is unable to keep pace with the regular overburdened preservation tasks due to lack of funds, resources, manpower and limited time for authentication of all the cultures. Additional research on optimization of cryoprotectants, preservation protocols and storage conditions using different groups of

microorganisms is essential to obtain more conclusive data about the specific practices that must be undertaken to ensure that bring the preserved cultures undergo minimum change.

Although validation is a tough task and needs sophisticated instruments, knowledgeable scientists and skilled technicians, it can be anticipated that use of next generation sequencing elucidating whole genome sequences of microbes and matrix-assisted laser desorption/ionization time of flight, mass spectrometry in microbial typing will reduce the cost, labor and typing time. These high throughput techniques will provide more rapid and extensive comparisons of genotypic and phenotypic integrity of pre- and post-preserved microorganisms.

## Concept of cell-dormancy, cell-resuscitation and viable but non-culturable state

Dormancy is a well known mechanism of cell survival in response to starvation and environmental stress (Lennon & Jones, 2011). It is an essential mechanism of evolution, diversity, succession and community dynamics in natural ecosystems. Dormancy also provides a mechanism for the maintenance of microbial 'seed banks' (Lennon & Jones, 2011). Every kind of environment maintains some fraction of its cellular diversity in a dormant or inactive state. The 'Great plate count anomaly' and 'Non-culturability' can be partially explained by the concepts of cell dormancy and the viable but non-culturable (VBNC) state of bacteria (Lennon & Jones, 2011; Hoefman *et al.*, 2012). Long-term preservation methods such as cryopreservation and lyophilization induce a state of dormancy within the cells and completely halt the cellular metabolism without a change in the physiological and genetic features of microorganisms. In addition, preservation can create a condition of stress within the cell and induce a VBNC state in some fraction of preserved cells (Hoefman *et al.*, 2012). Epstein (2009) highlighted the concept of microbial dormancy and discussed cracking the dormancy of microbes in order to get the active population of the cells (scouts). Understanding the mechanisms and signaling molecules responsible for cracking cell dormancy will assist in solving problems related to post-treatment disease recurrence and disease suppression. The knowledge of signaling molecules and environmental conditions responsible for cracking cell dormancy is also applicable to culture collections. The work of Hoefman *et al.* (2012) showed that preservation induces a VBNC state in methane-oxidizing bacteria and cells could be resuscitated using trypticase soy agar medium. The findings of Epstein (2009) and Hoefman *et al.* (2012) substantiate each other, and suggest that an understanding of signaling molecules and suitable resuscitation media may help increase viability during post-preservation resuscitation of the cells. Some researchers have investigated the effect of rehydration media on post-preservation recovery of cells and have focused on how to increase the culturability of non-culturable or fastidious types of bacteria. However, no systematic work on signaling molecules, media components, and the mechanisms responsible for cracking dormancy and VBNC state has been done so far (Costa *et al.*, 2000; Abadias *et al.*, 2001; Bruns *et al.*, 2002; Nichols *et al.*, 2008; Vartoukian *et al.*, 2010; Heylen *et al.*, 2012; Hoefman *et al.*, 2012). Despite the immense importance of microbial dormancy and viability-related research for culture collections and industries, this area is often neglected. As discussed earlier, the discovery of novel mechanisms and signaling molecules responsible for induction of active cell formation (scout formation), study of the effect of components of rehydration medium on cell viability, and role of signaling molecules (e.g. c-AMP, homoserine lactone, short peptides) on breaking the post-preservation cell dormancy of VBNC microbes will be very promising for long-term preservation and post-preservation revival of the cells.

## Prospects of preservation research

Preservation of the ecosystems and ecosystem services are the top research priority in life science. Unlike for plants and animals, little attention has been given to the conservation of microbial diversity in the diversity conservation agenda (Cockell & Jones, 2009; Bodelier, 2011; Bhardwaj & Garg, 2012; Griffith, 2012; Heylen *et al.*, 2012). The smaller priority is partially based on the Baas–Becking hypothesis (Everything is everywhere, but the environment selects) and also due to rudimentary knowledge about microbial ecology and diversity (Bodelier, 2011). Existing methods work well for pure cultures, but protocols for ecosystem and community level preservation need more work. Almost all MRCs are focusing on *ex situ* preservation of pure cultures, but ecosystem and community level preservation is still in its infancy. In the future, researchers should focus on preservation of intact communities such as co-cultures, enrichment cultures and natural communities (Emerson and Wilson, 2009; Heylen *et al.*, 2012). In reality, conservation of all sorts of habitats is a difficult task in terms of cost and labor. Therefore, using technical advances of the field microbiologists should first generate an in-depth knowledge about structure and structure-related functions of the microbial communities of different ecosystems. On the basis of the accumulated data, we should then prioritize the habitats (polar region, hot spring, alkaline lake, marine ecosystems) of ecological and biotechnological significance and

focus on their conservation. Along with preserving the geochemical features (salinity, pH) of the sites, strategies for conservation should also include restricted entry of pollutants, grazing, and preservation of host-plants and animals that support microbial growth.

Community and ecosystem level preservation is difficult using the current technology of MRCs, but gene pools in the form of extracted DNA could also be preserved instead of intact ecosystems or communities. This would allow for development of hypotheses regarding the effect of pollution, climate change and ecological perturbation on community structure. In addition, strict anaerobes, archaea, extremophiles, fastidious bacteria, newly discovered taxa, and non-sporulating fungi require more extensive research in terms of growth optimization. Major areas for future preservation research include: (1) development of cheap and less labor-intensive protocols for existing organisms, (2) optimization of media, cryoprotectants and signaling molecules for cracking the preservation-induced dormancy, (3) work to unveil the molecular mechanisms that induce morphological, physiological and genetic changes during the course of preservation; and (4) research on ecosystem and community level preservation. In addition, research is necessary on the response of different phases of the cultures to different preservation strategies and optimization of initial cell densities for preservation in order to provide more viability. Instead of using the widely accepted protocols, microbiologists should seek alternative support or carrier materials that reduce the cost of preservation, provide extended shelf-life to microorganisms, and provide less expensive storage for low budget culture collections. This may help reduce costs associated with liquid nitrogen and lyophilization. Use of inexpensive carrier materials that can provide extended shelf-life to microorganisms for bulk commercial formulation in bio-fertilizers, biopesticides and bio-inoculants is also a future need in preservation (Trivedi *et al.*, 2012).

## Conclusion

Despite the immense importance of microbial preservation, the research related to this area needs more attention. Microbial culture preservation is currently limited to the culture collections or bioresource centers. There are many possibilities of research available in the area of microbial preservation, but there is little interest in the optimization of preservation methods, even with novel taxa. Although a little bit research is going on in the area of microbial preservation and a few papers appear every year using ecologically and medically important groups, results are not promising. Research efforts should focus on the preservation of problematic groups such as *Campylobacter*, *Neisseria*, *Helicobacter*, vegetative non-spore forming fungi and fastidious and oligotrophic bacteria. Furthermore, newly discovered taxa need more attention because preservation using the existing methods without optimization of protocols may lead to extinction of organisms or loss of valuable traits. It has been found that different groups of microorganisms give widely varied responses to the same preservation method. Even different strains of the same species may show different responses in terms of survival and durability with the same preservation strategy. Due to the vast diversity of microbial life and the time-consuming nature of preservation research, it is not possible to optimize the preservation of all the species of the same genus or all the strains of same species. However, microbiologists should consider the preservation of medically, ecologically and industrially important strains as paramount. If not all strains, then at least representative members from each group should be optimized for routine preservation. The state of anaerobic preservation techniques requires even more attention because only a few culture collections in the world are dealing with anaerobic preservation. In the future, microbiologists should focus on the development and optimization of robust preservation methods for strict anaerobes and archaea in order to ensure the long-term viability of these microorganisms.

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