Abstract: The present invention relates to preservation of genetic constituents.
FIELD OF THE INVENTION

The present invention relates to preservation of biomaterials.

Particularly, the present invention relates to preservation of genetic constituents.

Still more particularly, the present invention relates to preservation of DNA.

BACKGROUND OF THE INVENTION & PRIOR ART

Deoxyribonucleic acid (DNA) is a nucleic acid which contains the genetic instructions used in the development and functioning of all known living organisms. Chemically, DNA is a long polymer of simple units called nucleotides, with a backbone made of sugars and phosphate groups joined by ester bonds. The main role of DNA molecules is the long-term storage of information.

Preservation of DNA is of wide interest to researchers in different fields from biorepository management to pharmaceutical sciences. Although the term "preservation" is used by researchers in all these fields and refers to the maintenance of chemical and physical integrity of the DNA molecule, it should not be surprising that the perspective of scientists from these distinct fields differs significantly. Most notably, the time frame for stability of a pharmaceutical product is approximately 2 years, whereas meaningful stability for an evolutionary biologist is measured in the hundreds of millions of years. Such divergent viewpoints not only have a
significant effect on the time span of preservation, but also on what criteria are used to assess "stability" (Anchordoquy and Molina 2007).

The storage and preservation of DNA is receiving increasing attention from the point of conservation of biodiversity. Efforts are under way to store DNA from every endangered (or important) microbial, plant or animal species on the earth for the sake of posterity. With the automated DNA sequencing methods, our descendants will be able to derive the sequence of any organism whose DNA has been appropriately collected and stockpiled. The medical value of such DNA banks cannot be overlooked and will undoubtedly provide materials that will contribute to advances in medicine. Some of the more futuristic aspects of long-term DNA preservation include the use of DNA for data storage and the possibility of human colonization in outer space (Shapiro R. 1999).

DNA is considered to be a stable molecule. However, the stability of DNA is affected by various factors in vivo and in vitro. In vivo, the damaged DNA is repaired by the cellular machinery, which is well equipped with numerous repair mechanisms like 'Base excision', 'Nucleotide excision' and 'Mismatch repair' for each kind of DNA damage. Thus, isolated and purified DNA (in vitro) is far more vulnerable to damages than the DNA in vivo, and hence demands more care.
DNA damage (Figure 1) affects the primary structure of the double helix; that is, the bases themselves are chemically modified. These modifications can, in turn, disrupt the regular helical structure of the molecule by introducing non-native chemical bonds or bulky adducts that do not fit in the standard double helix. Unlike proteins and RNA, DNA usually lacks tertiary structure and therefore damage or disturbance does not occur at that level. DNA is, however, super coiled and wound around "packaging" proteins called 'histones' (in eukaryotes), and both the superstructures are vulnerable to the effects of DNA damage. DNA needs protection from various damaging causes, which affect DNA both structurally and functionally. Various forms of exogenous agents may damage the DNA, some of which are as provided below:

**Ultra-Violet (UV) Light:**

- UV-B light causes cross linking between adjacent cytosine and thymine bases creating pyrimidine dimers. This is called 'direct DNA damage'.
- UV-A light creates mostly free radicals. The damage caused by free radicals is called 'indirect DNA damage'.

**Ionizing radiations:**

- Ionizing radiations such as those created by radioactive decay or those present in cosmic rays cause breaks in DNA strands.

**Elevated temperature:**
- At elevated temperature, thermal disruption increases the rate of depurination and single strand breaks in the DNA.

Chemical agents:
- Industrial chemicals such as vinyl chloride and hydrogen peroxide, and environmental chemicals such as polycyclic hydrocarbons found in smoke, soot and tar create a huge diversity of DNA adducts- ethenobases, oxidized bases, alkylated phosphotriesters and cross-linking of DNA just to name a few.

As mentioned above, there are various types of damaging factors that can pose a threat to the isolated, purified and stored DNA. These causative agents may not be of much danger when the time frame of storage is short and includes one or the other cryogenic or non-cryogenic techniques involved in preservation. But with time, any kind of damage can get magnified leading to completely dysfunctional DNA, because of the structural modifications in the DNA molecule. Hence, the long-term preservation of DNA requires greater attention from the perspective of vulnerability of the DNA to the various damaging factors.

Damage caused in the isolated and purified DNA has to be avoided by some techniques as it is required to use the DNA for various downstream applications. Methods that are practiced usually during DNA extraction and subsequent usage are maintaining sterile conditions, use of nuclease free solvents, nuclease free apparatus and maintenance of low temperature (typically
4 -10°C). For short term to long term storage of DNA, usually refrigeration is recommended. DNA is stored in T_{10}EI buffer (4°C to -20°C), 95% Ethanol (-20°C / -80°C), or methanol (-196°C in liquid nitrogen) depending on the time to be stored (Anchordoquy and Molina 2007).

A study by Eiseman and Haga (Eiseman and Haga 1999) on human tissue storage estimated that approximately 307 million samples were stored in the frozen state in the United States. If we assume that half of these samples are stored at -80°C and the other half in liquid nitrogen (-196°C), it is possible to estimate that it would cost approximately $120 million to purchase the freezers necessary to store these samples, and an additional $30 million is spent each year to maintain those samples. Furthermore, they estimated that samples are accumulating at approximately 20 million per year, and according to calculations, it costs an additional $10 million for freezers and maintenance of newly generated samples each year. Using these numbers, it was estimated that an annual cost of approximately $54 million (in 2007) would be incurred just to maintain frozen samples in the United States. It is estimated that half of the global tissue storage occurs in the United States, and thus the global cost to maintain frozen samples each year likely exceeds $100 million. With such an astronomical amount of money spent on storage and preservation of biomolecules and biomaterials, there is an urgent need to think outside the ice box.
Thinking outside the ice box is to think of a method of preservation that is non-cryogenic (Dutton 2005). Only a non-cryogenic method will be useful in preservation of all kinds of samples for any long time period without spending too much. As non-cryogenic method of DNA preservation is not studied extensively the urgent need of it was felt. Various attempts have been made in an endeavour to bring down the cost of preservation of DNA by non-cryogenic means.

For an instance, 'Encapsulation technique' for preservation, which involves the use of biodegradable polymers such as poly(lacto-co-glycolide) (PLG), poly(lactide), poly(caprolactone), poly(hydroxybutyrate) and similar copolymers. Provided herein below is a list of US patent documents which disclose processes for preservation of genetic constituents by encapsulation.

For example, US 6,461,571 disclose a method for prolonged storage of DNA molecules and packaging which involves encapsulation in a sealed corrosion proof metallic capsule. The method employs degradable bio-polymer.

Again, US patent Application 2004/0009941 discloses a method of encapsulation of nucleic acid molecule in microspheres using biodegradable polymers such as poly (lacto-co-glycolide) (PEG), poly (lactide), poly (caprolactone).
US 6,667,294 discloses encapsulation of DNA in polymer, selected from lactide containing polymer, a glycolide-containing polymer and a polymer containing lactide and glycolide.

Further, US 6,254,890 discloses biodegradable polymer nano-sphere capable of encapsulating and transporting of nucleic acids.

US 6,627,226 discloses a dry solid medium for storage and analysis of genetic material. It provides a device for obtaining and storing genetic material. The device has a support and a head portion. The head portion is composed of a solid matrix for sorbing genetic material and a preserving mechanism for the protection of genetic material from degradation. Additionally, it provides a method of storing a sample of genetic material onto the swab device. In the preferred embodiment, it utilizes polyester material as the solid matrix.

US 20060099567 discloses compositions and methods for automated storing, tracking, retrieving and analyzing biological samples, including dry storage at ambient temperatures of nucleic acids, proteins (including enzymes), and cells using a dissolvable dry storage matrix that permits recovery of biologically active materials.
Bacterial spores, fungal spores, tardigrades and pollen grains get preserved in desiccated state under harsh environmental conditions and revive in favorable conditions. This phenomenon is called 'anhydrobiosis' (Buitink and Leprince 2004), and can be seen in pollen grains because of the presence of plasticizing disaccharide content and structural polymer sporopollenin. Sporopollenins are nonhydrolysable biomacromolecules (biopolymers) and are the major components of the tough outer walls (exine) of spores and pollen grains. Due to their structural arrangement and chemical properties like high stability, sporopollenins provide a very effective shield against environmental stress.

The exine shell may be derived from any suitable naturally occurring spore, whether fungal, algal or plant origin. It can be isolated from the spore by successive treatments with organic solvents, alkali and acid so as to remove the other components of the spore such as the cellulosic intine layer and lipid, protein and nucleic acid components that may be attached to or contained within the exine shell. The resulting exine shell, which takes the form of an essentially hollow capsule, typically contains Sporopollenin. Sporopollenins are extremely resistant to physical, biological and chemical degradation and are also inert and non-toxic.

Sporopollenin's resistance to physico-chemical and biological degradation has resulted in preservation of pollens in fossils, for e.g. the palynomorphs of Ordovician era; 400 million years old (Blokker et al. 2006) are structurally and chemically unchanged. Thus, sporopollenin being
the major content of pollen wall was thought to play a major role in preserving pollen and their contents.

The exact chemical composition of sporopollenin is still not known, but evidences indicate that it may be composed of aliphatic polymers containing aromatic or conjugated side chains (Dominguez 1999) that vary among the different plant groups. Lignin, Cutin, suberin, and sporopollenin form a family of biopolymers that are biosynthetically related. The unusual properties of sporopollenin make it a novel material for exploring different applications.

Various applications of sporopollenin are being explored by different research groups for using it as ion exchange resin as it is chemically inert and can serve as matrix in ion-exchange columns (Shaw et al. 1988).

Sporopollenin is also used for preservation of oils as it acts as 'antioxidant', and hence even can be used in food preservation as disclosed in PCT Application No. PCT/GB2006/002800 (WO 2007/012856 Al).
Sporopollenin being non-allergenic and biocompatible is used as an oral drug delivery vehicle for loading and delivery of active components (pharmaceuticals and neutraceuticals) as disclosed in PCT Application No. PCT/GB2004/002775 (WO 2005/000280 A2).

Sporopollenin is capable of absorbing UV B radiation because of the presence of p-coumaric acid residues (Rozema et al 2001). Attempts have been made for using sporopollenin in topical formulations as disclosed in PCT Application No. PCT/GB2006/002802 (WO 2007/012857 Al). Isolated sporopollenin microcapsules are hollow and porous and various active substances can be loaded into it for immediate use (Guedeau-Boudeville et al 1995).

United States Patent Application 20080188572 discloses the use of an exine shell of a naturally occurring spore, or a fragment thereof, as an antioxidant, for instance in a composition or formulation containing an active substance. Also provided is a method for reducing rancidity, or other oxidative degradation, of a substance, composition, or formulation, by encapsulating the substance, composition, or formulation in, or chemically binding it to, or mixing it with, an exine shell of a naturally occurring spore or a fragment thereof. However, the active substance as disclosed in this prior art document is not a genetic material.

The cryogenic methods of preservation of genetic constituents discussed above are expensive. Various non-cryogenic methods including encapsulation as disclosed in the prior art are
complicated and not much effective in the preservation of genetic material for prolonged period of time. Thus, an inexpensive and reliable method of preservation of genetic material is the need of the hour.

OBJECTS OF THE PRESENT INVENTION

It is an object of the present invention to provide a process for safeguarding the genetic information by the preservation of genetic constituents.

It is another object of the present invention to provide a process for preservation of genetic constituents that employs a biopolymer for preservation of the genetic constituents.

It is a further object of the present invention to provide a process for preservation of genetic constituents which is simple and reliable.

It is yet another object of the present invention to provide a process for preservation of genetic constituents which provides structurally intact genetic constituents even after accidental or prolonged exposure to UV light.
It is still another object of the present invention to provide a process for preservation of genetic constituents which provides protection of genetic constituents against unfavourable atmospheric effects such as fluctuating global ambient temperature during transportation.

It is still another object of the present invention to provide a process for preservation of genetic constituents which provides protection of genetic constituents against unfavorable atmospheric effects such as high temperature.

It is still another object of the present invention to provide a process for preservation of genetic constituents that does not alter the functional properties of the genetic constituents besides preserving their structural integrity.

It is still another object of the present invention to provide a process for preservation of genetic constituents which enables easy recovery of the genetic constituents whenever the need arises.

It is still another object of the present invention to provide a process for preservation of genetic constituents which is cost-effective.
In accordance with the present invention, there is provided a method of preservation of genetic constituents, said method comprising encapsulating the genetic material into purified sporopollenin microcapsules.

Typically, the genetic constituent is at least one selected from a group of genetic constituents consisting of single genes, oligomers, plasmids, cosmids, fosmids, single stranded genomic deoxyribonucleic acid (ssDNA), double stranded genomic DNA (dsDNA), ribonucleic acid (RNA), synthetic gene sequences and natural gene sequences.

Typically, the sporopollenin microcapsules are obtained from exines of naturally occurring spores of organisms selected from a group consisting of microorganisms, bryophytes, pteridophytes and gymnosperms, pollen grains of angiosperms or fragment thereof.

Typically, the sporopollenin microcapsule is at least one selected from a group consisting of granules, pellets, tablets, multi-layer and dry powder.

Typically, the microcapsules have a diameter in the range of about 2 to about 200 microns depending on the source.
Typically, the sporopollenin microcapsules obtained from a particular source have a uniform shape and diameter and are thus called as mono-disperse.

Typically, the sporopollenin microcapsules used for encapsulation of the genetic constituent in accordance with this invention are mono-disperse.

Typically, the step of encapsulation of genetic constituent is carried out by a process selected from a group consisting of electroporation; liposome mediated transfer, micromanipulation, re-dispersion of compressed sporopollenin pellet and controlled vacuum filtration.

Preferably, the step of encapsulation of genetic constituent is carried out by controlled vacuum filtration.

In accordance with the present invention, the isolation and purification of sporopollenin microcapsules is carried out in the following manner:

i) suspending spores in acetone and stirring under reflux typically for about 4 hours;

ii) filtering the refluxed acetone to obtain solid residue;

iii) washing the filtered solid residue with fresh acetone;
iv) suspending the washed residue in KOH solution (6% w/v in water) and stirring under reflux (100°C) for 6 h;

v) filtering the residue;

vi) washing the filtered residue with hot water at a temperature ranging from about 45-50°C;

vii) resuspending the washed residue in KOH solution (6% w/v in water) and stirring under reflux (100°C) for 6 h;

viii) filtering the residue and washing with hot water at a temperature ranging from about 45-50°C, hot absolute ethanol at a temperature of about 45°C followed by washing with water again;

ix) stirring the residue under reflux at a temperature ranging from about 60-70°C in ethanol for about 2 hours, filtering and washing sequentially with fresh ethanol and dichloromethane.

x) resuspending the resulting solid in dichloromethane and stirring under reflux at a temperature ranging from about 50-55°C for about 2 hours;

xi) removing the residue by filtration, drying in air for about 24 hours at room temperature, and filtering the sporopollenin microcapsules;

xii) treating the filtered sporopollenin microcapsules with 1% cellulase and 1% pectinase in citrate phosphate buffer of pH of about 4.8 at a temperature of about 45°C for about 24 hours with shaking at about 100 rpm to obtain purified sporopollenin microcapsules;
xiii) washing said sporopollenin microcapsules several times with hot water and hot ethanol;

xiv) refluxing washed sporopollenin microcapsules in dichloromethane for about 4 hours; and

xv) filtering said sporopollenin microcapsules and vacuum drying to obtain purified sporopollenin microcapsules.

The present invention also provides a method of encapsulating genetic constituent in sporopollenin microcapsules, said method comprising the following steps:

i) preparing a compact multilayered mass of purified sporopollenin microcapsules;

ii) exercising a negative pressure typically below atmospheric pressure but not less than -1 Barr in said multilayered mass for about 30-40 seconds;

iii) absorbing the genetic material contained in buffer within said microcapsules and encapsulating by further applying said negative pressure for about 30-40 seconds to said multilayered mass; and

iv) desiccating said sporopollenin microcapsules to permanently encapsulate the genetic material within said sporopollenin microcapsules.

Typically, the buffer used for containing the genetic material is Tris-EDTA buffer (Tris buffer).
Typically, the desiccation of the genetic material loaded sporopollenin microcapsules is carried out by slow dehydration.

Typically, the encapsulated genetic material can be recovered from the sporopollenin microcapsules whenever the need arises by mixing the encapsulated material with Tris-EDTA buffer.

Typically, the method of encapsulation further comprises a step of coating a layer of hydrophobic substance, typically lipid layer on to the desiccated microcapsule as a means to provide an extra protection to the encapsulated genetic material.

The present invention also provides an apparatus for encapsulation of substances including genetic constituents, said apparatus comprising:

i) a filter tube with silica membrane on to which sporopollenin microcapsules are loaded to receive the substance to be encapsulated;

ii) said filter tube introduced into a slightly larger tube with two or more holes on its surface;

iii) said slightly larger tube introduced into a still larger tube with cut open bottom; and

iv) said cut open bottom connected to vacuum pump.

Typically, the tubes are made up of polymer.
Typically, the cut open bottom is connected to vacuum pump by means of silicon tubing.

DETAILED DESCRIPTION OF THE INVENTION

The drawings and the description thereto are merely illustrative and only exemplify the invention and in no way limit the scope thereof.

The present invention relates to a method of preservation of genetic constituents, said method comprising encapsulating the genetic material into purified sporopollenin microcapsules.

Typically, in accordance with the present invention, the genetic constituent is at least one selected from a group of genetic constituents consisting of single genes, oligomers, plasmids, cosmids, fosmids, single stranded genomic deoxyribonucleic acid (ssDNA), double stranded genomic DNA (dsDNA), ribonucleic acid (RNA), synthetic gene sequences and natural gene sequences.

Typically, the sporopollenin microcapsules are obtained from exines of naturally occurring spores of organisms selected from a group consisting of microorganisms, bryophytes, pteridophytes and gymnosperms, pollen grains of angiosperms or fragment thereof.
Typically, the sporopollenin microcapsule is at least one selected from a group consisting of granules, pellets, tablets, multi-layer and dry powder.

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Typically, the sporopollenin microcapsules obtained from a particular source have a uniform shape and diameter and are thus called as mono-disperse.

Typically, the sporopollenin microcapsules used for encapsulation of the genetic material in accordance with this invention are mono-disperse.

Typically, the step of encapsulation of genetic material is carried out by a process selected from a group consisting of electroporation; liposome mediated transfer, micromanipulation, re-dispersion of compressed sporopollenin pellet and controlled vacuum filtration.

In accordance with the present invention, the isolation and purification of sporopollenin microcapsules is carried out in the following manner:

ii) suspending spores in acetone and stirring under reflux typically for about 4 hours;
iii) filtering the refluxed acetone to obtain solid residue;
iv) washing the filtered solid residue with fresh acetone;
v) suspending the washed residue in KOH solution (6% w/v in water) and stirring under reflux (100°C) for 6 h;
vi) filtering the residue;
vii) washing the filtered residue with hot water at a temperature ranging from about 45-50°C;
viii) resuspending the washed residue in KOH solution (6% w/v in water) and stirring under reflux (100°C) for 6 h;
ix) filtering the residue and washing with hot water at a temperature ranging from about 45-50°C, hot absolute ethanol at a temperature of about 45°C followed by washing with water again;
x) stirring the residue under reflux at a temperature ranging from about 60-70°C in ethanol for about 2 hours, filtering and washing sequentially with fresh ethanol and dichloromethane.
xi) resuspending the resulting solid in dichloromethane and stirring under reflux at a temperature ranging from about 50-55°C for about 2 hours;
xii) removing the residue by filtration, drying in air for about 24 hours at room temperature, and filtering the sporopollenin microcapsules;
xiii) treating the filtered sporopollenin microcapsules with 1% cellulase and 1% pectinase in citrate phosphate buffer of pH of about 4.8 at a temperature of about 45°C for
about 24 hours with shaking at about 100 rpm to obtain purified sporopollenin microcapsules;

xiv) washing said sporopollenin microcapsules several times with hot water and hot ethanol;

 xv) refluxing washed sporopollenin microcapsules in dichloromethane for about 4 hours; and

 xvi) filtering said sporopollenin microcapsules and vacuum drying to obtain purified sporopollenin microcapsules.

The present invention also relates to a method of encapsulating genetic material in sporopollenin microcapsules, said method comprising the following steps:

 i) preparing a compact multilayered mass of purified sporopollenin microcapsules;

 ii) exercising a negative pressure typically below atmospheric pressure but not less than -1 Barr in said multilayered mass for about 30-40 seconds;

 iii) absorbing the genetic material contained in buffer within said microcapsules and encapsulating by further applying said negative pressure for about 30-40 seconds to said multilayered mass; and

 iv) desiccating said sporopollenin microcapsules to permanently encapsulate the genetic material within said sporopollenin microcapsules.

Typically, the buffer used for containing the genetic material is Tris-EDTA buffer (Ti0E1 buffer).
Typically, the desiccation of the genetic material loaded sporopollenin microcapsules is carried out by slow dehydration.

Typically, the encapsulated genetic material can be recovered from the sporopollenin microcapsules whenever the need arises by mixing the encapsulated material with Tris-EDTA buffer.

Typically, the method of encapsulation further comprises a step of coating a layer of hydrophobic substance, typically lipid layer on to the desiccated microcapsule as a means to provide an extra protection to the encapsulated genetic material.

The present invention also provides an apparatus for encapsulation of substances including genetic constituents, said apparatus comprising:

i) a filter tube with silica membrane on to which sporopollenin microcapsules are loaded to receive the substance to be encapsulated;

ii) said filter tube introduced into a slightly larger tube with two or more holes on its surface;

iii) said slightly larger tube introduced into a still larger tube with cut open bottom; and
iv) said cut open bottom connected to vacuum pump.

Typically, the tubes are made up of polymer.

Typically, the cut open bottom is connected to vacuum pump by means of silicon tubing.

In accordance with the present invention, the properties of sporopollenin biopolymer like elasticity, high tensile strength, thermal resistance, UV absorbance, antioxidant nature and structural features are utilized for encapsulation of genetic constituents in sporopollenin microcapsules and preservation thereof.

Typically, sporopollenin microcapsules are selected from spores of pteridophytes like *Lycopodium clavatum* or pollen grains of angiosperms. The steps involve the isolation and purification of these microcapsules of sporopollenin.

Typically, the isolation of sporopollenin microcapsules was carried out according to modified Zetzsche's non-oxidative extraction hydrolysis method. The modification involves the enzymatic degradation of cellulose and pectin using a mixture of cellulase and pectinase enzymes (Example 1).

This invention is supported by the fact that sporopollenin does not harm the structural properties or any functional efficiency of the genetic constituents loaded onto or into the...
sporopollenin microcapsules, which was investigated as mentioned in the examples (Examples 2-5).

This invention involves loading of genetic constituents on sporopollenin microcapsules by physical adsorption or loading inside the hollow cavities of these capsules by encapsulation, preferably encapsulation.

The present invention also includes the method of release of loaded genetic constituents from sporopollenin microcapsules using appropriate buffers.

Typically, in accordance with this invention, all the experimental designs are based on statistically valid orthogonal arrays.

The present invention describes the process of encapsulation of genetic constituents and its preservation from thermal disruption. The genetic constituents encapsulated within are protected from structural and functional damage from varying temperatures even above the melting point of the molecule. The preserved genetic constituents do not undergo any strand breakages, strand separation, change in the sequence integrity and hence remain functionally viable.
The present invention also discloses the process of encapsulation of genetic constituents and its preservation from background ultraviolet radiation. The genetic constituents protected from ultraviolet radiation by sporopollenin microcapsules retain their structural and functional integrity.

The main aspect of this invention is a biomimetic approach of long term preservation of biomaterials, more particularly, the genetic constituent DNA. This invention discloses a process of prolonged preservation of genetic constituents.

Applications of preserved DNA

The DNA preserved in sporopollenin microcapsules can be useful in transporting DNA at room temperature in forensic research and various other applications in ecological field works.

Examples

The invention will now be described with the help of following examples; however, these examples should not be construed to limit the scope of the present invention.
Example 1: Isolation and purification of sporopollenin from *Lycopodium clavatum* by modified Zetzsche's method

Sporopollenin was isolated using Zetzsche's non-oxidative Extraction Hydrolysis Method (Zetzsche 1928). Spores of *Lycopodium clavatum* (Figure 2b) were suspended in acetone and stirred under reflux for 4 h followed by filtration. The filtered solid residue was washed with fresh acetone and transferred back to the reaction flask, resuspended in KOH solution (6% w/v in water) and stirred under reflux (100°C) for 6 h. The residue was filtered, washed copiously with hot water (45-50°C), and transferred back to the reaction flask. The KOH treatment was repeated. After filtration, the solid material was washed with hot water (45-50°C), hot absolute ethanol (45°C), and water again. The residue was stirred under reflux (60-70°C) in ethanol for 2 h, filtered and washed sequentially with fresh ethanol and dichloromethane. The resulting solid was resuspended in fresh dichloromethane, stirred under reflux (50-55°C) for 2 h removed by filtration, dried in air for 24 h at RT, and filtered particles were then suspended in 85% orthophosphoric acid, stirred under gentle reflux (25-30°C) for 5 days and filtered. The residue was washed with copious amounts of hot water (45-50°C) and vacuum dried. The particles were washed with hot water (45-50°C), ethanol (45-50°C), and dichloromethane (45-50°C). Finally, the solid was stirred under reflux (60-70°C) in ethanol for 2 h, filtered and washed with dichloromethane and vacuum dried and were imaged by SEM (Figure 2c). In comparison with spores (Figure 2b) the particles (Figure 2c) revealed the structure of sporopollenin, which is of the same size, shape and symmetry of the spores depending on the species from which it is extracted. The illustration (Figure 2a) shows the trilayered nature of sporopollenin and a hollow
inner space. Confocal Scanning Micrograph of Sporopollenin microcapsules isolated from
*Lycopodium clavatum*, exhibiting auto fluorescence is shown in Figure 3.

The sporopollenin microcapsules were imaged by light microscopy after every step of isolation
and it was found that only after the H$_3$PO$_4$ treatment, the percentage of breakage is more
whereas KOH treatment yielded completely intact grain. The isolated sporopollenin from both
the methods i.e. KOH treatment and H$_3$PO$_4$ step, were compared using FTIR, and found to be
chemically similar with residual cellulose component. But the demerit of using H$_3$PO$_4$ was the
yield of non-intact microcapsules. Hence to replace the H$_3$PO$_4$ as a cellulose and pectin removal
chemical, a mixture of cellulase and pectinase was used.

The spores treated with KOH after washings were treated with 1% Cellulase and 1% pectinase
in citrate phosphate buffer pH 4.8 at 45°C for 24hours with 100 rpm shaking. This purified
sporopollenin was further washed several times with hot water and hot ethanol. Further, the
sporopollenin was refluxed in dichloromethane for 4hrs.

This was a major modification in the Zetzsche's method of sporopollenin isolation. The method
disclosed in the present invention yields intact and hollow sporopollenin microcapsules with
uniform pores suitable for DNA loading in a short time. This major modification not only saves
time but avoids usage of hazardous and corrosive acid. The chemical purity of sporopollenin
isolated by both these methods is comparable and hence the modified method is most suitable
for isolating sporopollenin microcapsules for encapsulation of DNA or any other genetic constituent.

Investigations were carried out to confirm the suitability of sporopollenin in preservation of biomaterials, by studying the effect of sporopollenin on DNA in various different conditions.

Example 2: Effect of sporopollenin on structural intactness of DNA

The effect of sporopollenin on structural intactness of Plasmid DNA, Lambda DNA and Lambda DNA Eco RI / Hind III double digest marker was checked by its electrophoretic mobility.

Plasmid DNA (pUC18), Lambda DNA and Lambda DNA Eco RI / Hind III double digest marker of 10ng/µL, 25ng/µL, 50ng/µL were mixed with 10µg/µl sporopollenin. The mixture was kept at 4°C for 24hrs to check the effect sporopollenin on DNA. Similarly, a range of sporopollenin concentration of 5µg/µL, 10µg/µL and 50µg/µL was taken and mixed with 25ng/µL plasmid DNA, Lambda DNA and Lambda DNA Eco RI /Hind III double digest marker. In both the experiments, the total reaction volume was 10µL and the solvent used was T10E buffer at pH 8.0. Electrophoresis of these samples was done using 1% agarose gel, with 10µg/mL EtBr at 80V for 60-90 min.
Analysis of these gels shows the presence of intact DNA fragments of appropriate size in the experimental sets, comparable to that of control DNA. It shows that sporopollenin does not cause any kind of strand breakages (Figure 4). These results with various types of DNA show the structural intactness of DNA and confirm that sporopollenin is safe to DNA. Hence sporopollenin microcapsules can be used for DNA preservation.

Example 3: Effect of sporopollenin on functional efficiency of plasmid DNA

The functional efficiency of DNA with sporopollenin was investigated by transformation. Plasmid DNA pUC18 (50ng\*L) was mixed with 10\(\mu\)g/\(\mu\)L sporopollenin and incubated at 4°C for 24hrs. Transformation of 100ng plasmid DNA was carried out using 50\(\mu\)L competent cells of E. coli strain DH5a by heat shock at 42°C/2min. The cell suspension (25\(\mu\)L, 50 \(\mu\)L & 100 \(\mu\)L) was spread onto the Luria Bertani Agar plates with 100\(\mu\)g/mL ampicillin. The plates were incubated at 37°C for 16-18hours. Colonies of E.coli were seen on the plates because of the expression of Amp\(^R\) gene in pUC18 which was used in transformation. This confirms the successful transformation and functional integrity of the plasmid DNA incubated with sporopollenin.

Efficiency of transformation was calculated by the formula: \(\text{Transformation efficiency} = \frac{\text{Number of colonies} \times 1000}{\text{amount DNA plated.}}\)

The transformation efficiency of plasmid DNA incubated with sporopollenin was found to be 12640 CFU\(^g\) which is comparable with 12920 CFU\(^g\) of the control plasmid. This shows that
sporopollenin does not harm the DNA as plasmid DNA is being functionally efficient. Even the presence of sporopollenin in the mixture does not affect transformation. Hence sporopollenin microcapsules can be used for DNA preservation.

Example 4: Effect of sporopollenin on restriction enzyme digestion of plasmid DNA (pUC18)

The effect of sporopollenin on the activity of restriction enzymes was investigated to further confirm the suitability of sporopollenin for DNA preservation. Plasmid DNA (50ng/VL) was loaded on to sporopollenin (10μg/μL). This was mixed with restriction enzyme Bgl I, and incubated at 37°C for 2 hours. This enzyme Bgl I has two restriction sites in pUC18 and if acted properly would provide two fragments of the plasmid DNA on electrophoresis. The digested control DNA and plasmid DNA with sporopollenin were electrophoresed using 1.5% agarose gel at 80V for 60min.

The appearance of two bands of 1568 bp and 1118 bp (Figure 5) indicates the restriction digestion at two restriction sites 265 bp and 1816 bp by Bgl I. This result further confirms the suitability of sporopollenin for DNA preservation as it does not adversely affect the restriction digestion.

Example 5: Effect of sporopollenin on polymerase chain reaction
The effect of sporopollenin on polymerase chain reaction was investigated to confirm the suitability of sporopollenin in DNA preservation. A range of concentration of Sporopollenin (1, 5 & 10 µg/µL) was added to master mix of 800bp template DNA (100ng/µL), reverse primer, forward primer and Taq polymerase. Amplification by PCR was carried out for 30 cycles. After PCR, the mixture was electrophoresed using 1.5% agarose gel, at 80V for 30min.

The result shows proper amplification of template DNA (800bp), which indicates the absence of any adverse effect of sporopollenin on PCR process (Figure 6). The experimental result also proves that the activity of Taq polymerase is not adversely affected. This again confirms the suitability of sporopollenin in DNA preservation.

**Example 6: Binding and release of DNA from sporopollenin**

Another major issue in using sporopollenin for DNA preservation is, understanding the type of binding between the DNA and the sporopollenin. As sporopollenin is chemically inert, it was assumed that DNA does not have any chemical binding with sporopollenin. To verify this hypothesis, DNA release experiment was carried out using Ti₀El buffer. In carrying out this experiment, orthogonal array was used. The design includes L₄ orthogonal array based on 3 factors with 2 levels of each viz. State of DNA-sporopollenin (dehydrated and in solution), concentration of Lambda DNA Eco RI / Hind III double digest marker (50ng/µL, 100ng/µL), concentration of sporopollenin (5µg/µL, 50µg/µL). DNA was loaded on to sporopollenin by the controlled vacuum filtration method disclosed in this invention and desiccated under sterile
condition or kept in solution. The dried samples were then kept at room temperature and the samples in T10E1 were kept at 4°C. Tris EDTA (T_{10}E_{1}) buffer was added to the dried samples.

Electrophoresis of these DNA samples was carried out using 1% agarose gel with 10μg/μL EtBr in it, at 80V for 30min. The results show intact bands of DNA fragments comparable to that of control sets indicating structurally intact DNA released from sporopollenin (Figure 7). This confirms the hypothesis of DNA not getting bound to sporopollenin. As simple addition and mixing of T_{10}E_{1} buffer releases DNA from sporopollenin, it can be confirmed that, the binding between DNA and sporopollenin is weak and easily reversible. Hence sporopollenin can be used for DNA preservation.

Example 7: DNA loading on sporopollenin microcapsules

Adsorption of DNA on sporopollenin was done by mixing dry powder of sporopollenin with appropriate concentration and volume of DNA solution in T_{10}E_{1} buffer. The loaded DNA was stained using 2μg/mL DAPI (4′6-Diamidino-2-phenylindole). Confocal micrographs were obtained using Leica Microsystems. Differential contrast image of sporopollenin microcapsules (Figure 8a) show the presence of numerous spherical particles of about 20μm which were loaded with DNA. Confocal microscopy was carried out (Figure 8b), DAPI gets excited at 310nm and emits at 455nm, revealing the presence of DNA. Composite image of Figure 8a and 8b reveals that DNA is onto sporopollenin microcapsules (Figure 8c). Three dimensional re-
construction of the confocal image shows the presence of DNA all over the surface of sporopollenin microcapsules (Figure 8d). This result shows that when DNA is loaded on to sporopollenin and dehydrated, DNA remains on the surface.

Encapsulation of DNA was thought to be possible as sporopollenin microcapsules isolated from *Lycopodium clavatum* have nanopores of 100-200 nm spread all over its surface leading to the inner hollow space.

The following methods of encapsulation were experimented:

**Example 8: DNA encapsulation by micro-injector**

20µL of plasmid DNA was injected in to a single sporopollenin microcapsule using a micro injector by holding it with the vacuum hold in micromanipulation system. This method is successful in injecting higher volume and higher concentration of DNA precisely inside the hollow sporopollenin microcapsules as shown in Figure 9a.

**Example 9: Re-dispersion of compressed sporopollenin pellet:** Compressed sporopollenin pellet or granule was re-dispersed in DNA solution for encapsulation of DNA. Sporopollenin pellet of 200mg was re-dispersed in 200pL of 50ng/µL DNA solution. Fluorescent microscopic
images show the loading of DNA on the surface of the sporopollenin microcapsule as shown in Figure 9b.

Example 10: Controlled vacuum filtration of DNA multilayered sporopollenin microcapsules:

The need of practical and reliable methods of encapsulation of DNA lead to the discovery of a completely new method of encapsulation using an apparatus specifically designed and fabricated for the purpose. This method is being reported for the first time to the best of our knowledge. The multilayered and compact sporopollenin microcapsules were subjected to vacuum suction from one side and appropriate volume of DNA was added from the other side. The controlled vacuum and proper addition of DNA solution lead to almost complete encapsulation of DNA into sporopollenin microcapsules. This method can be used to encapsulate any amount of DNA. Different DNA to sporopollenin ratios were maintained and slow dehydration was carried out to get desiccated DNA loaded sporopollenin.

Apparatus design for DNA encapsulation in sporopollenin microcapsules

It is disclosed here with that sporopollenin in the form of multilayer in the filter apparatus can be used for encapsulation of DNA based on the amount of genetic constituents (DNA) to be encapsulated and the expected time duration and the purpose of DNA after preservation. Compact sporopollenin multilayer can be prepared using filter plates (microtitre plates) or filter
tubes fitted with silica membranes using suction by vacuum pump as shown in Figures 10 a & 10 b respectively.

This apparatus is a make shift device made at the inventor's lab using regularly used micro centrifuge tubes, filter tubes, filter tips and a vacuum pump. Sporopollenin in 100% ethanol was added on to the filter tube under suction by vacuum pump to get a multilayered compact sporopollenin of required thickness. DNA solution was added onto this multilayered sporopollenin in the filter tube, under vacuum suction. DNA encapsulation in sporopollenin microcapsules was confirmed by confocal laser scanning microscopy (Figure 11 a & lib).

Example 11: Process optimization of DNA release from sporopollenin microcapsules using L9 orthogonal array

Optimization of DNA release from sporopollenin microcapsules was carried out to standardize the process. DNA release method from sporopollenin was standardized using orthogonal array. Four factors viz. Time (10, 30, 60min), Temperature (25, 37, 50°C), Medium for release (De-ionized sterile water, TioEi buffer, Elution buffer) and mixing by pipetting (5, 10, 20 times) were selected with three levels each (Table 1a). L9 orthogonal array (Table 1b) was selected based on the selected factors and levels. Nine experiments were according to the following table. Plasmid DNA (50ng*L) was loaded on to sporopollenin microcapsules (10µg/µL) and desiccated by slow dehydration. DNA release from these sporopollenin microcapsules was
carried out according to the L9 design. The released DNA was electrophoresed using 1% agarose gel, with 10µg/µl EtBr in it, at 80V for 60min. The results show that all the 9 combinations yield DNA release from sporopollenin microcapsules (Figure 12).

Nine conditions were followed to release the DNA from sporopollenin microcapsules, using untreated DNA-sporopollenin microcapsules and temperature treated DNA-sporopollenin microcapsules (80°C for 30min), to confirm the absence of any kind of binding at higher temperature. The results show that DNA can be released from sporopollenin even after temperature treatments. One optimum condition from these 9 sets was selected, even though all the conditions show similar results, based on the following reasons. DNA can be successfully released without any damage using TiOEt buffer by incubating the sample at 37°C for 30-60min. As for all the analyses and applications of DNA need to be carried out using TiOEt, this was preferred over water and elution buffer. Higher temperature may result in fragmentation of DNA or loss of super coiled content in case of plasmids, hence 37°C (<50°C) can be used. Temperature slightly higher than room temperature will speed up the process of dissolution of dehydrated TiOEt, leading to the release of DNA from sporopollenin microcapsules. In all the experiments, this method of DNA release was used.
Table la: Factors and levels for L9 experiment for optimization of release process (DIW = de ionized water)

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<td>50</td>
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<td>DIW</td>
<td>T10E1</td>
<td>Elution Buffer</td>
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<td>4 Mixing by pipetting</td>
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Table lb: L9 Orthogonal array

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<td>60min/50°C/Te/5times</td>
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Preservation experiments based on orthogonal array designs

The requirement of huge amount of DNA and sporopollenin in large number of experiments was a constraint in the conventional designs of experiments. To avoid this constraint and to get statistically valid data, statistically valid designs were required in these experiments. The Taguchi design of experiments [DOE] methodology involves establishment of a large number of experimental situations described as orthogonal arrays to reduce experimental errors and to enhance efficiency and reproducibility of laboratory experiments.

Example 12: Preservation of plasmid DNA from thermal disruption

Preservation of plasmid DNA (pUC18) by sporopollenin microcapsules was investigated in this experiment. This experiment was designed based on the orthogonal array. Four factors viz. concentration of DNA (ng/µL), concentration of sporopollenin (µg/µL), Time (min) and Temperature (°C) were selected as these factors together decide the effectiveness of DNA preservation at any level in a range. Three levels for each factor were decided as it would help in deciding the efficiency of DNA preservation (Table 2a).

Orthogonal array L9 (3^4) was selected and experiments were carried out as in (Table 2b). Nine experiments were carried out along with nine control experimental sets.
Table 2a: Factors and levels for L9 experiment for preservation of plasmid DNA

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<td>factor 2: concentration of sporopollenin (μg/μL)</td>
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<td>50</td>
</tr>
<tr>
<td>factor 3: Time (min)</td>
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<td>60</td>
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<td>factor 4: Temperature (°C)</td>
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<td>120</td>
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</table>

Table 2b: L9 orthogonal array (this experiment was done using 9 experimental conditions along with 9 similar conditions with only DNA as control)

<table>
<thead>
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<th>Factors</th>
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<td>E6</td>
<td>2 3 1 2</td>
</tr>
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<td>E7</td>
<td>3 1 3 2</td>
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</table>
The DNA was released from sporopollenin microcapsule using TiOE buffer by incubating at 37°C for 60 min. These DNA samples were electrophoresed using 1.0% agarose gel at 80V/60min. The results show (Figure 13) the structural intactness of temperature treated desiccated DNA-sporopollenin, unlike the smears in the unprotected control DNA. In this experiment, the 3rd and 4th condition convincingly proved that DNA gets preserved from thermal disruption even after heating at 120°C for 10min (15 & 16 in Figure 13), 30 min (5 & 6 in Figure 13), and 60 min (7 & 8 in Figure 13). Whereas unprotected DNA gets damaged and either it is seen as small remnants of DNA or cannot be observed on the gel as it gets completely sheared.

The structural intactness of DNA samples were further analyzed by restriction digestions using BglI. The pUC18 samples and controls from L9 experiment were digested by restriction enzyme BglI by incubating at 37°C for 2 hours. This enzyme BglI has two restriction sites in pUC18 and if acted properly would provide two fragments of the plasmid DNA on electrophoresis. The digested control DNA and the plasmid DNA with sporopollenin was electrophoresed using 1.5% agarose gel at 80V for 60 min. The appearance of two bands of 1568 bp and 1118 bp indicates the restriction digestion at 265 bp and 1816 bp by BglI.
The results show the presence of two bands in DNA-sporopollenin after temperature treatment (Figure 14). As there wasn't any DNA left intact in the 5th & 7th sample, there are no two bands which were seen in the 6th & 8th sample which was plasmid DNA encapsulated in sporopollenin before temperature treatment. This reiterates that preserved DNA is structurally intact as the highly site specific restriction endonuclease can cut at specific site to give rise to two distinct bands. This result confirms structural integrity of preserved DNA.

These samples were further used for transforming competent cells of DH5a strain. Transformation of 100ng plasmid DNA was carried out using 50µL competent cells of E. coli DH5a by heat shock at 42°C for 2min. The cell suspension (25µL, 50 µL & 100 µL) was spread on to the Luria Bertani Agar plates with 100µg/µL ampicillin. The plates were incubated at 37°C for 16-18hours. Colonies of E.coli were seen on plates because of the expression of AmpR gene in pUC18 which was used in transformation. This confirms the successful transformation and functional integrity of the preserved plasmid DNA. The Efficiency of transformation was calculated based on the following formula:

\[
\text{Transformation efficiency} = \frac{\text{Number of colonies} \times 1000}{\text{amount DNA plated}}
\]

The transformation efficiency in the 2nd set was distinctly more in the DNA encapsulated in sporopollenin than the unprotected DNA even after 100°C treatment for 30min (Figure 15). In
the 3rd, 4th, 6th, 7th and 8th sets, the transformation efficiency of DNA encapsulated in sporopollenin was more than that from unprotected DNA, which confirms the results observed in gel electrophoresis and restriction enzyme digestion. These results confirm that DNA encapsulated in sporopollenin microcapsules get preserved even after treatment at higher temperatures.

**Example 13: Preservation of structural and functional integrity of shuttle vector**

Preservation of structural integrity and functional efficiency of shuttle vector from thermal disruption was carried out using p416TEF (5 kb) which can express both in *E. coli* a prokaryotic as well as *S. cerevisiae*, a eukaryotic system.

This experiment was designed based on orthogonal array. Four factors *viz.* concentration of DNA (ng/µL), concentration of sporopollenin (µg/µL), Time (min) and Temperature (°C) were selected as these factors together decide the effectiveness of DNA preservation at any level in a range. Three levels for each factor were decided as it would help in deciding the efficiency of DNA preservation (Table 3a).

Orthogonal array L9 (3⁴) is selected and experiments were carried out as in (Table 3b). Nine experiments were carried out along with nine control experimental sets.
Table 3a: Factors and levels for L9 experiment for preservation of plasmid DNA (p416TEF)

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<td>concentration of sporopollenin (μg/μL)</td>
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<td></td>
</tr>
<tr>
<td>factor 3</td>
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<tr>
<td>Time (min)</td>
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<td>factor 4</td>
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<td>Temperature (°C)</td>
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Table 3b: L9 orthogonal array (this experiment was done using 9 experimental conditions along with 9 similar conditions with only DNA as control)

<table>
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<th>Factors</th>
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<td>Experiment</td>
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<td>E1</td>
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<td>E2</td>
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<tr>
<td>E7</td>
<td>3</td>
</tr>
<tr>
<td>E8</td>
<td>3</td>
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</tbody>
</table>
After the temperature treatment, the DNA was released from sporopollenin microcapsule using T20E buffer by incubating at 37°C for 60 min. These DNA samples were electrophoresed using 1.0% agarose gel at 80V/60 min. The results show the structural intactness of temperature treated desiccated DNA-sporopollenin, unlike the smears in the unprotected control DNA (Figure 16). The unprotected DNA gets thermally disrupted whereas the DNA encapsulated in sporopollenin gets preserved at higher temperatures for longer time (120°C up to 1 hr).

The structural intactness of DNA samples were further analyzed by restriction digestions using Bgl I. The pUC18 samples and controls from L9 experiment were digested by restriction enzymes Bam HI and Pst I by incubating at 37°C for 2 hours.

The results show the presence of two bands in DNA-sporopollenin after temperature treatment (Figure 17). This result confirms structural integrity of preserved DNA as two bands each in well nos. 2, 4, 6, 8, 10, 12, 14, 16 and 18 show the protection of plasmid DNA by sporopollenin.

Example 14: Preservation of genomic DNA from thermal disruption
Preservation of genomic DNA from thermal disruption by sporopollenin microcapsules was investigated using genomic DNA isolated from yeast 5. cerviceae. The genomic DNA (SOng/µL) was encapsulated in sporopollenin microcapsules (10µg/µL) using our novel apparatus.

Encapsulated DNA samples were dried at room temperature under laminar airflow. These dried and desiccated samples were treated at 50-120°C with interval of 10°C for 10, 30, 60 and 120 min along with control sets containing only DNA of same concentration at same volume. After the treatment, the encapsulated DNA was released from sporopollenin microcapsules by incubating at 37°C in Ti9Ei.

Gel electrophoresis of these samples was carried out to confirm the structural integrity after temperature treatment. Electrophoresis of these samples was done using 1% agarose gel, with ^g/mL EtBr at 80V for 60-90min.

The results show that at 50°C DNA remains stable for 10, 30, 60 and 120min, whereas only DNA treated at same temperature for same time duration shows smearing of DNA. This indicates that sporopollenin microcapsules provide protection to DNA against thermal disruption. Similar results can be observed after treating the DNA encapsulated in sporopollenin microcapsules at 60°C (Figure 18). The temperature was increased to 70°C and 80°C for treatment at same time duration. The results become clearer showing that even after higher temperature and longer
time duration, the encapsulated DNA is safer than the non-encapsulated DNA (Figures 19 and 20).

This confirms that sporopollenin microcapsules protect DNA from thermal disruption in a wide range of temperature fluctuations for any time duration.

Example 15: Preservation of Lambda DNA at higher temperature

Similarly, lambda DNA was used to investigate the DNA preservation by sporopollenin microcapsules, at still higher temperature for a different time durations. Lambda DNA loaded onto sporopollenin microcapsules were treated at 50-100°C with 10°C interval for 10, 30 and 60min. Further, the same DNA was treated at 100-150°C with 10°C interval for 5, 15 and 45 min. The dried DNA-sporopollenin were treated at above mentioned temperatures and then released from sporopollenin using TiE buffer by incubating at 37°C for 60min. Electrophoresis of these samples was done using 1% agarose gel, with 10µg/mL EtBr at 80V for 60-90min.

The results confirm the preservation of DNA in sporopollenin microcapsules from thermal disruption as it can be seen in the image (Figures 21 and 22). The DNA is stable even upto 120°C, as it forms a single band after electrophoresis, which is of same intensity showing the comparable DNA concentration. As it is seen at 130°C and higher temperature there is smearing
even in case of experimental sets. But there was a difference between the smear found in the experimental sets and the control sets. DNA gets completely denatured at 130°C and higher temperature in case of control sets but the DNA fragmentation and smearing in experimental sets shows that, if sporopollenin concentration is increased subsequently, complete DNA preservation could be seen. It means that sporopollenin can be used to preserve DNA even at very high temperatures.

Example 16: Preservation of plasmid DNA from ultraviolet radiation

Preservation of plasmid DNA (pUC18) by sporopollenin microcapsules was investigated in this experiment. This experiment was designed based on the orthogonal array. Four factors viz. concentration of DNA (ng/µL), concentration of sporopollenin (µg/µL), Time (min) and Distance from UV source (cm) were selected as these factors together decide the effectiveness of DNA preservation at any level in a range. Three levels for each factor were decided as it would help in deciding the efficiency of DNA preservation (Table 4a).

Orthogonal array L9 (3^4) was selected and experiments were carried out as in (Table 4b). Nine experiments were carried out along with nine control experimental sets.

Table 4a: Factors and levels for L9 experiment for preservation of plasmid DNA
### Table 4b: L9 orthogonal array (this experiment was done using 9 experimental conditions along with 9 similar conditions with only DNA as control)

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The DNA was released from sporopollenin microcapsule using Ti0Ei buffer by incubating at 37°C for 60 min. These DNA samples were electrophoresed using 1.0% agarose gel at 80V/60min. The results show DNA denaturation in case of control or unprotected DNA as indicated by absence of band in Well no. 9 (slight bands can be seen) which was exposed to UV B for 60 min. In similar exposure, intact bands were seen in Well no. 10, which indicates the protection of DNA by sporopollenin as shown in Figure 23.

The structural intactness of DNA samples were further analyzed by restriction digestions using Bgl I. The pUC18 samples and controls from L9 experiment were digested by restriction enzyme Bgl I by incubating at 37°C for 2 hours. This enzyme Bgl I has two restriction sites in pUC18 and if acted properly would provide two fragments of the plasmid DNA on electrophoresis. Digested control DNA and plasmid DNA with sporopollenin was electrophoresed using 1.5% agarose gel at 80V for 60 min. The appearance of two bands of 1568 bp and 1118 bp indicates the restriction digestion at 265 bp and 1816 bp by Bgl I.

The results show the presence of two bands in DNA-sporopollenin after temperature treatment (Figure 24). As there wasn't any DNA left intact in the 9th sample there are no two bands (there are faint two bands) which are seen in the 10th sample which was plasmid DNA encapsulated in sporopollenin before UV treatment. This reiterates that preserved DNA is structurally intact as
the highly site specific restriction endonuclease can cut at specific site to give rise to two distinct bands. This result confirms structural integrity of preserved DNA.

Additional experiments to confirm the sequence intactness, quantification of T=T dimer formation, AP site quantification and gene expression of UV exposed DNA encapsulated in sporopollenin microcapsule are being done and the results shall be provided on completion of the experiments.

Accelerated Aging Experiment for investigating long term preservation of DNA by sporopollenin microcapsules

As proved till now, sporopollenin can be used to preserve DNA under fluctuating temperatures. But the main objective of this invention is to preserve the DNA for a very long time. To prove the DNA preservation for a long time, it is required to carry out the experiment, for as long as possible, i.e. to prove that sporopollenin can preserve DNA for 10 years, theoretically the samples have to be kept for those many years. When it was intended to prove DNA preservation for a "very long time", the time frame had to be defined. Very long time may be described as 100 - 500 or thousands of years. As described earlier, there is a need for preservation of DNA for such a long time which cannot be practically defined, based on the technologies of utilization of DNA present today. But conventionally it is impossible to prove DNA preservation for such a long time period.
The need of different methods to prove DNA preservation for a long time was understood.

There are no methods reported till date to prove the preservation possibility of anything, up to thousands of years to the best of our knowledge.

In pharmaceutical industries and other manufacturing industries, reliability testing based on accelerated aging conditions are used to prove the reliability and maximum life of the products.

The introduction of new or modified products to the medical marketplace requires the assurance that they can be stored for an extended period (from one to five years) without any decrease in performance that may affect safety and efficacy when the products are used.

Because full-period, ambient-aged samples usually do not exist for such products, it is generally necessary to conduct accelerated-aging tests to provide experimental data in support of performance and shelf-life claims for these products until full-period samples become available.

Many accelerated-aging techniques used for the qualification testing of polymer medical devices are based on the assumption of zero-, first-, and pseudo-first-order chemical reactions following the Arrhenius reaction rate function. This function long the basis for studying most chemical reactions, it states that an increase or decrease in the reaction rate at which a chemical reaction proceeds, changes according to the following equation:

\[
r = \frac{dq}{dt} = Ae^{-\Phi/kT},
\]

Where \( r \) = the rate at which the reaction proceeds; \( A \) = the constant for the material (frequency factor); \( \Phi \) = apparent activation energy (eV); \( k \) = Boltzmann’s constant \((0.8617 \times K \Gamma^{-4} eV/K)\); and \( T \)
**absolute temperature.** With appropriate substitutions, the simplified expression for the 10-degree rule can be derived:

\[
    r = \frac{dq}{dt} = C_2 [T_2 - T_1]^{1/10}.
\]

It should be noted that the 10-degree rule provides a conservative acceleration factor at room temperature for activation energies less than 0.7 eV. Because of the exponential effect, this can be conservative by orders of magnitude.

This kind of accelerated test model could be used to prove DNA preservation by sporopollenin microcapsules. But there are only two reports on DNA stability studies using accelerated aging experiment based on Arrhenius model. But these studies deal with DNA in solutions, and hence the physical data about reactivity of DNA in solution can be incorporated in the model.

Whereas our final product in our studies is a desiccated and sealed one, where relative humidity factor is insignificant and general data on DNA reactivity in solutions with ions, free radicals etc. cannot be used to test the preservation time.
Most of these accelerated test models are not developed for any biomolecule particularly as it becomes difficult to rely. Hence there is a definite need of a new model for accelerated life tests of biomolecules either in solution or in desiccated form.

Possibility of stochastic and empirical models was also studied, but these models can be developed to model physical data rather than actual data generated by experiments like DNA preservation by sporopollenin microcapsules. Further explorations on this regard indicated an Artificial Neural Network model (ANN) for this purpose. This ANN model has to be written based on the data generated by DNA preservation experiments.

Example 17: Preservation of Lambda DNA Eco RI / Hind III double digest marker at accelerated conditions

Lambda DNA Eco RI Hind III double digest marker has 13 fragments (54bp -29kb) hence a good model to prove the preservation of various sizes of DNA molecules in one experiment. These samples (50ng/pL) were encapsulated in sporopollenin microcapsules (10μg/μL) and kept for 1, 7, and 32 days at 4°C, room temperature and 60°C. After the treatment electrophoresis of these samples was done using 1% agarose gel, with 10pg/mL EtBr at 80V for 60-90min.
The results show that (Figure 25), (well# 5 & 6, 11 & 12, 17 & 18) DNA stored for 32 days at 4°C and 30°C (room temperature) was stable with or without sporopollenin but the unprotected DNA (well# 17) got completely degraded when kept at 60°C for 32 days whereas DNA encapsulated in sporopollenin microcapsules kept at 60°C for 32 days (well# 18) was completely intact. This shows that unprotected DNA cannot remain stable at room temperature for a year but DNA encapsulated in sporopollenin microcapsules remain stable even for a complete year. These interpretations are based on the following formula:

\[ r = \Delta q/\Delta t = C_2 \left[ T_2 - T_1 \right]^{10} \]

\[ R = C_2^{(60°C-25°C)/10} \]

\[ R = C_2^{55} = 11.31 \text{ therefore} \]

Accelerated aging time = \( \frac{365 \text{days}}{11.31} = 32.27 \)

i.e. 32.27 days at 60°C = 365 days room temperature

Hence our result show that DNA encapsulated in sporopollenin microcapsules stays stable for a year. Further studies and analysis are being carried out.

Example 18: Preservation of Lambda DNA Eco RI / Hind III double digest marker for 5 days at higher temperature
Preservation of lambda DNA Eco RI/ Hind III double digest marker at higher temperatures up to 5 days at 60°C and 80°C remain stable as they are encapsulated in sporopollenin microcapsules.

After the treatment, electrophoresis of these samples was done using 1% agarose gel, with 10μg/mL EtBr at 80V for 60-90min. The results confirm the structural integrity of DNA hence its preservation by sporopollenin microcapsules (Figure 26).

Example 19: Preservation of plasmid DNA for a long term

Preservation of plasmid DNA for a long term was carried out based on accelerated aging experiment using an orthogonal array to investigate the structural and functional integrity of plasmid DNA.

Preservation of plasmid DNA (pUC18) by sporopollenin microcapsules was investigated in this experiment. This experiment was designed based on the orthogonal array (Table nos. 5a & 5b). Nine experiments were carried out along with nine control experimental sets in triplicates.

Table 5a: Factors and levels for L9 experiment for preservation of plasmid DNA

<table>
<thead>
<tr>
<th>Factors</th>
<th>level 1</th>
<th>level 2</th>
<th>level 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>factor 1 concentration of sporopollenin (μg/μL)</td>
<td>5</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>factor 2 concentration of DNA (ng/μL)</td>
<td>25</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 5b: L9 orthogonal array (this experiment was done using 9 experimental conditions along with 9 similar conditions with only DNA as control)

<table>
<thead>
<tr>
<th>Factors</th>
<th>9 conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiments 1 2 3 4</td>
<td>conc. Of DNA (ng/μL) conc. spP (μg/μL) Time (days) Temperature (°C)</td>
</tr>
<tr>
<td>E1</td>
<td>1 1 1 1</td>
</tr>
<tr>
<td>E2</td>
<td>1 2 2 2</td>
</tr>
<tr>
<td>E3</td>
<td>1 3 3 3</td>
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<tr>
<td>E4</td>
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<tr>
<td>E8</td>
<td>3 2 1 3</td>
</tr>
<tr>
<td>E9</td>
<td>3 3 2 1</td>
</tr>
</tbody>
</table>

The DNA was released from sporopollenin microcapsule using TioEi buffer by incubating at 37°C for 60min. These DNA samples were electrophoresed using 1.0% agarose gel at 80V/60min.
The results show the structural intactness of temperature treated desiccated DNA sporopollenin, unlike the smears in the unprotected control DNA (Figure 27). In this experiment, the 3rd and 4th condition (well# 5 & 6, 7 and 8) convincingly prove that DNA gets preserved from thermal disruption even after keeping at 60°C for 1 week and 1 month, whereas unprotected DNA gets damaged and either is seen as small remnants of DNA or cannot be observed on the gel as it gets completely shear.

The structural intactness of DNA samples were further analyzed by restriction digestions using Bgl I. The pUC18 samples and controls from L9 experiment were digested by restriction enzyme Bgl I by incubating at 37°C for 2 hours. This enzyme Bgl I has two restriction sites in pUC18 and if acted properly would provide two fragments of the plasmid DNA on electrophoresis. Digested control DNA and plasmid DNA with sporopollenin were electrophoresed using 1.5% agarose gel at 80V for 60min. The appearance of two bands of 1568 bp and 1118 bp indicated the restriction digestion at 265 bp and 1816 bp by Bgl I.

The results show the presence of two bands in DNA-sporopollenin after temperature treatment (Figure 28). As there wasn’t any DNA left intact in the 5th sample there are no two bands which are seen in the 6th sample which was plasmid DNA encapsulated in sporopollenin before temperature treatment. This reiterates that preserved DNA is structurally intact as the highly site specific restriction endonuclease can cut at specific site to give rise to two distinct bands. This result confirms structural integrity of preserved DNA.

These samples were further used for transforming competent cells of JM109 strain. Transformation of lOOn plasmid DNA was carried out using 50µL competent cells of E. coli.
O1H5α by heat shock at 42°C for 2min. The cell suspension (100 μL) was spread on to the Luria Bertani Agar plates with 100μg/μL ampicillin. The plates were incubated at 37°C for 16-18hours. Colonies of E.coli were seen on plates because of the expression of Amp<sup>R</sup> gene in pUC18 which was used in transformation. This confirms the successful transformation and functional integrity of the preserved plasmid DNA. Efficiency of transformation was calculated based on:

\[
\text{Transformation efficiency} = \frac{\text{Number of colonies} \times 1000}{\text{amount DNA plated}}.
\]

Transformation efficiency of control plasmid is comparable with that of plasmid DNA incubated with sporopollenin as shown in Figure 29.

In the 3<sup>rd</sup> condition of L9, the plasmid DNA encapsulated in sporopollenin microcapsules and kept at 60°C for 32 days remained stable whereas the unprotected DNA got completely denatured. This shows that if DNA samples encapsulated in sporopollenin microcapsules are kept at room temperature they remain stable for a year but unprotected DNA cannot remain stable. This confirms that DNA can be preserved for a long time using sporopollenin microcapsules.

**Example 20: Preservation of different types of DNA at accelerated conditions for prediction of long term preservation using mathematical models**

Preservation of DNA for a long time can be predicted using an artificial neural network (ANN), a predictive mathematical model. Two types of DNA viz. linear long double stranded genomic DNA and closed circular super coiled plasmid DNA were selected for this experiment. These
DNA were encapsulated in sporopollenin microcapsules. These sporopollenin microcapsules were then kept at 30°C and 60°C for 100 days. After every third day, the samples were analyzed for their structural and functional integrity by gel electrophoresis and real time PCR. This data was quantitated as percentage DNA preservation. These quantitative values of DNA preservation are used to train the ANN model. Further prediction of time of DNA preservation by sporopollenin is tested and validated by real experiments. Hence, we are generating an ANN model based on real data for predicting mathematically the time duration of DNA preservation.

TECHNICAL ADVANCEMENTS AND ECONOMIC SIGNIFICANCE

The present invention provides several technical advancements and economic significance as discussed below:

The present invention provides an efficient process for safeguarding the genetic information by the preservation of genetic constituents.

The process for preservation of genetic constituents employs a biopolymer for preservation of the genetic constituents and hence the process is eco-friendly.

Further, the process for preservation of genetic constituents is simple and reliable.

The process for preservation of genetic constituents provides structurally intact genetic constituents even after accidental or prolonged exposure to UV light.
The process for preservation of genetic constituents disclosed in the present invention provides protection of genetic constituents against unfavorable atmospheric effects such as fluctuating global ambient temperature during transportation.

The process for preservation of genetic constituents provides protection of genetic constituents against unfavorable atmospheric effects such as high temperature.

The process for preservation of genetic constituents does not alter the functional properties of the genetic constituents besides preserving their structural integrity.

The process for preservation of genetic constituents enables easy recovery of the genetic constituents whenever the need arises.

The process for preservation of genetic constituents is cost-effective.

While considerable emphasis has been placed herein on the various features of the preferred embodiment, it will be appreciated that many alterations can be made and that many modifications can be made in the preferred embodiment without departing from the principles of the invention. These and other changes in the preferred embodiment as well as other embodiments of the invention will be apparent to those skilled in the art from the disclosure herein, whereby it is to be distinctly understood that the foregoing descriptive matter is to be interpreted merely as illustrative of the invention and not as a limitation.
BRIEF DESCRIPTION OF ACCOMPANYING DRAWINGS

The invention will now be described with the help of accompanying drawings in which:

Figure 1 illustrates the various types of DNA damage.

Figure 2 illustrates (a) sporopoUenin microcapsules and its layered nature, (b) spores of *Lycopodium clavatum*, (c) sporopoUenin isolated from *L. clavatum*.

Figure 3 illustrates Confocal Scanning Micrograph of SporopoUenin microcapsules isolated from *Lycopodium clavatum*, exhibiting auto fluorescence (Excitation = 344 nm and Emission = 516 nm).

Figure 4 illustrates the effect of sporopoUenin on structural intactness of DNA; (a) range of cone of plasmid DNA pUC18 (10, 25, 50ng/µL) with constant sporopoUenin cone, of 10µg/µL; (b) 25ng/µL of pUC18 with 5, 10 and 50µg/µL sporopoUenin; (c) a range of cone, of Lambda DNA (10, 25, 50ng/µL) with constant sporopollenin cone. Of 10µg/µL; (d) 25ng/µL of Lambda DNA with 5, 10 and 50µg/µL sporopoUenin; (e) a range of cone, of Lambda DNA and Lambda DNA Eco RI/Hind III double digest marker (10, 25, 50ng/µL) with constant sporopoUenin cone, of 10µg/µL; 25ng/µL of Lambda DNA and Lambda DNA Eco RI/Hind III double digest marker with 5, 10 and 50µg/µL sporopoUenin; M= marker, C=control, 1,5,9=controls of respective cone. of DNA in the set.

Figure 5 illustrates the effect of sporopoUenin on restriction enzyme digestion (m =marker, l=standard, 2,3,4=DNA samples with sporopoUenin).
Figure 6 illustrates the effect of sporopollenin on PCR amplification (M=100bp Marker, negative control without sporopollenin, positive control without DNA template, 1µg/µL, 5 µg/µL, 10 µg/µL sporopollenin in 3, 4, and 5 respectively.

Figure 7 illustrates intact bands of DNA fragments comparable to that of control sets indicating structurally intact DNA released from sporopollenin, where M = Marker, S = Standard, 1 = hydrated control (50ng/µL), 2 = dehydrated control (50ng/µL), 3 & 5 = 50ng/µL DNA to 5 µg/µL sporopollenin, 4 & 6 = 100ng/µL DNA to 50µg/µL sporopollenin, A = dehydrated, B = Hydrated and then released.

Figure 8 illustrates (a) Differential Contrast Image of sporopollenin microcapsules; (b) Confocal image showing only DAPI signal from DNA; (c) Composite image of a & b, showing that DNA is adsorbed on sporopollenin microcapsules; (d) 3D image of b, showing the presence of DNA on the surface of sporopollenin microcapsules.

Figure 9 illustrates (a) light microscopic image showing microinjection of DNA and (b) Fluorescent microscopic image showing DAPI labeled DNA on the surface, loaded by redispersing the compressed pellet;

Figure 10 illustrates (a) microtitre plate with silica membrane and sporopollenin microcapsules, said plate connected to vacuum manifold in order to obtain sporopollenin multilayer, and (b) the DNA encapsulating apparatus in accordance with this invention (1 = DNA, 2 = sporopollenin multilayer, 3 = silica membrane, 4 = 0.5mL filter tube, 5 = 1.5mL tube, 6 = two holes for vacuum, 7 = 2mL tube with cut open bottom, 8 = silicone tubing). The size and shape of the various components of the apparatus may vary according to the quantity of genetic constituent to be encapsulated within the sporopollenin microcapsules.
**Figure 11** illustrates (a) CLSM image showing encapsulated DNA by controlled vacuum filtration method; and (b) CLSM optical scan showing encapsulated DNA by controlled vacuum filtration method.

**Figure 12** illustrates Process optimization of DNA release from sporopollenin microcapsules using L9 orthogonal array, wherein Set I was released after 80°C/30min treatment. M=marker, well # 1, 3, 5, 7, 9, 11, 13, 15, 17 = set I was released without any temperature treatment. 2, 4, 6, 8, 10, 12, 14, 16, 18 = released.

Figure 13 illustrates Gel Electrophoresis of 1, 3, 5, 7, 9, 11, 13, 15, 17 control sets and 2, 4, 6, 8, 10, 12, 14, 16, 18 experimental sets. The experiment was carried as per 9 conditions based on L9 orthogonal array (Table 2a and 2b).

Figure 14 illustrates Restriction digestion of preserved DNA (1, 3, 5, 7, 9, 11, 13, 15, 17 control sets and 2, 4, 6, 8, 10, 12, 14, 16, 18 experimental sets. Experiment was carried as per 9 conditions based on L9 orthogonal array (Table 2a and 2b). Temperature treated DNA samples were digested using Bgl I. M= marker and S= Stock.

Figure 15 illustrates the Transformation efficiency of plasmids preserved using sporopollenin microcapsules.

**Figure 16** illustrates the electrophoresed gel indicating the structural intactness of temperature treated desiccated DNA-sporopollenin, unlike the smears in the unprotected control DNA. Well nos. 1, 3, 5, 7, 9, 11, 13, 15 and 17 are control sets and well nos. 2, 4, 6, 8, 10, 12, 14, 16 and 18 are experimental sets. Experiment was carried as per 9 conditions based on L9 orthogonal array.
(Table 2a and 2b). The unprotected DNA gets thermally disrupted whereas DNA encapsulated in sporopollenin gets preserved at higher temperatures for longer time (120°C up to 1 hr).

**Figure 17** illustrates restriction digestion of preserved plasmid DNA p416TEF (1, 3, 5, 7, 9, 11, 13, 15, 17 control sets and 2, 4, 6, 8, 10, 12, 14, 16, 18 experimental sets. Experiment was carried as per 9 conditions based on 9 conditions based on L9 orthogonal array (Table 2a and 2b). Temperature treated DNA samples were digested using PstI and BamHI. Two bands in 2, 4, 6, 8, 10, 12, 14, 16 and 18 show the protection of plasmid DNA by sporopollenin.

**Figure 18** illustrates the preservation of yeast genomic DNA at 50°C and 60°C, (M=Marker, S=standard, 1, 3, 5, 7, 9, 11, 15 = DNA encapsulated in sporopollenin microcapsules, 2, 4, 6, 8, 10, 12, 14, 16 =Control DNA sets).

**Figure 19** illustrates the preservation of yeast genomic DNA at 70°C and 80°C (M=marker, S = standard, 1, 3, 5, 7, 9, 11 are DNA samples encapsulated in sporopollenin and 2, 4, 6, 8, 10, 12 are unprotected control DNA.

**Figure 20** illustrates the preservation of yeast genomic DNA at 80°C (M=marker, S=Standard, 1 & 3 are DNA samples protected by sporopollenin microcapsules, 2 & 4 are unprotected DNA samples.

**Figure 21** illustrates the preservation of Lambda DNA from temperatures ranging from 50°C to 100°C (M = marker, 3, 6, 9, 12, 15, 18 are unprotected DNA samples and 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17 are DNA samples protected by sporopollenin.
Figure 22 illustrates the preservation of Lambda DNA from temperatures ranging from 100°C to 150°C (M = marker; 3, 6, 9, 12, 15, 18 are unprotected DNA samples and 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16 and 17 are DNA samples protected by sporopollenin.

Figure 23 illustrates Gel Electrophoresis of UV treated pUC18 (L9 orthogonal array). Unprotected control DNA sets 1, 3, 5, 7, 9, 11, 13, 15, 17 and Experimental sporopollenin protected sets 2, 4, 6, 8, 10, 12, 14, 16, 18

Figure 24 illustrates Restriction enzyme digestion of UV treated pUC18 (L9 orthogonal array). Unprotected control DNA sets 1, 3, 5, 7, 9, 11, 13, 15, 17 and Experimental sporopollenin protected sets 2, 4, 6, 8, 10, 12, 14, 16, 18

Figure 25 illustrates the Preservation of lambda DNA marker (50ng/µL DNA was encapsulated into 10µg/µL sporopollenin and treated for 1 day, 1 week and 1 month at 4°C, 25°C, 60°C. M=marker, 1, 3, 5, 7, 9, 11, 13, 15, 17 are controls, and 2, 4, 6, 8, 10, 12, 14, 16, 18 are experimental sets).

Figure 26 illustrates the Preservation of lambda DNA Eco RI/Hind III double digest marker at higher temperatures for a long time, a: lhr, 6hr, b: 12hr, 24hr, c: 2day, 3day, d: 4day, 5day (M= marker, SI = 50ng/L stock, S2 = 100 ng/µL stock, 1,3,5,7 Unprotected DNA, 2,4,6,8= DNA encapsulated in sporopollenin).

Figure 27 illustrates the Preservation of plasmid DNA for a long term. (M= marker, 1, 3, 5, 7, 9, 11, 13, 15, 17, are control sets and 2, 4, 6, 8, 10, 12, 14, 16, 18 are experimental sets) (25ng/L = 1-6, 50 ng/µL = 7-12, 100 ng/µL = 13-18).
**Figure 28** illustrates the Restriction enzyme digestion of preserved plasmid DNA for a long term. (M=marker, 1, 3, 5, 7, 9, 11, 13, 15, 17 are control sets and 2, 4, 6, 8, 10, 12, 14, 16, 18 are experimental sets) (25ng^L^ = 1-6, 50 ng/µL = 7-12, 100 ng/µL = 13-18).

**Figure 29** illustrates the Transformation efficiency of plasmid DNA (pUC18) kept for long term at higher temperature.
We Claim:

1. A method of preservation of genetic constituents, said method comprising encapsulating the genetic constituents into purified sporopollenin microcapsules.

2. The method as claimed in claim 1, wherein the genetic constituent is at least one selected from a group of genetic constituents consisting of single genes, oligomers, plasmids, cosmids, fosmids, single stranded genomic deoxyribonucleic acid (ssDNA), double stranded genomic DNA (dsDNA), ribonucleic acid (RNA), synthetic gene sequences and natural gene sequences.

3. The method as claimed in claim 1, wherein said sporopollenin microcapsule is at least one selected from a group consisting of granules, pellets, tablets, multi-layer and dry powder.

4. The method as claimed in claim 1, wherein said sporopollenin microcapsules are obtained from exines of naturally occurring spores of organisms selected from a group consisting of microorganisms, bryophytes, pteridophyets and gymnosperms, pollen grains of angiosperms or fragment thereof.

5. The method as claimed in claim 1, wherein the step of encapsulation of genetic material is carried out by a process selected from a group consisting of electroporation, liposome mediated transfer, micromanipulation, re-dispersion of compressed sporopollenin pellet and controlled vacuum filtration, preferably controlled vacuum filtration.
6. The method as claimed in claim 1, wherein said microcapsules have a diameter in the range of about 2 to about 200 microns.

7. The method as claimed in claim 1, wherein isolation and purification of sporopollenin microcapsules is carried out by:

   i) suspending the spores in acetone and stirring under reflux for about 4 hours;
   ii) filtering the refluxed acetone to obtain solid residue;
   iii) washing the filtered solid residue with fresh acetone;
   iv) suspending the washed residue in KOH solution (6% w/v in water) and stirring under reflux (100°C) for 6 h;
   v) filtering the residue;
   vi) washing the filtered residue with hot water at a temperature ranging from about 45-50°C;
   vii) resuspending the washed residue in KOH solution (6% w/v in water) and stirring under reflux (100°C) for 6 h;
   viii) filtering the residue and washing with hot water at a temperature ranging from about 45-50°C, hot absolute ethanol at a temperature of about 45°C followed by washing with water again;
   ix) stirring the residue under reflux at a temperature ranging from about 60-70°C in ethanol for about 2 hours, filtering and washing sequentially with fresh ethanol and dichloromethane.
   x) resuspending the resulting solid in fresh dichloromethane and stirring under reflux at a temperature ranging from about 50-55°C for about 2 hours;
   xi) removing the residue by filtration, drying in air for about 24 hours at room temperature, and filtering the sporopollenin microcapsules;
xii) treating the filtered sporopollenin microcapsules with 1% cellulase and 1% pectinase in citrate phosphate buffer of pH of about 4.8 at a temperature of about 45°C for about 24 hours with shaking at about 100 rpm to obtain purified sporopollenin microcapsules;

xiii) washing said sporopollenin microcapsules several times with hot water and hot ethanol;

xiv) refluxing washed sporopollenin microcapsules in dichloromethane for about 4 hours; and

xv) filtering said sporopollenin microcapsules and vacuum drying to obtain purified sporopollenin microcapsules.


9. The method as claimed in claim 8, wherein said loading is carried out by physical adsorption or dipping said purified sporopollenin microcapsules, preferably granules of purified sporopollenin microcapsules into a solution containing the genetic constituent.

10. A method of encapsulating genetic material in sporopollenin microcapsules, said method comprising the following steps:

i) preparing a compact multilayered mass of purified sporopollenin microcapsules;

ii) exercising a negative pressure typically below atmospheric pressure but not less than -1 Barr in said multilayered mass for about 30-40 seconds;

iii) absorbing the genetic material contained in buffer within said microcapsules and encapsulating by further applying said negative pressure for about 30-40 seconds to said multilayered mass; and
iv) desiccating said sporopollenin microcapsules to permanently encapsulate the genetic material within said sporopollenin microcapsules.

11. The method of encapsulating genetic material as claimed in claim 10, wherein said buffer is Tris-EDTA buffer.

12. The method of encapsulating genetic material as claimed in claim 10, wherein said desiccation is carried out by slow dehydration.

13. The method of encapsulating genetic material as claimed in claim 10, wherein said method further comprises the step of coating a layer of hydrophobic substance onto the desiccated microcapsule.

14. The method of encapsulating genetic material as claimed in claim 13, wherein said hydrophobic substance is preferably a lipid.

15. The method of encapsulating genetic material as claimed in claim 10, wherein the encapsulated genetic material can be recovered from the sporopollenin microcapsules by mixing the encapsulated material with a suitable buffer, preferably Tris-EDTA buffer.

16. An apparatus for encapsulation of substances including genetic constituents, said apparatus comprising:
i) a filter tube with silica membrane on to which sporopollenin microcapsules are loaded to receive the substance to be encapsulated;
ii) said filter tube introduced into a slightly larger tube with two or more holes on its surface;
iii) said slightly larger tube introduced into a still larger tube with cut open bottom; and
iv) said cut open bottom connected to vacuum pump.

17. The apparatus as claimed in claim 16, wherein said tubes are made up of polymer.

18. The apparatus as claimed in claim 16, wherein said cut open bottom is connected to vacuum pump by means of silicon tubing.

19. The methods of preservation of genetic constituents, the method of encapsulating genetic material and the apparatus as substantially herein described with reference to the accompanying examples and drawings.
Figure 19

Figure 20
A. CLASSIFICATION OF SUBJECT MATTER

**IPC(8)** - A61 K 36/00, A61 K 47/00 (2010.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELD(S) SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC - 424/778, 424/439

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched


Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST/PGPB,USPT,USOC,EPAB,JPAB; Dialog Classic Files - 654, 652, 351, 349, 6, 35, 65, 155; Google Scholar Google Patents; USPTO Web Page: encapsulation, sporopollenin, nucleic acid, vacuum filtration, silica filter, desiccator, bryophyte exines, lipid coating,

DNA preservation, citrate-phosphate buffer, reflux, wash, ethanol

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>X</td>
<td>US 2005/0002963 A1 (BECKETT et al.) 06 January 2005 (06.01.2005) para [0006], [0012], [0014], [0016] [0017], [0019]-[0021], [0024], [0026], [0033], [0039], [0056]-[0057], [0060], [0062], [0065], [0097], [0133]-[0184]</td>
<td>1-6, 8-10, 12-14</td>
</tr>
<tr>
<td>Y</td>
<td>US 2004/0079709 A1 (LISA et al.) 29 April 2004 (29.04.2004) para [0013]-[0015], [0018], Fig 1</td>
<td>16-18</td>
</tr>
</tbody>
</table>

[ ] Further documents are listed in the continuation of Box C.

* Special categories of cited documents:
  “A” document defining the general state of the art which is not considered to be of particular relevance
  “E” earlier application or patent but published on or after the international filing date
  “L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  “O” document referring to an oral disclosure, use, exhibition or other means
  “P” document published prior to the international filing date but later than the priority date claimed

“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

“X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Date of the actual completion of the international search

18 April 2010 (18.04.2010)

Date of mailing of the international search report

28 APR 2010

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-272-3281

Authorized officer: Lee W. Young

PCT Helpdesk: 571-272-4300
PCT Update: 571-272-774

Form PCT/ISA/2 10 (second sheet) (July 2009)
### Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 19
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
   Claim 19 is an omnibus type claim, and is not drafted in accordance with PCT Rule 6.2(a). The claim is indefinite as it is unclear what is included or excluded by the claim language.

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box No. III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)