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Isolation, preservation and revival of *Trichoderma Viride* in culture media

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Abstract
The present study was conducted in the year 2015-2017; different methods for isolation and preservation methods were tested in CABI lab for 2 years to standardize the long term less expensive preservation technique for *Trichoderma viride* fungus. Multiple serial dilutions and multiple serial dilutions with 0.05% of Twen-20 were tested and proved to be best for isolation on PDA media. For long term, inexpensive storage; different techniques were tested for preservation of *Trichoderma viride* which were previously proved to be best for short term and long term storage to other group of fungus. Drying method proved to be best for the preservation of *Trichoderma viride* for long period of time. It is an easy, convenient, economical, and effective tool for the preservation of *Trichoderma viride* isolate. Longer monitoring of *Trichoderma viride* isolate and storage would further validate the reliability strains. Also, the stability of *Trichoderma viride* should be further assessed by molecular parameters. Drying is not only reliable but is very inexpensive and easy to use in any laboratory with few resources

Keywords: *Trichoderma viride*, Isolation, Preservation

1. Introduction
Trichoderma is a filamentous, asexual spore producing ascomycetic fungi belongs to class Deuteromycetes. T*richoderma viride* is one of many species of mold which is almost found in all types of soils and is most prevalent and culturable fungi [9, 33, 5]. *Trichoderma* is a very useful fungus for industry and as a biocontrol; it has shown little host specificity, colonizing most plants and it is widely studied fungi and most commonly used as biological control agents in agriculture and their products as alternatives to synthetic agro-chemicals [34].
Trichoderma spp. isolated from the soil is often difficult to quantify because of the relatively rapid growth of other soil fungi on conventional agar media. In spite of the growing interest in these soil-inhabiting antagonists, no special selective medium for their isolation has been reported [10]. However, *Trichoderma* spp. have been reported among soil fungi growing on versions of Martin's rose-bengal agar medium. On Martin's medium, however, some *Trichoderma* isolates grow more rapidly than others, forming larger colonies which suppress the growth of other isolates, thus reducing colony counts [5, 9]. TSM was developed for quantitative isolation of *Trichoderma* spp. from soil [10]. Selectivity was obtained by using chlor-amphenicol as a bacterial inhibitor, and pentachloronitrobenzene, p-dimethyl-aminobenzenediazo sodium sulfonate and rose-bengal as selective fungal inhibitors. The TSM medium have low concentration of glucose but it is good for relatively rapid growth and sporulation of *Trichoderma*, enables for the rapid identification of trichoderma colonies [9]. Recovery of *Trichoderma* from artificially inoculated soils was high and unaffected by soil type [5].
In the last decay many scientists adopted different methods for fungal preservation to enhance its duration to live long but safe storage of numerous fungi of industrial strain is essential for future molecular studies. Among the several approaches known, storage of microorganism at low temperature in frozen form has several advantages [8] as freezing enhance the shelf life of microorganism but freezing and thawing again can decrease the viability but this problem was reduced by using glass beads, porous ceramic beads in a cryoprotectant to facilitate absorption [13, 17]. Freeze storage preservation is applicable for a large number of bacterial and some of yeast species [13]. However, no filamentous fungi have been reported to be preserved by freeze method. Scientist aimed is to test different strains of filamentous fungi and to optimize the procedure to enhance the storage conditions in freeze preservation method [1, 8].

Some low-cost effective methods of preservation; in distilled water and the silica gel are good, but none are best for long term duration/ permanent. The maximum duration of storage varies between 8-10 years by using specific preservation method and microorganism preserved [2, 1]. Permanent preservation is essential for strains with critically important characteristics and for type specimens. Cultures that are permanently preserved in metabolically inactive states now can serve as type specimens [29, 41].

Maintaining and preserving fungal cultures are essential for systematic and biodiversity studies. Because fungi are such a diverse group of microorganism and several methods for cultivation and preservation/storage. Inexpensive and more durable method must also consider for preservation of fungal culture [36, 38]. Culture collection is very expensive because special equipment and continuous attention is required to maintain fungal cultures without losing its effectiveness or virulence because fungus has a high rate of production as well high rate of mutation with the passage of time so scientists are more interested to preserve particular important and numerous industrial strains [17, 29]. Therefore the present experiments were conducted to evaluate several of preservation methods to establish a simple and reliable preservation method for long-term maintenance of *Trichoderma viride* strains isolated from vegetable growing soil. Main objective of study was to isolate and preserve *Trichoderma viride* for long term to ensure the viability and morphological, physiological, and genetic integrity of the cultures over time and safe storage is essential further to validate the reliability of strains their in-depth study.

2. Materials and Methods

2.1 Surveys and sampling

Surveys were conducted in the year 2015-2016 in the vegetables production areas of Punjab, Pakistan. 100 gm of samples were taken from the root zone area of different vegetable host plants and brought to Biotechnology Lab, CABI Rawalpindi, Pakistan.

2.2 Isolation and Identification of *Trichoderma viride*

Trichoderma fungal species were isolated from soil samples by using potato dextrose agar (PDA) medium. PDA media were prepared and autoclaved at 121 ºC @15psi for 20mints. Penicillin @100,000 units L-1 and streptomycin @0.2g L-1were added to sterilized stock media just before pouring to inhibit the bacterial growth. The media were poured in 9cm diam. Petri plates @ 10 ml per plate. Samples were inoculated over plates by multiple tube dilution technique (MTDT) and the plates were incubated at 26˚C for 4-7 days. Inoculated plate shows a mixture of multiple fungi and bacteria cultures. The desire fungal colonies were then picked and purified by streaking or by single spore and incubated at 26˚C for 7-8 days. 1ml of distilled water spread over the fully grown culture of *Trichoderma* with a spade and then transfer to 99 ml of distilled water to make a suspension. Take a drop from the diluted fungal suspension over the side and observe *Trichoderma* sp. under the microscope at 10-40X magnification.

2.3 Maintenance and preservation of *Trichoderma viride* cultures on PDA

Different methods were adopted for the preservation of *Trichoderma viride* isolate for long period. Ten vials of *Trichoderma viride* was used for each preservation protocol in order to estimate the less expensive, long duration protocol and what conditions are best for preservation and revival of *Trichoderma viride* on PDA. There are several methods for long-term preservation have been developed [17].

2.3.1 Serial transfer preservation

Mostly fungal cultures can be maintained for some period by serial transfer. There are two methods for serial transfer preservation for short duration

2.3.1.1 Fresh cultures preservation

Single spores technique was used for *Trichoderma viride* to get a pure culture, from the mother culture a plug of media were taken and placed on fresh media. Properly wrapped and incubate the plates at 25 ºC for 7days and repeat after 15-20 days to maintain the culture for longer period of time. This protocol is very laborious, hectic and time wasting and of course prove to be very costly.

2.3.2.2 Slants preservation

Autoclaved PDA media at 121 ºC for 15min at 15psi. Poured autoclaved media in already sterilized test tubes/vials. Allow media to solidify in test tubes/vials at an angel of 65^0. Loopful inoculums of *Trichoderma viride* was transferred to Potato Dextrose Agar (PDA) slants, keep the tubes in an incubators at 25 ºC for 7 days and maintained at 4 ºC as pure culture.

2.3.2.2.1 Recovery

Small loop of inoculums was placed on fresh PDA media and incubate for 7 days and check the recovery and viability with three months interval

2.3.3 Water preservation

There are two methods for preservation *of Trichoderma viride* in distilled water at room temperature [10]

2.3.3.1 Spore suspension

1ml of sterilized autoclaved distilled water added on fully grown fresh mother culture of *Trichoderma viride* and scraped with a spade to produce slurry and then transfer to 99 ml of distilled water to make a suspension. Take 1000 µl in a sterile glass vial/ eppendorf tubes, tie cap tightened and store at room temperature.

2.3.3.1.1 Recovery

Take 100µl of the suspension from the vial and placed on fresh medium to check its recovery after 3 months relatively.

2.3.3.2 Plug in distilled water

3 ml of autoclaved distilled were taken in small vials. A plug of *Trichoderma viride* from fresh mother culture were taken and placed in the vials contains DDH_2O and maintain at room temperature. This is another inexpensive and low-maintenance method for storing fungal cultures for 6 months to 1 year without getting contamination.

2.3.3.2.1 Recovery

Picked the plug from vials and placed on PDA media. Incubate for 7 days and check the recovery and viability with three months interval

2.3.4 Dry methods/High temperature preservation

At certain maximum high temperature fungi can b preserved. There are lot of method which were previously used by other scientist for many other fungi

2.3.4.1 Direct harvest of fresh culture
After complete sporrulation of *trichoderma viride* from the mother cultures were harvested with the help of a small sterile metal spatula on already autoclaved filter paper. Harvested conidia were air dried for two days under laminar air flow and stored in a small air tight screw cap vials (10 cm with 2.5 cm diameter) at room temperature or in refrigerator at 4 °C before using for further studies.

2.3.4.1.1 Recovery
Dried spores were spread on fresh PDA medium. Incubate for 7 days and check the recovery and viability after three months interval

2.3.4.2 Preserve on toothpick sticks/wood chips
50 pieces of toothpicks were dipped in 2% PDA broth and sterilized for 20 minutes at 121 °C (Delatour 1991). After that all toothpicks were drained and placed on fresh culture of T*richoderma viride* growing on PDA agar. Sealed with parafilm and incubate at 25ºC for 5days to allow the *Trichoderma viride* to colonize the toothpick stick. After 10-15 days, the inoculated wood chips were transferred to sterile test tubes (18 × 180 mm). The tubes were stored at 4 °C.

2.3.4.2.1 Revive
A single toothpick stick was removed from the test tubes and placed on fresh agar medium. The tube is resealed and returned to the refrigerator. Incubate the plate for 7days and check the recovery and viability after three months interval

2.3.4.3 Agar strip method
Trichoderma viride cultures were grown in petri dishes containing PDA media. Strip of 1cm long were cut from the mother culture and placed in sterile petri dish, incubate at room temperature for 1-1.5 weeks. Agar media get dried and transfer this dried strips into a separate vials and sealed.

2.3.4.3.1 Revive
Dried agar strips of *Trichoderma viride* were placed on fresh medium of choice. Incubate the plates for 7 days and check the recovery and viability with three months interval

2.3.4.4 Preservation in sand
5gm of sand were taken in bottle were sterilized at 121ºC for 15 mint and maintain moisture level 20%. The bottles were allowed to cool and then sterilized again. 5ml of sterilized distilled water was added in fresh mother culture of *Trichoderma viride*, and scraped gently to produce spore suspension. One milliliter of the suspension was added to each bottle of sterilized sand. After 2-14 days of growth at room temperature and stored in the refrigerator at 4 °C.

2.3.4.4.1 Revive
Few grains of soil were sprinkled onto fresh agar medium. Inoculated plates were incubated for 7 days to check the recovery and viability after three months interval. Test tubes or vials can be used in place of glass bottles to save space.

2.3.4.5 Filter paper preservation
Autoclaved the filter paper and cut into small pieces; arrange these filter paper pieces in a plate containing PDA media. A small clump of mycelium of *Trichoderma viride* was placed in each piece of filter paper. Proper wrap the petri plate with parafilm, and incubated at 25oC for 8 days because fungus grow slowly on filter paper. Once the fungus begins to sporulate on the filter paper, each piece of filter paper bearing fungus culture were separated form one another and placed in a new petri dish without the PDA media/empty plate. After that again put the petri plates in an incubator for 30days until the filter paper and fungi dry completely. When the fungus dried completely; these filter paper containing *Trichoderma viride* culture were properly sealed in small sized zipper bags. Properly label and store at 4 ºC.

2.3.4.5.1 Recovery
Each piece of filter paper was placed on the specific fresh PDA media. Incubate the plate for 7days and check the recovery and viability after three months interval

2.3.5 Low temperature preservation
Lots of fungus culture can be preserved at low temperature, in order to inactivate their metabolism for short and long period of time. I used only two methods for the preservation of *Trichoderma viride* culture

2.3.5.1 Freeze in liquid nitrogen
1ml of sterile solution of 10% glycerol was taken in eppendorf tubes. Small plugs 2mm in diameter are cut from fresh vigorously growing mother culture *of Trichoderma viride* by using a sterilized surgical blade. Several plugs were placed in a vial, the cap is tightened with parafilm and the tubes were placed directly into a liquid-nitrogen tank for more than 2 years.

2.3.5.1.1 Recovery
Removing the vials from the liquid nitrogen tank or from -80 freezer and rapidly thawing them in a 37 °C water bath and placed thawed agar plugs on PDA agar plates. Viability of the cultures should be checked from 2-7 days after storage.

2.3.5.2 Direct freezing of cultures
Cultures grown on agar slants in bottles or test tubes with screw caps were placed directly in the freezer [6, 7]. The cultures were preserved successfully at -80 °C for up to 2 years. In general, vigorously growing and sporrulating cultures survive the freezing process better than less vigorous strains. We do not recommend repeated freezing and thawing, which will significantly reduce viability of the cultures. But this method can reduce the variability of Trichoderma viride, morphological and physiology can be change by continuous thawing and melting.

2.3.6 Suffocation method
In this method fungus culture were preserved by creating anaerobic condition for the fungus not to sporrulate.

2.3.6.1 Preserve in skim milk and slica gel
3.5g powdered milk were mixed in 50 ml of deionized water and sterilized by boiling it for 20 minutes. You can also used condense milk instead of powdered milk. Sterilization in autoclave at 15psi for 20mint should be avoided because it carmelizes the milk. Screw-cap tubes were half filled with 6- to 22-mesh silica gel [26, 25, 28, 29]. *Trichoderma viride* spores suspension was prepared in 10ml of already prepared skim milk solution, previously cooled at 4 °C. The silica gel was also chilled at 4 °C. 100µl spore suspension was added to wet about three-fourths of the silica gel and left it for 30 minutes. Tubes were stored at room temperature for 1-2 weeks.

2.3.6.1.1Revival

Revival of cultures from silica gel was so easy; few silica gel crystals were scattered on the suitable agar media. Kept in incubator for at 25 ºC 7days; check the recovery and viability after three months interval. If the cultures are viable, caps are tightened, and the tubes are stored in a tightly sealed container at 4 °C.

2.3.6.2 Oil drop cover

Trichoderma viride culture was grown in slants; 10ul of vegetable oil was dropped over the fungi culture and properly submerged in the oil. It is low-cost and low-maintenance method for preserving cultures in slants. Cultures can be preserved for many years and one of the main advantage is from the attack of mites.

2.3.6.2.1 Revival

A small amount of preserved *Trichoderma viride* culture were taken and placed on the PDA media in order to drain extra oil, culture and then sub-culture *Trichoderma viride* 2-3times to get oil free colony. Incubate the plates for 7days and check the recovery and viability after three months interval Oil level in the tubes/vials must be checked periodically or after culturing and add more vegetative oil if necessary.

3. Results and Discussion

Survey for the isolation of *trichoderma* fungi has been conducted for continuously two years, as Elad used specific medium (TSM) a low nutrient media for isoating *trichoderma* spp but the TSM for isolation does not works in our lab conditions as the growth was very slow or restricted. Only the enrich PDA media was proved to be best for the isolation of pure colonies of *trichoderma.* As spores of *trichoderma viride* are very small in size and lighter in weight; it is very difficult to harvest from the soil, Multiple dilution was also proved to get pure colony at dilution 5, 6, and 7. Somehow twen-20 as of its high consistency to grabs spores was used for the isolation. But this method was also good to some extent for getting pure isolations.

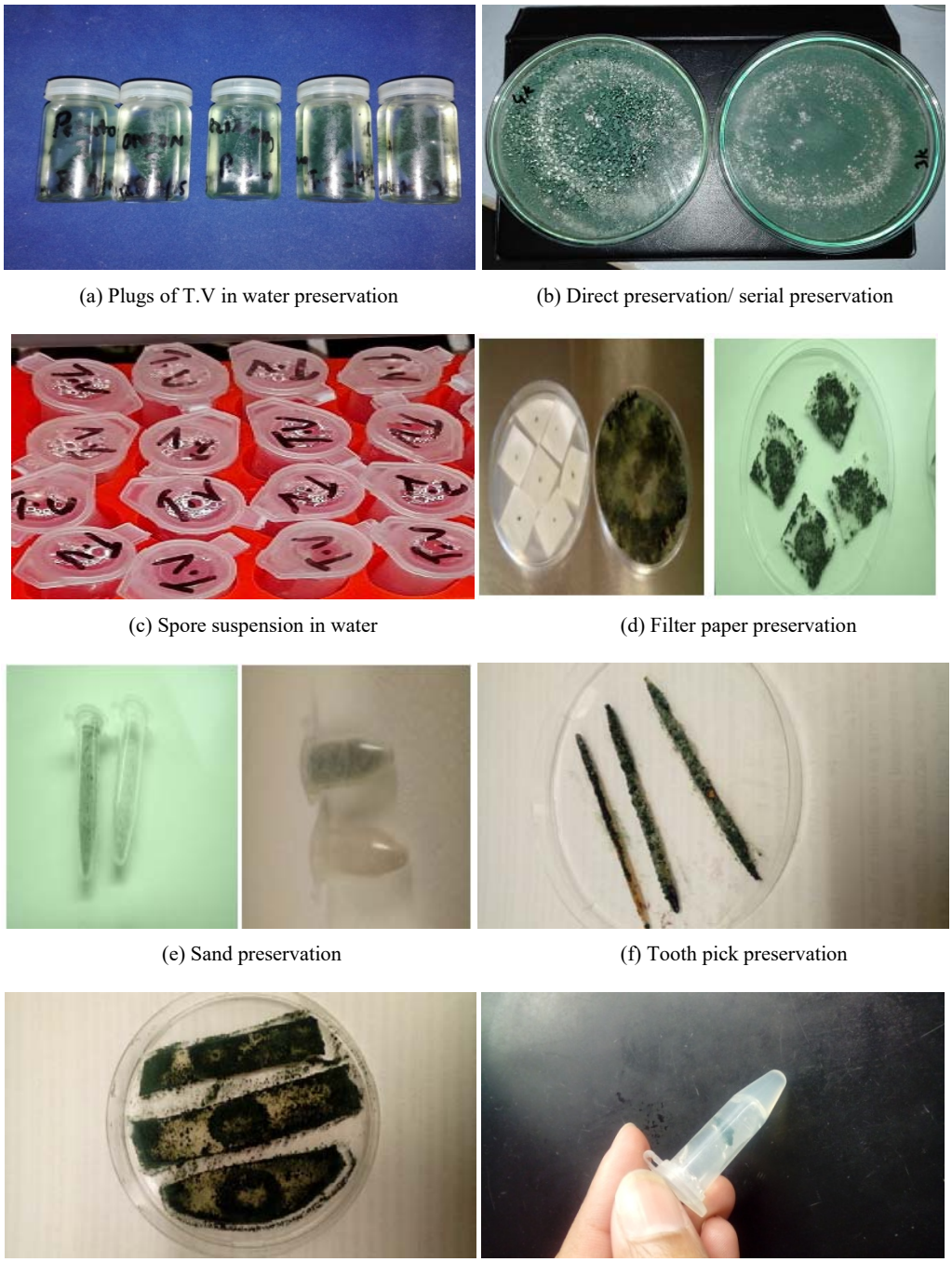
The growth rate of fungi is slow as compared to bacteria in culture media contrarily fungus culture is mostly contaminated by bacteria or other fungi, which effects the preservation of the colonies. The existent techniques for the collection and maintenance of fungus cultures are difficult, costly and frequently inefficient. Preservation methods are more important because the rates of mutation in fungus are very high due to rapid multiplication of cell division and metabolic activity. Proper storage and preservation helps to stop cell division completely and totally arrest metabolism, to retaining viability. The choice of preservation method depends on the species of concern, and to develop a technique which is not only reliable but should be very inexpensive and easy to use in any laboratory with few resources. Different methods of preservation were adopted to check the effectiveness of fungi. Stability of fungal cells was not ensured by simple procedure, other methods have also been suggested, such as preservation in soil or on oil or water-covered slants, cryopreservation either in liquid nitrogen or at low temperature (−20 and −80 °C) [2, 5, 7, 9, 11, 14, 16] and lyophilization [1, 5]. Cryopreservation in liquid nitrogen and lyophilization are the methods recommended and used by the American Type Culture Collection [1]. The preservation on fresh and slant culture are simple, inexpensive, and widely used. Although time consuming and labor intensive. These methods are good when you have to maintain one or two isolates and constantly use for short periods (less than 1 year). But these methods also have several disadvantages, contamination by mites and other is drying/death of cells before use in future. In addition, the morphology and physiology of a cultured fungus may change over time. Although some fungus of Ascomycetes, including their mitosporic forms can survive in H_2O up to 10 years when stored at 20 °C [15]. Fully dried fungus culture keeping in view 10% moisture level is very good for the long term storage of *trichderma viride* at room temperature. The culture can remain viable for more than 10 years without changing its morphology and physiology. Liquid nitrogen preservation is more expensive, so no one can afford liquid nitrogen in bulk amount. Storage of fungus in liquid nitrogen is an effective way to preserve that cannot be lyophilized. It costs somewhat more than lyophilization. Liquid-nitrogen storage is recommended for the preservation of dictyostelids [32, 31], amoebae [11]. Zygomycetes including Entomophthorales [14], oomycetes [34] phytopathogenic fungi [11] and yeasts [17]. Ascomycetes which sporulate poorly in culture, and basidiomycetes that generally grow only as mycelia in culture can also be stored in liquid nitrogen. The major advantages of liquid nitrogen storage include prevention of increased genetic variability of culture; timesaving, reduced labor, prevention of culture loss from contamination; and increased assurance of long-term availability of cultures. Various alternative techniques for liquid-nitrogen storage, such as using plastic straws instead of vials or tubes, have been reported [32, 17]. Wood chip/ tooth pick culture is best for the wood fungi, as *Trichoderma* also infect or present on the surface of tree and can easily be isolated from trunk. Agar strip method is also very cheap and reliable for the preservation of *Trichoderma viride* culture. Nuzum (1989) described a method of vacuum-drying fungal cultures on agar strips. *Pythium, Rhizoctonia,* and some basidiomycete species survived 18 months with this method, whereas ascomycetes and their mitosporic forms survived from 3-5 years [31]. Skim milk method was developed by Perkins (1962) for *Neurospora* species. He found that [25, 28, 30] sporulating fungi protected by skim milk and stored on silica gel remain viable for 4-5 years. Spores and microcysts of other fungi can be preserved for up to 11 years on silica gel [40]. In general, viability after storage on silica gel depends on the strain of fungus and the medium on which it was grown before storage. The advantage of silica gel is that it prevents all fungal growth and metabolism. Some researchers use glass beads instead of silica gel. Silica gel can be used to preserve at least nonconidiating strains, but only with special effort, using suspensions of mulled mycelia or preparing as described by Metzenberg [39]. But when i used this method against Trichoderma viride, the spore colour totally changed and I also face lot of bacterial contamination during revival. After three months I get only bacterial colonies instead of *Trichoderma* culture. So this is not a good method for preservation because Trichoderma viride produce certain enzymes and antibiotic with react with milk lactose which favors the growth of fungi.

Fungi preservation in sterile sand is easy and cost-effective method and is appropriate for fungi such as *Rhizoctonia* [35], *Septoria* [33], and *Pseudocercosporella.* Dormancy caused by dryness can take time to develop, however, and morphological changes in some fungi have been recorded. Mostly basidiomycetes and some of the oomycetes were maintained on slants by oil drop cover method but growth rates of the cultures was slow as storage times increase [15, 4,

[11]. The major disadvantage of the oil overlay technique is that the fungi continue to grow, and thus, selection for mutants that can grow under adverse conditions may occur.

Drying process is most crucial because if it is too fast, the fungus can lose effectiveness and virulence or be killed; and if it is too slow it can become contaminated by other fungi or bacteria. Cultures must be checked periodically for contamination and desiccation. Both the viability and the morphological characteristics of each culture were observed. Filter paper method is a new technique is not only reliable; it is very inexpensive and easy to use in any laboratory with few resources.

Microbial cultures always checked for both purity and viability after storage. It is noteworthy that there was no detectable decrease in viability of any fungal strain. The viability and purity of the isolate was monitored and evaluated when the storage begins immediately after 3months of storage, 6 months, 9months, 12months, 15 months, 18months, 21months and after 24months of storage subsequently as shown in the table 1 and graph 1. The fungal subcultures were evaluated on the basis of whether or not they grew after the different storage at specific time interval and kept original morphological characteristic. This graph also predicts that drying method is best for the long time storage of *Trichoderma viride* at room temperature and all the strain have maintain their original macroscopic characteristics after storage.



(a) Plugs of T.V in water preservation

(b) Direct preservation/ serial preservation

(c) Spore suspension in water

(d) Filter paper preservation

(e) Sand preservation

(f) Tooth pick preservation

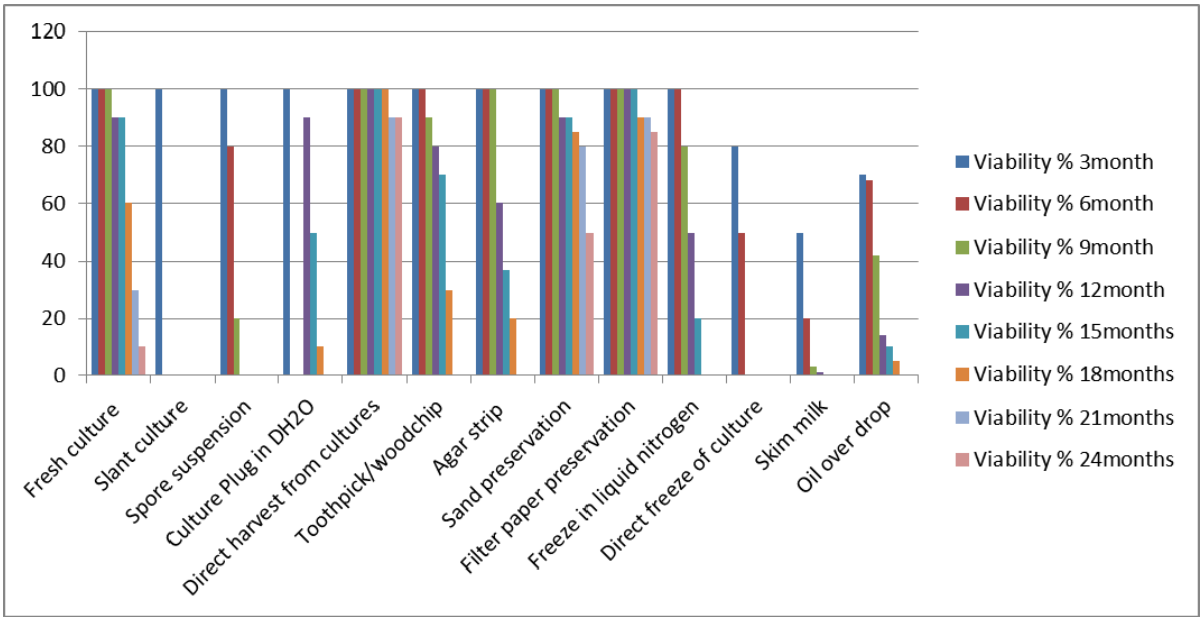
(g) Agar stirp preservation

(h) Slant method

Fig 1: Preservation of *Trichoderma viride* by using different methods (a-h)

Table 1: Percentage recovery of *Trichoderma viride* with the passage of time by adopting different preservation methods

Methods of preservation	Viability %							
	3month	6month	9month	12month	15months	18months	21months	24months
Fresh culture	100	100	100	90	90	60	30	10
Slant culture	100							
Spore suspension	100	80	20	0	0	0	0	0
Culture Plug in DH_2O	100			90	50	10	0	0
Direct harvest from cultures	100	100	100	100	100	100	90	90
Toothpick/woodchip	100	100	90	80	70	30	0	0
Agar strip	100	100	100	60	37	20	0	0
Sand preservation	100	100	100	90	90	85	80	50
Filter paper preservation	100	100	100	100	100	90	90	85
Freeze in liquid nitrogen	100	100	80	50	20	0	0	0
Direct freeze of culture	80	50	0	0	0	0	0	0
Skim milk	50	20	3	1	0	0	0	0
Oil over drop	70	68	42	14	10	5	0	0



Graph 1: Viability of *Trichoderma viride* by using different methods of preservation with three months interval for two years

4. Conclusion

Different methods for isolation and preservation were tested for optimization against *Trichoderma viride* fungus. Isolation method serial dilution with 0.5% twen-20 proved to be best for the isolation and for colony count. As in case of the preservation different methods were tried to check the long term preservation and revival of *Trichoderma viride* isolate. Sand preservation, filter paper, dry method and DDH_2O prove to be best for two years. All the above result shows that *Trichoderma viride* can survive in dry form/ inactive form for more than 2 years but drying is most crucial because if drying is too fast, fungus can loose its efficacy/ pathogenicity can cause the death of biocontrol fungal spores and if it is too slow it can become contaminated by other fungi or bacteria whereas in case of H_2O preservation cultures revived after 12months without losing its efficacy.

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