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VIABILITY ASSESSMENT OF PURE CULTURES OF MUCORALES PRESERVED AT 4°C

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A B S T R A C T

Mucorales (Zygomycetes) are among the most ancient groups of fungi. Although they are fast growing in culture but preservation of these cultures is rather sensitive as compared to other filamentous fungi. First Fungal Culture Bank of Pakistan maintained a large number of isolates of this group. Recently viability of members of mucorales was assessed that were isolated and purified from a variety of substrates between the years 2003-2006. Agar slants of pure cultures of these strains were preserved at 4 °C. These include seven species each of the genus *Absidia* and *Mucor,* four species of *Rhizopus,* two of *Cunninghamella* and one species belonging to genus *Phycomyces.* Results indicated that most of the strains lost their viability during storage period even none of the species from genus *Absidia* and *Phycomyces* survived. Therefore maintaining cultures of mucorales by this method is not recommended for their long term preservation. For long term preservation of fungal cultures, cryopreservation, lyophilization or silica gel preservation could be the alternate methods.

Keywords: Fungal culture, viability, stability, preservation

INTRODUCTION

Microbes are not only valuable due to their environmental, industrial and research applications but also they are gene pools and of course such gene pools must not be lost. Such unique significance suggested collection and preservation of microbes in systematic and organizational way (Watanabe *et al.,* 2004). Keeping the point in view, it is believed that microorganisms are cultural heritage as well as cultural property that must be transferred to next generation in healthy condition. Microbial culture collections are fulfilling this requirement all over the world. Generally the objectives of establishing the microbial culture collection centers are; deposition of microbial cultures, their supply to researchers and teachers and also to provide the training related to microbial culture isolation, identification and their maintenance. For the study of fungal biodiversity as well as systematic, preservation of fungal cultures are essential. Being a versatile group of organisms, fungi are cultivated and preserved as per requirement of a strain and also considering the cost and convenience of the method needed. However the core point is to ensure morphological, physiological, and genetic integrity as well as the viability of fungal cultures (Fennell, 1960; Smith and Onions, 1994). Generally cultures are maintained by continuous growing or sub-culturing after a specific time to fresh growth medium. Sub-culturing is for short term preservation and need a considerable labor and data recording. Also cultures must be regularly examined for microbial contamination, mites or desiccation. Other methods of culture maintenance include drying and freezing. Although drying is useful for long term preservation but works only with the fungal cultures that heavily sporulate for example *Aspergilli* and *Penicilli*. A cost effective and simple method for preserving relatively longer period is oil overlay on cultures growing on agar slants. Cultures can be preserved by this method for several years at room temperature however this method is best for mycelial or non sporulating cultures.

Institute of Mycology and Plant Pathology (presently Institute of Agricultural Sciences) University of the Punjab, Lahore, took initiative in laying the foundation of Fungal Culture Bank of Pakistan (FCBP)

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in 2003 with the aim to collect, identify, and preserve indigenous fungal flora of Pakistan; collaboration with local and international culture collection centers and providing the authentic identified fungal cultures to scientists for academic purpose. The objective behind this project was highly appreciated by the scientific communities and FCBP started working under the supervision of Dr. Jaffar Hussain Mirza (Late). During 10 years of FCBP establishment, progress is being made to build the inventory of fungal cultures, relevant literature and expertise needed to solve identification problems as well as up-gradation of FCBP. Present study is designed to investigate the viability of cultures of mucorales that were preserved at 4 °C.

MATERIALS AND METHODS

Strains used for this present study were isolated from a variety of sources, for example air, soil, water, different plant parts, dung etc. Information regarding type of substrate, site from where samples were collected and date of sampling was recorded.

Fungal growth and incubation conditions: For the isolation of fungal strains from different substrates, malt extract medium (2% malt extract, 2% agar) was used as described by Dhingra and Sinclair (1985). Strains were grown at 25 °C for 3-4 days.

Isolation techniques: Appropriate isolation method based on the nature of substrate was applied. Gravitational Petriplate method (Sen and Asan, 2009) for the isolation of airborne mucorales, and dilution plate method for soil mycoflora was employed. From the rotten fruits or vegetables, direct platting or wash off methods were tried (Hindi *et al.,* 2011). Coprophilous strains were isolated by incubating the dung of animals in moist chambers. All the fungal isolates were purified by single spore isolation technique (Choi *et al.,* 1999). Pure fungal cultures were used for identification and preservation.

Identification preservation of strains: Fungal cultures were identified morphologically and complete description of each isolate based on macro and micro morphological characters was prepared. Species were key out by comparing their description with relevant taxonomic literature (Mirza *et al.,* 1979). Malt extract agar slants of pure cultures of fungal strains were preserved at 4 °C for future study.

Viability assessment of strains: Inoculums from the culture tubes that were preserved at 4 °C were transferred aseptically onto the surface of fresh malt extract agar (MEA) slants. Inoculated culture tubes were preserved at 25 °C for 3 - 4 days. Pure cultures were examined under stereoscope for mites and other microbial contamination. Data was maintained for all viable and not-viable fungal strains.

RESULTS

FCBP maintains collection of more than 1200 cultures. Mucorales isolated and preserved during the years 2003-2006 were tested for their viability or stability in cultures. Mucorales are considered to be more susceptible for preservation due to their coenocytic hyphae as well as long mycelium. The members of order Mucorales studied for this present viability survey were isolated from a great variety of substrates. From their preservation till the end of 2008 these strains were sub-cultured on fresh MEA slants after every three months and always rejuvenated from the previous cultures.

Viability of *Absidia* species: Viability of twenty isolates of genus *Absidia* belonging to seven different species namely; *A. blakesleeana, A. corymbifera, A. cylindrospora, A. hesseltinii, A. heterospora, A. ramosa, A. repens,* and two isolates that were not identified upto species level was tested. These isolates of *Absidia* were purified from a great variety of substrates and preserved during the period of 2004-2005 (Table 1) hence belonging to age group 8 - 9 years. None of the isolate from twenty different cultures was found to be living.

Viability of *Mucor* species: Strain record of FCBP documented 13 isolates of taxon *Mucor*. Data represented in the Table 2 demonstrated that 11 isolates of *Mucor* out of 13 could not be revived from their stock cultures. These died *Mucor* strains belong to seven different species viz. *M. attenuates*, *M. corticolus, M. hiemalis, M. philippowi, M. pusillus*, *M. subtilissimus* and *M. varians*. These cultures were processed and preserved on agar slants during the years 2003-2005. Although belonging to same age group, two isolates, *Mucor* sp. (FCBP041) isolated from *Glycine max* seed and *Mucor* sp. (FCBP042) purified from *Luffa cylindrica* seed survived.

Viability of *Cunninghamella* species: Viability of fifteen strains of genus *Cunninghamella* belonging to two species i.e. C. *echinulata* and *C. elegans* were assessed. When these strains were transferred to fresh media

slants that were fresh by the end of 2008, only three strains FCBP0103, FCBP0104 and FCBP0462 managed to grow and rest of the strains (Table 3) found to be non-viable.

Viability of *Phycomyces* species: All isolates of *Phycomyces blakesleeanus* (Table 4) lost their viability when tested recently.

Viability of *Rhizopus* species: Sixteen isolates belonging to four different species of genus *Rhizopus viz., R. arrhizus, R. nigricans, R. oligosporus, R. oryzae* and two unidentified species were studied for their potential viability when maintained at 4 °C. Detail information of the dead strains is summarized in the form of Table 5. Only three strains (unidentified species) of *Rhizopus* i.e. FCBP039 isolated from *Glycine max* seed, FCBP040 *Helianthus annuus* seed and FCBP0531 isolated from *Zea mays* seed were manage to grow on fresh agar medium.

Table 1. Details of non-viable FCBP strains belonging to genus *Absidia.*

Sr No.	Strain ID	Fungus	Substrate/locality	Preservation date
1	FCBP449	A. blakesleeana	Air, Lahore	26.06.05
2	FCBP0283	A. corymbifera	Buffalo dung, Lahore	11.07.04
3	FCBP0288	A. corymbifera	Buffalo dung, Lahore	10.08.04
4	FCBP0302	A. corymbifera	Horse dung, Lahore	15.08.04
5	FCBP0304	A. corymbifera	Leather, Lahore	01.09.04
6	FCBP0310	A. corymbifera	*Grewia asiatica* fruit, Lahore	26.08.04
7	FCBP0214	A. cylindrospora	Cotton cloth, Lahore	16.03.04
8	FCBP0554	A. cylindrospora	*Dabergia sissoo* root, Lahore	12.12.05
9	FCBP0314	A. hesseltinii	Horse dung, Lahore	16.08.04
10	FCBP 0266	A. heterospora	Field soil, Lahore	05.06.04
11	FCBP0286	A. ramosa	Buffalo dung, Lahore	28.08.04
12	FCBP0299	A. ramosa	Goat dung, Lahore	15.07.04
13	FCBP0306	A. ramosa	Donkey dung, Lahore	25.07.04
14	FCBP0308	A. ramosa	Cow dung, Lahore	25.08.04
15	FCBP0307	A. ramosa	Horse dung, Lahore	28.08.04
16	FCBP0303	A. repens	Bread, Lahore	15.08.04
17	FCBP0358	A. repens	*Cucumis melo* pulp, Lahore	25.05.05
18	FCBP0272	A. repens	Field soil, Lahore	05.06.04
19	FCBP0334	Absidia *sp.*	Picture wall, Lahore Fort, Lahore	20.10.04
20	FCBP0332	Absidia *sp.*	Horse dung, Lahore	03.08.04

Table 2. Details of non-viable FCBP strains belonging to genus *Mucor.*

Sr No.	Strain ID	Fungus	Substrate/locality	Preservation date
1	FCBP485	*M. attenuatus*	*Grewia asiatica*, Lahore	15.07.05
2	FCBP0135	*M. corticolus*	*Valeriana walichi* leaf, Donga Gali	30.09.03
3	FCBP0398	*M. corticolus*	Soil, Kasur	07.04.05
4	FCBP0531	*M. hiemalis*	*Dalbergia sissoo* root bark, Lahore	01.12.05
5	FCBP0425	*M. hiemalis*	*Cucumis melo* pulp, Lahore	17.05.05
6	FCBP0388	*M. philippowi*	*Brassica rapa* root, Lahore	28.03.05
7	FCBP0431	*M. pusillus*	*Eucalyptus citriodora* soil, Lahore	27.05.05
8	FCBP0563	*M. subtilissimus*	*Spinacia oleracea* leaf, Lahore	14.12.05
9	FCBP0465	*M. varians*	*Solanum tuberosum* tubers, Lahore	07.07.05
10	FCBP0532	*M. varians*	*Zea mays* seed, Okara	09.12.05
11	FCBP0300	*Mucor* sp.	Cow dung, Lahore	01.09.03

Table 3. Details of non-viable FCBP strains belonging to genus *Cunninghamella.*

Sr No.	Strain ID	Fungus	Substrate/locality	Preservation date
1	FCBP0105	*C. echinulata*	*Pinus* sp. wood, Nathia Gali	27.08.03
2	FCBP0132	*C. echinulata*	*Acacia arabica* root, Lahore	30.09.03
3	FCBP0347	*C. echinulata*	*Erythrina suberosa*, Lahore	18.11.04
4	FCBP0138	*C. echinulata*	*Acacia arabica* root, Lahore	13.10.03
5	FCBP0112	*C. elegans*	*Acacia arabica* soil, Sialkot	06.09.03
6	FCBP0171	*C. elegans*	Decaying wood, Lahore	22.11.03
7	FCBP0172	*C. elegans*	Air, Lahore	22.11.03
8	FCBP0199	*C. elegans*	Air, Lahore	20.01.04
9	FCBP0218	*C. elegans*	Soil, Lahore	20.03.04
10	FCBP0463	*C. elegans*	Soil, TobaTek Singh	06.07.05
11	FCBP0504	*C. elegans*	*Dalbergia sissoo* soil, Jhelum	23.08.05
12	FCBP0464	*Cunninghamella* sp.	Soil, Jhang	07.07.05

Table 4. Details of non-viable FCBP strains belonging to genus *Phycomyces.*

Sr No.	Strain ID	Fungus	Substrate/locality	Preservation date
1	FCBP0227	*P. blakesleeanus*	*Litchi chinensis* soil, Lahore	03.04.04
2	FCBP0230	*P. blakesleeanus*	Paper slime, Sheikhupura	24.02.04
3	FCBP0270	*P. blakesleeanus*	Polluted water, Lahore	05.06.04
4	FCBP0315	*P. blakesleeanus*	*Prunus persica* Fruit, Murree	26.09.04
5	FCBP0354	*P. blakesleeanus*	*Psium sativum*, Lahore	17.12.04
6	FCBP0357	*P. blakesleeanus*	Horse dung, Lahore	03.01.05

Table 5. Details of non-viable FCBP strains belonging to genus *Rhizopus.*

Sr No.	Strain ID	Fungus	Substrate/locality	Preservation date
1	FCBP0251	*R. arrhizus*	*Helianthus annuus*, seed, Lahore	26.05.04
2	FCBP0255	*R. arrhizus*	*Malpighia emarginata* fruit, Lahore	10.06.04
3	FCBP0269	*R. arrhizus*	*Lathyrus sativus* seed, Lahore	05.06.04
4	FCBP0525	*R. arrhizus*	*Zea mays* seed, Lahore	12.09.05
5	FCBP0279	*R. arrhizus*	*Lycopersicon esculentum* fruit, Lahore	10.07.04
6	FCBP0476	*R. arrhizus*	Soil, Lahore	11.07.05
7	FCBP0555	*R. nigricans*	*Dalbergia sissoo* root, Lahore	12.12.05
8	FCBP0257	*R. oligosporus*	*Prunus persica* Fruit, Lahore	15.05.04
9	FCBP0563	*R. oligosporus*	*Zea mays* seed, Lahore	14.12.05
10	FCBP0256	*R. oligosporus*	*Lathyrus sativus* seed, Lahore	15.05.04
11	FCBP0268	*R. oryzae*	*Brassica* sp. leaf, Lahore	05.06.04
12	FCBP0250	*Rhizopus* sp.	*Brassica oleracea* leaf, Lahore	26.05.04
13	FCBP0399	*Rhizopus* sp.	Air, Lahore	27.03.05

DISCUSSION

Subculturing is the oldest method of preserving microbial cultures but still is in use in most of the microbiological laboratories. Strains of Mucorales deposited in FCBP were preserved at 4 °C and revived by continuous growth or sub-culturing. The strategy was in agreement with espinel-ingroff *et al.* (2004) who suggested viability and the stability of the fungal cultures in collection should be regularly monitored during the preservation period. In subculturing a small inoculum from the culture is transferred onto the fresh gar slant and after growth such slants are kept in a refrigerator. The cultures are periodically transferred to fresh media to retain viability hence are maintained by giving alternate periods of growth and storage. However, to increase the viability of cultures, and decrease the frequency of subculture it is important to use an appropriate medium as well as storage temperature (Winters & Winn, 2010; Prakash *et al.,* 2013). Members of Order Mucorales are rapid growing and have long aseptate mycelia. Hence there is aways

problem in maintaining the cultures of this group by single method. Benny (2008) reported that even preserved by cryoprotectants, cultures of zygomycetes survive only for 3-5 years.

For present study cultures of fungal strains stored in a refrigerator and sub-cultured on fresh growth medium at regular interval of six months from 2003 to 2008 . Sub culturing is a short term preservation method and is unable to preserve the cultures for a long period. Storing fungal cultures at 4 °C although simple, inexpensive and widely used technique but do not ensure the long viability and stability of cultures. During preservation actually cell dormancy is created so remains viable but its cell metabolism is slowed down or completely halt (Lennon and Jones, 2011; Hoefman *et al.,* 2012). Most of filamentous fungi can survive at least 1-2 years at 4 °C (von Arx and Schipper 1978). Vigorous, sporulating cultures also can be sealed tightly and saved in a freezer at -20°C (Carmichael, 1962) or stored at -70°C (Pasarell and McGinnis 1992) to improve and increase the time period required for reculturing. Phenotypic and genotypic characters of stored fungi may affect by the choice of preservation method (Ward *et al.,* 2001). It has been reported that sub-culturing of active cultures have more chances of mutations than lyophilization and cryopreservation (Simione, 1992; Lang and Malik, 1996; Muller *et al.,* 2007). Therefore alternate methods, for example cryopreservation (Hwang *et al.,* 1976; Stalpers *et al.,* 1987, Prakash *et al.,* 2013) should be employed for long term preservation of cultures.

CONCLUSION

Results of present study have shown that maintaining strains belonging to order mucorales at 4 °C is although simple and rapid method but not reliable for long term preservation of this fungal group.

REFERENCES

Benny, G. L. 2008. The methods used by Dr R.K. Benjamin and other mycologists to isolate Zygomycetes. Aliso, 26: 37–61.

Carmichael, J. W. 1962. *Chrysosporium*. Canad. J. Bot. 40: 1137–1173.

Choi, Y. W., K. Hyde, W.H. Ho. 1999. Single spore isolation of fungi. Fungal Div. 3: 29-38.

Dhingra, O. D., J. B. Sinclair. 1985. Basic Plant Pathology Methods. CRC press, Inc. of Boca Raton, Florida.

Espinel-Ingroff, A., D. Montero, E. Martin-Mazuelos. 2004. Long-term preservation of fungal isolates in commercially prepared cryogenic microbank vials. J. Clin. Microbiol. 42: 1257-1259.

Fennell, D.I. 1960. Conservation of fungus cultures. Bot. Rev. 26: 79-141.

Hindi, R.R., A.R. Al-Najad, S. A. Mohamed. 2011. Isolation and identification of some fruit spoilage fungi: Screening of plant cell wall degrading enzymes, African J. Microbiol. Res. 5: 443-448.

Hoefman, S., K. Van Hoorde, N. Boon, P. Vandamme, P. De Vos, K. Heylen. 2012. Survival or revival: long-term preservation induces a reversible viable but non-culturable state in methane oxidizing bacteria. PLoS ONE. 7: 1–9.

Hwang, S., W. F. Kwolek, W. C. Haynes. 1976. Investigation of ultra low temperature for fungal cultures. III. Viability and growth rate of mycelial cultures following cryogenic storage. Mycologia. 68: 377-387.

Lang, E., K. A. Malik. 1996. Maintenance of biodegradation capacities of aerobic bacteria during long-term preservation. Biodegrad. 7: 65–71.

Lennon, J. T., S. E. Jones. 2011. Microbial seed banks: the ecological and evolutionary implications of dormancy. Nat. Rev. Microbiol. 9: 119–130.

Mirza, J. H., S. M. Khan, S. Begum, S. Shugufta. 1979. Mucorales of Pakistan. Uni. of Agric., Fsd, Pakistan.

Muller, J., J. G. Day, K. Harding, D. Hepperle, M. Lorenz, T. Friedl. 2007. Assessing genetic stability of a range of terrestrial microalage after cryopreservation using amplified fragment length polymorphism (AFLP). Am. J. Bot. 94: 799–808.

Pasarell, L., M. R. McGinnis. 1992. Viability of fungal cultures maintained at −70 °C. J. Clin. Microbiol. 30: 1000-1004.

Prakash, O., Y. Nimonkar, Y. S. Shouche. 2013. Practice and prospects of microbial preservation. FEMS Microbiol. Lett. 339: 1–9.

Sen, B., A. Asan. 2009. Fungal flora in indoor and outdoor air of different residential houses in Tekirdag City (Turkey): Seasonal distribution and relationship with climatic factors. Environ. Monitoring Assess. 151: 209-219.

Simione, F.P. Jr. 1992. Key issues relating to the genetic stability and preservation of cells and cell banks. J. Parenter Sci. Technol. 46: 226–232.

Smith, D., A. H. S. Onions. 1994. The preservation and maintenance of living fungi. Second edition. IMI

Technical Handbooks. Wallingford, UK: CAB International.

Stalpers, J.A., A. De Hoog, I. J. Vlug. 1987. Improvement of the straw technique for the preservation of fungi in liquid nitrogen. Mycologia. 79: 82-89.

Von Arx, J.A., M. A. A. Schipper. 1978. The CBS fungus collection. Adv. App. Microbiol. 24: 215-236.

Ward, N., J. Eisen, C. Fraser, E. Stackebrandt. 2001. Sequenced strains must be saved from extinction. Nature. 414: 148.

Watanabe, M. M., K. Suzuki, T. Seki. 2004. Innovative Roles of Biological Resource Centers. In Proceedings ICCC10. Tsukuba, Japan.

Winters, R.D., W. C. Jr. Winn. 2010. A simple effective method for bacterial culture storage: a brief technical report. J. Bacteriol. Virol. 40: 99–101.