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Sampling, baiting techniques and culture methods for the study of Watermoulds

S. K. Prabhuji*, Ausaf Ahmad[1], Gaurav K. Srivastava & Richa Srivastava

**Biotechnology and Molecular Biology Centre, M.G. Post Graduate College, Gorakhpur – 273 001, India*
[1]Amity Institute of Biotechnology, Amity University, Lucknow, India

***e-mail :** *shaktiprabhuji@rediffmail.com*

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ABSTRACT

The isolation and procuring pure cultures of the watermoulds has been a tedious process and therefore, requires special attention. Various methods of sample collection and isolation of watermoulds from water bodies and soils together with pathogenic conditions have been reviewed. Culture methods and techniques for obtaining unifungal contaminant-free cultures have also been discussed.

Keywords : Sampling and culture techniques, isolation methods, watermoulds.

Watermoulds, particularly the Aquatic Phycomycetes, form part of the rich aquatic fungal flora of ponds and rivers and the soils of nearby areas. Among aquatic phycomycetes the members of family: Saprolegniaceae are of special interest because of their facultative parasitic nature, i.e., they are saprophytes as well as parasites of fishes under certain set of conditions. The isolation and procuring pure cultures of the watermoulds has been a tedious process and therefore, requires special attention.

The first collections of specimens of watermoulds were from fish and floating dead flies (Ledermüeller 1760, Wrisberg, 1765, Spallanzani 1777, Schränk 1789). Later, proper sampling methods and baiting techniques were developed by the aquatic mycologists working on watermoulds (Petersen 1909, 1910, Minden 1916, Johnson 1956, Durbin, 1961, McKay 1967, Seymour 1970, Stevens 1974, Dick 1976). Most of the aquatic mycologists working on watermoulds prefer the simple aliquot water sampling technique in which 1-2 litres of water is collected from the sampling site, thoroughly mixed, and 10-15 ml aliquots are placed in different Petri dishes baited with boiled hempseed cotyledons. These are incubated for 2 – 4 days to observe the growth of watermould hyphae (Srivastava 1967 a, b, Milanez 1967, Lund 1978, Khulbe 1980, Prabhuji 2005). For soils, the sampling technique and isolation methods are slightly different. About 10 g of dry or wet soil is collected and placed in a Petri-plate and covered with pre-distilled, ion-exchanged water to a depth of 1 cm and mixed properly. Following the settlement of particulate matter, it is baited with boiled hemp-seed cotyledons and incubated at 18–20ºC for 2–4 days prior to the observation for the growth of any watermoulds (Johnson 1956, Dick & Newby 1961, Seymour 1970). The trapping technique, devised by Cooke & Bartsch (1959, 1960), has been generally a successful method for collecting saprolegnoid fungi directly from aquatic habitats.

Basically, the following three culture methods are used to study the watermoulds :

i. Water and soil samples are brought to the laboratory and organic substrates (dead flies or seeds) are added.
ii. Baits are submerged in the aquatic habitats and, following a particular incubation period there, are examined in the laboratory.
iii. Naturally submerged or floating organic debris is examined directly with or without prior incubation period.

Watermoulds have been isolated from water–mostly stagnant water; and from inundated, wet or dry soils, and therefore, sampling techniques must be considered separately for water bodies and soils.

FROM WATER SAMPLES

Most of the aquatic mycologists working on watermoulds prefer the simple water sampling technique in which 1-2 litres of water is collected from

the sampling site, thoroughly mixed, and 10-15 ml aliquots are placed in different Petri dishes baited with boiled hempseed or water-melon cotyledons (for the isolation of keratinophilous forms defatted and boiled hairs and nails are used as baits). These are incubated for 2 – 4 days to observe the growth of watermould hyphae (Khulbe 1980, Lund 1978, Milanez 1967, Prabhuji 2005, Srivastava 1967 a, b).

Park (1972) tried to collect quantitative data on the occurrence of zoosporic fungi by dilution plating and particle plating. However, neither the dilution (1:1, 00,000 to 1:7, 00,000) nor the particle plating approach yielded members of the Saprolegniaceae. While investigating the occurrence of saprolegniaceous fungi in a river system, Ho (1975) prepared discs of hempseed extract agar, put these into Petri dishes containing water samples and incubated them at 18 - 22ºC. The number of such discs that supported growth of watermould hyphae was taken as the "isolation percentage". Similarly, in a study of the frequency of watermoulds in a Newfoundland river, Maestres (1977) centrifuged 20 litres of river water during each sampling, and the concentrate was used to prepare various dilutions with sterile distilled water. She plated these dilutions on nutrient agar, incubated the plates at 18 - 20ºC and analyzed the yield quantitatively to relate approximate number of propagules in the original volume of water sample. The results, however, could not be considered reliably quantitative. Willoughby (1962, 1965) collected a large volume of water (5 – 10 liters) that was shaken thoroughly, and aliquots were mixed with molten oatmeal agar and poured into sterile Petri dishes. As soon as the medium solidified, it was cut into eight equal parts; each section was placed in a new Petri dish containing sterile distilled water and incubated for 24-48 h. The edge of each part, following incubation, developed a fringe of watermould mycelia. Willoughby observed that in each agar section the morphology of the mycelium was consistent over the entire surface of that section, indicating that the fringe mycelium came from a single propagule. In cases where more than one species was isolated on a single section, Willoughby introduced a "correction factor" (the number of sections that could be expected to harbor more than one propagule) which he developed and tested using the spores of a non-sexual *Saprolegnia* and claimed to be able to calculate with reasonable accuracy the number of propagules per liter of water sample. Later, Thakurji & Dayal (1966) combined Willoughby's quantitative technique with sample dilution.

To overcome problems with assessing the distribution of watermoulds within various vertical strata of a water body, Johannes (1957) devised a novel bait submergence technique. He stuck ant eggs onto thick paper slips covered with a paraffin layer and attached these slips at measured distances on a pole. The poles were submerged upright in such a fashion as to position the eggs at various depth intervals (Fig. 1). Following an average submergence time of five days, the eggs were removed and transferred to sterile Petri dishes containing sterile distilled water and observed for growth of watermoulds.

FROM SOIL SAMPLES

The earliest investigation on the collection and isolation of watermoulds from soils is that of Raper (1928), following Harvey (1925), who tried to determine the abundance of these fungi in a given area and volume of soil. He simply prepared suspension of known quantities of soil with distilled water and, following the settling of soil particles, baited the supernatant liquid for isolates. He obtained data showing the frequency with which particular species were isolated from various sites.

Dick & Newby (1961) and Dick (1962), investigating the distribution and abundance of Saprolegniaceae in the soils of southeastern England, developed a "quadrat sampling" method. A quadrat with an area of 90 cm^2 was permanently fixed on a particular soil sampling area, and divided it into 16 square subdivisions. From each subdivision soil samples were taken from five fixed points and baited for watermoulds by conventional methods. Prabhuji (1979, 1984a, b) has also employed this method for his studies on some lower fungi occurring in soils of Gorakhpur (India). Dick (1966) developed a technique for quantitatively determining the watermould populations in soil based on fractionation of the samples. A soil sample was mixed with water, stirred mechanically, and three portions were selected for plating and baiting: the coarse plant material, supernatant fluid, and the slurry. The supernatant portion yielded a species composition unlike that recovered from the slurry; by contrast, the spectrum

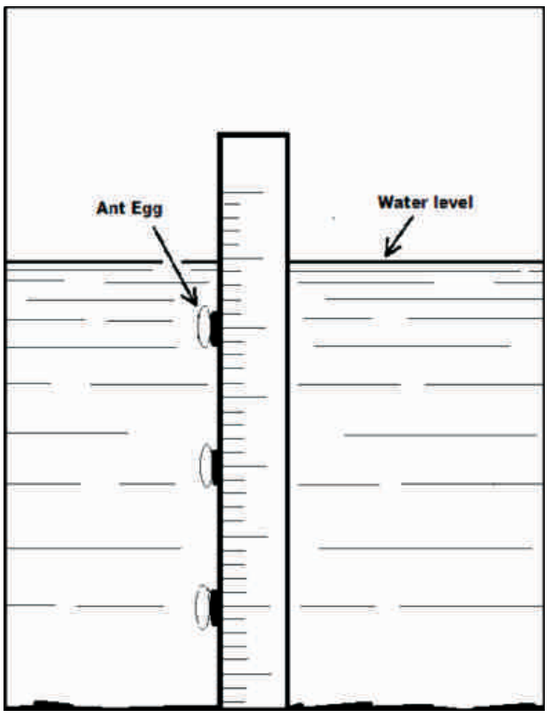

Fig. 1 – Assessing the distribution of watermoulds within vertical strata of a water body (after Johannes 1957)

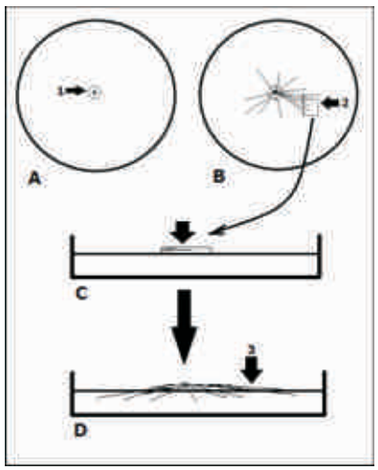

Fig. 2 A – D: Method of obtaining contaminant-free watermould culture.

A: Inoculum in the centre of glucose-glutamate agar medium and a "van Tieghem cell" or "sterling silver ring" (1) is placed around it.

B: agar block (2) is cut outside the ring containing hyphal tips.

C: the cut block of agar is transferred to new medium upside down.

D: watermould grows and contaminant-free hyphae (3) develop. (After Raper 1937, Powell et al. 1972)

of watermould species found in the coarse plant material was very similar to that in the baited slurry.

After the initial culturing period, the soil samples may be kept and simply allowed to dry out in the Petri dishes with covers in place. These dried samples can again be wetted and baited for culturing. Occasionally such second growth cultures yield watermould species not recovered initially. However, the longevity of some watermoulds is drastically shortened by desiccation (Remy 1950, Apinis 1964).

SAMPLING OF PATHOGENIC FORMS

Majority of the watermoulds are facultative parasites and, therefore, are the causal organisms of diseases of aquatic animals exhibiting symptoms like cottony outgrowths on the body surface. The pathogenic forms are isolated from the particular hosts just like the isolation from water samples, however, the basic difference lies in taking the inoculum from the affected regions or the symptomatic areas and its transfer onto the boiled hemp-seeds or melon-seeds or synthetic media. The watermoulds, particularly *Aphanomyces* sp., have also been found to attack a variety of vascular and non-vascular plants. The plant pathogenic watermoulds require special attention. Small bits of affected tissues are repeatedly washed with sterile distilled water and kept in moist place for 1–2 days with which extrametrical hyphae will appear. These washed pieces are blotted on to sterile filter paper and transferred to suitable media; and bacteria-free cultures may be obtained by using Brown's method (Drechsler 1929).

CULTURE METHODS

CULTURE ON SEMISOLID MEDIA

Usually unifungal and contaminant-free cultures are maintained on chemically defined media for a longer duration. However, this method is also used to isolate watermoulds as well as for preparing contaminant-free cultures. Different culture media (Tables 1 – 6) are generally used for culturing the watermoulds, however, best results may be obtained using glucose-glutamate medium (Table 1) and nutrient agar (Table 3).

Table 1—Glucose-glutamate agar medium (Seymour 1970)

Particulars	Amount (mg)
EDTA	200
K_2HPO_4	87
KH_2PO	68
$MgCl_2.6H_2O$	160
CaCl	66
ZnCl	40
$FeCl_3.6H_2O$	1.3
dl-methionine	50
Monosodium glutamate	500
d-glucose	3000
$MnCl_2.4H_2O$	75

Dissolved ingredients in 500 ml distilled and deionized water, brought final volume to 1 lit; Adjusted pH to 6.5 with KOH. Added 1.5 g Difco Agar for culturing; 15 g for isolation.

Table 2 – Glycerin-peptone agar medium

Particulars	Amount
Glucose	20 g
Peptone	10 – 15 g
Glycerin	5 ml
Beef serum	10 ml
Agar-agar	12 – 15 g

Dissolved ingredients in 500 ml distilled and deionized water, brought final volume to 1 lit;
Adjusted pH to 6.5 with KOH.

Table 3 – Nutrient agar medium

Particulars	Amount
Peptone	5.0 g
Beef Extract	3.0 g
Sodium Chloride	5.0 g
Agar-agar	12 – 15 g

Dissolved ingredients in 500 ml distilled and deionized water, brought final volume to 1 lit; Adjusted pH to 7.0.

Table 4 – Potato-dextrose agar medium

Particulars	Amount
Peeled and cut potato	250 g
Dextrose	20 g
Agar-agar	12 – 15 g

Small pieces of potato were boiled to softness in 500 ml of distilled water, filtered through muslin cloth, dissolved other ingredients and brought final volume to 1 lit; Adjusted pH to 6.5.

Table 5 – Oat meal agar medium

Particulars	Amount
Oat meal	60 g
Agar-agar	20 g

Oat meal was boiled to softness in 500 ml of distilled water, filtered through muslin cloth, dissolved agar-agar and brought final volume to 1 lit; Adjusted pH to 6.5.

Table 6 – Czepak-Dox agar medium

Particulars	Amount
Sodium Nitrate	2.0 g
Potassium dihydrogen phosphate	1.0 g
Magnesium Sulphate	0.5 g
Potassium Chloride	0.5 g
Ferrous Sulphate	0.01 g
Sucros	30.0 g
Agar-agar	20.0 g

Dissolved ingredients in 500 ml distilled and deionized water, brought final volume to 1 lit; Adjusted pH to 7.3 with KOH.

UNIFUNGAL CONTAMINANT-FREE CULTURES

In general, the various methods of isolation and culture involve either some mechanical manipulation or the incorporation of a chemical adjuvant to suppress the contaminants. DeBary (1884) who was among the first few to realize the need for pure cultures; mentioned methods very briefly and not clearly enough that they could be duplicated precisely. Trow (1895) provided a well-illustrated account of the method and obtained what would now be recognized as a unifungal culture by

placing a single hypha bearing oögonium in a drop of boiled water and adding a fly-leg to it.

Weston (1917) described a technique that provided the basic manipulation for development of single spore culture. Using an atomizer, he sprayed a spore suspension onto beef-extract agar, then located single germinated spores and transferred them to new media. He also obtained unifungal and contaminant-free cultures by serially diluting suspensions of gemmae. Afanasiev (1948) achieved the same results with spore suspensions. Brown (1924) employed a new technique and inoculated the centre of the surface of the plated nutrient agar. After 5-6 days, the medium was cut in advance of the hyphal tips, and the agar turned over. Hyphae growing into the agar below the colony could then be picked out along with the adherent medium and transferred to new plate. Modification of this technique was used by Johnson (1956) who cut plates of 2% water agar into triangular sections and placed a bit of inoculum under each section. Contaminant-free hyphae grew into these agar blocks and subsequently could be transferred to water cultures.

Raper (1937) devised a method combining a mechanical barrier to the spread of contaminating organisms and inducing growth of hyphae into the agar medium. A glucose-glutamate medium (Table 1) poured aseptically half-full into sterile Petri plate; a van Tieghem cell (a glass cylinder approximately 12 mm diameter and 12 mm deep) is placed on the semisolid surface, after dipping in 95% alcohol and touched to a flame to burn off alcohol, and gently pushed about half way into the medium. Spores or hyphal tips are then placed within the ring and incubated it at 18 - 20ºC. Hyphae grow inside the medium outside the ring. Small blocks of the medium are cut outside the ring containing hyphal tips and transferred onto new media to get contaminant-free cultures. Powell *et al.* (1972) used sterling silver rings of 15 mm diameter and 10 mm deep, instead of glass, for the same purpose. Blank & Tiffney (1936) used UV-irradiated (3 hours irradiation prior to inoculation) plated media and noted that the bacterial populations around the growing hyphae were substantially reduced.

We, on the basis of our personal experience, recommend certain effective methods of freeing watermould cultures from bacterial contamination which are less cumbersome and less time consuming. Usually sporangia are initiated in 24 h old cultures in sterilized distilled water. Single spores (spore balls in case of *Achlya* and *Aphanomyces*) are aseptically taken out using sterile glass capillary tubes under stereoscopic dissecting microscope and transferred to media or on the baits (boiled hempseed cotyledons) in sterile distilled water. Other easy to perform methods (Fig. 2) are given by Blank & Tiffney (1936) and Powell *et al.* (1972).

Following isolation of watermoulds, in gross culture, the most important part is to safeguard it from bacterial contamination. For this, most of the aquatic mycologists suggest the use of antibiotics and other chemicals like Potassium tellurite as a culture adjuvant. These adjuvants have been observed to suppress the sporulation in watermoulds and therefore, their use has been criticized (Dick 1976). Sub culturing at short duration, mostly fortnightly or monthly, will be suitable to overcome these problems for the study of watermoulds.

CONCLUSIONS

The isolation and procuring pure cultures of the watermoulds has been a tedious process and therefore, requires special attention. The isolation techniques should be carefully managed separately from water and soil samples and pathogenic samples together with the sampling methods. Precision is required in obtaining bacteria-free cultures of watermoulds and use of antibiotics should be avoided as far as possible.

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