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Surface spreading sporulation (SSS) technique for microfungal purification

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Abstract
A technique was used for the purpose of separating microscopic filamentous molds. The primary aim was to establish a method that will conveniently distribute vegetative spores across the surface of a culture medium allowing easy detection of desired strain. A contaminated Sabouraud Dextrose Agar (SDA) cultured plate previously used for isolation at 25 ºC after 5 days was used for the purpose of this investigation. The culture plate showed a mixture of probable isolates after incubation period signifying a case of cross contamination. Following standard laboratory procedure, the following isolates: *Aspergillus Niger, Aspergillus terreus, Penicillium funiculosum and Penicillium chrysogenum* were recovered using the surface spreading technique. The investigation confirmed the level of microbial co-existence which is often difficult to manage in cases of pollution caused by molds due to their growth pattern. On the other hand, the practise provided a simple and effective application in handling purification of multicellular sporulation.

Keywords: nutrients, cross contamination, purification, molds

Introduction
Green computing is the study in which discarding, recycling and building of computers and Molds are microscopic multicellular filamentous fungi with unique structural and functional properties. Their growth allow direct contact with the environment and based on different classification they form various types of effective nutritional habits enabling them cover a large surface area (Sun *et al.* 2019) [15]. These characteristics make diffusion of nutrient easy enabling the mold adapt to dehydration. Research activities have shown industrial relevance which has been justified in the proffering of enzymes, primary and secondary metabolites (Zhou *et al.* 2021; Suurbaar *et al.* 2017, Sunesen and Stahnke, 2003) [22, 17, 16] making them very significant to industrialization.
Their ability to grow in extreme conditions has been related to the protective structured vegetative spores, which provide high level of modifications for survival (Selbmann *et al.* 2005) [14]. Spores of molds have been recorded to be small in sizes and easily propagated by wind, rain, insects, animals, and man constituting frequent level of cross contamination (Maharachchikumbura *et al.* 2016) [9]. Furthermore, with relevance to their sizes and growth pattern; purification for the purpose of strain identification is often a challenge because: i. their filaments often easily growing interwoven forming a web across any environmental surface and ii. Undetected vegetative spores passing through filtration medium causing progressing contamination. Several techniques have been used for the purpose of micro-fungi purification and aligned to methods of isolation from samples with common principles involving: i. single spore isolation method often employed when the fungus produces spores, which are coloured and bold, and ii. Single hyphal tip method, often used when purifying fungi which either do not produce spores or produces small and hyaline spores (Fawzi, 2011) [2]. Either way, purification still remains a challenge resulting to consistent environmental pollution. The main objective of this investigation was to develop a simple and cost effective technique which overcomes the selection between spore or hyphae producers, and also reduce the time involved in the process of mold identification.

Materials and Methods
Specimen Collection
A contaminated culture plate was retrieved from a research institute located at longitude: 77°10′2.78″E (77.167441) and latitude: 28°31′43.82″N (28.52884) in the Northern part of India.

A Sabouraud Dextrose Agar (SDA) culture medium had been used for the isolation of microscopic filamentous fungi from soil sample at 25 ºC for 5 days. Further sub-culturing techniques had been carried out to separate the isolates for identification using the standard methods but there was a progressive recording of cross contamination which led to the application the surface spreading procedure.

Media Preparation

The following ingredients; Glucose 40 gm, Peptone10 gm, Agar15 gm weighed and dispensed into 1000 ml of distilled water. The mixture was stirred thoroughly to form a homogenous solution and finally sterilize at 110 ºC for 10 minutes, 15psi using a pressure steam sterilization autoclave. To further reduce rapid growth of hyphae and inhibit contaminating microorganisms, 20 gm of chloramphenicol was added to the solution at a final pH 5.6 +/- 0.2 at 25 ºC. 20 ml of the prepared medium was dispensed into sterile 15 × 100 mm Petri Dishes, and then allowed to solidify. The fresh culture plates were used for purification and further characterization of probable strains.

Surface Spreading Technique

Due to the interest in research diversification, an effective method was carried out to recover the probable isolates from the contaminated culture (SDA) plate. Using a sterile inoculating loop, a microgram unit from the sample was taken, turned over allowing the macroscopic structure direct contact with the surface of a freshly prepare culture medium earlier described above. The inoculating loop was used to spread the microgram unit of sample across the surface of the medium and it was later incubated for 72hr at 25 ºC. After the incubation period, the observed pure isolates were sub-cultured onto separate (SDA) culture plates for further analysis. The microgram unit weight was estimated using an analytical weighing balance and the surface spreading technique was used to disperse spores of probable strains present.

Microbial Identification

Microscopy is the key technique to obtain morphological characters and cellular structures except for some microscopic characters, which are not visible to the unaided eye. Method of Mailafia *et al.* (2017) [10] was carried out to characterize and further identify the probable mold isolates recovered.

Results

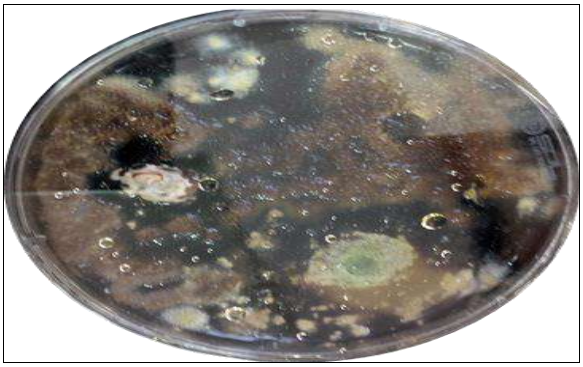

Fig 1: Contaminated Sabouraud Dextrose Agar (SDA) culture plate. The cultured plate showed differential colouration and mycelia textures indicating multicellular interactions of strains growing on nutrients available within the specific environment. The appearance also confirms the high level of cross pollution caused by uncontrolled and poor monitoring of filamentous microscopic fungi

Table 1: Macroscopic characteristics of recovered isolates

Codes	Macroscopic Characteristics	Probable Isolates
SDA1	Whitish wool colouration with complete marginal shape, rough hyphae turning blackish brown with the formation of conidia on the surface of the medium. Yellowish river pattern formed on reverse of the culture.	*Aspergillus niger*
SDA2	Whitish wool colouration with complete marginal shape. Growths with smooth like walls, fast dense sporulation, smooth hyphae turning cinnamon brownish colouration on the surface of the medium. Reverse pale colouration to bright yellow to deep brown	*Aspergillus terreus*
SDA3	Whitish wool colouration with rough marginal shape, fast growth sporulation, hyphae turned bluish-green in with a yellowish pigment on surface.	*Penicillium chrysogenum*
SDA4	Whitish wool to pale grey colouration, rough marginal shape, reverse green to brown colouration of diffused conidophopres.	*Penicillium funiculosum*

The following codes SDA1, SDA2, SDA3 and SDA4 represent each probable isolate recovered from specimen using Sabouraud Dextrose Agar (SDA). The descriptions were obtained while observing appearance of each isolate as shown on the surface and reverse of the culture plates.

Table 2: Microscopic characteristics of recovered probable isolates

Codes	Microscopic Characteristics	Probable Isolates
SDA1	Smooth conidia, protrusions of conidiophores from a septate, presence of hyaline hyphae, conidial appeared radial with biseriate, metulae supporting phialides (4um diameter) on the conidiophores. Smooth conidiophores (2500um long), globose (70um diameter).	*Aspergillus niger*
SDA2	Rough compact conidial heads with biseriate (up to 40µm diameter). Smooth conidiophores (5 µm in diameter). The conidia (up to 1.5µm in diameter), smooth globose, hyphae (6µm in diameter).	*Aspergillus terreus*
SDA3	Septate hyphae (up to 4µm in diameter), branched conidiophores, hyphalia phialides, conidia (up to 4µm in diameter) forming chains	*Penicillium chrysogenum*
SDA4	Conidia(up to 2µm length, and width), presence of globose and subglobose, Coarsely Roughened, Stipe (up to 30µm and 2.5µm in width) 2.7, phialide, branched conidiophores	*Penicillium funiculosum*

The following codes SDA1, SDA2, SDA3 and SDA4 represent each probable isolate recovered from specimen using Sabouraud Dextrose Agar (SDA). The descriptions were obtained using a fluorescence microscopy at X100 magnification while observing each specimen prepared using wet mount glass slides.

Discussion

Microscopic filamentous fungi affect all aspects of lives by way of co-existing in soils, air, food, water and any damped environment. They play important role in various industrial bioprocesses and influence the health and food sectors by causing some of the major respiratory diseases and also production of toxins. Due to their nature, molds can easily cross contaminate making monitoring and proper identification difficult using micro-filters and culture media (Promputtha *et al.* 2005; Noman *et al.* 2016) [12, 11]. In this investigation, a simple and unique method of monitoring and separating microfungal spores was exerted for the purpose of purification. The Figure 1 is a Sabouraud Dextrose Agar (SDA) culture plate utilized for isolation of starins from soil sample. From the figure, differential colouration and characteristics of mycelia texture was observed indicating multiple isolates growing on the medium due to availability of nutrients at suitable pH and temperature (°C).

Favourable growth conditions promote microbial development of mycelia necessary to perform periodic sub culturing but often during sub culturing, characteristics may change as a result of poor monitoring of environmental conditions, cross contamination leading to mixed culturing and pollution (Hyde *et al.* 2010). This occurrence is mostly associated with cross contamination common with microscopic filamentous fungi which are characterized by filament structures, size, punctiform, elevation, margin, surface and colour as shown in the Figure 1 above and similar to the reports described by (Lhan *et al.* 2006; Sammon *et al.* 2011; Talaiekhozani and Mohanadoss, 2015; Adhikari *et al.* 2019) [8, 13, 18, 1]. The technique described as surface spreading was used in this investigation to recover the following isolates: i. *Aspergillus Niger*, ii. *Aspergillus terreus*, iii. *Penicillium chrysogenum* and iv. *Penicillium funiculosum.*

The principal of this procedure can be related to the isolation and purification techniques often used for fungal investigation such as: dilution plating (Khuna *et al.* 2019; Tournas *et al.* 2017) [6], filtration (Warris *et al.* 2010) [20], wet sieving (Hoysted *et al.* 2018) [4], Single conidium (Zhang *et al.* 2003) [21], Spore suspension (Leyronas *et al.* 2012, Fei *et al.* 2019) [7, 3]. These practices have been used for isolation, enumeration and purification of molds but much activities and materials are involved in the practices as compared to the surface spreading technique which involve less procedure. Another advantage the surface spreading has over others is the high amount of vegetative spores recovered usually lost in the methods mentioned above. The observation recorded using the surface spreading technique proved to be effective in recovery of vegetative spores, direct screening of hypelia hypae, and cost effective. The method can be used to further recover several isolates from culture media that would have been dispatched as waste which often result to loose of time and materials during experiments.

Conclusion

An essential aspect of fungal strain collection and conservation from various samples and research environments is to utilize them for their industrial benefits. The surface spreading application in this investigation proved to be simple, cost effective and time saving. Its application in this experimental procedure was useful in recovering isolates that are relevant and important to industrial bioprocesses which are often disposed as waste. More so, monitoring of isolates causing damage in food, medical and agricultural industries can become more transparent in research activities involving microscopic filamentous fungi with the aid of the surface spreading practise.

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