Cultivation and Detection of Viable but Non-culturable Fungi in Soil using Yoghurt Whey Infusion agar

Wejdan A. Ali[1], Bahrouz M. A. AL-Jaff[2] and Sajid S. S. Al-Saeedi[1]

[1]Department of Biology, College of Science, University of Anbar, Ramadi, Iraq

[2]Department of Biology, College of Education, University of Sulaimani, Sulaimaniyah, Iraq

***Abstract*–Non-culturable and non-sporulating fungi represent a great challenge in studying biotrophic, endotrophic, and mycorrhizal fungal groups. In this study, a novel culture-dependent technique complemented with polymerase chain reaction was used to cultivate and identify non-culturable fungi in soil. To develop techniques for cultivation of non-culturable fungi for expanding studies on fungal biodiversity in soil, yoghurt whey infusion agar (YWIA) was developed. By this technique, from higher dilutions of soil, two tiny colonies (<1 mm in diameter) had grown on YWIA (1.0% and 2.0% filtration sterilized yoghurt whey) after incubation for 1–3 months at 28°C but had not shown growth when replicated on traditional mycological media, were selected, purified, studied culturally and microscopically, and identified by molecular methods. Growth of the two isolates on potato dextrose agar (PDA), but not Sabouraud dextrose agar, Malt extract agar, or Czapek Dox agar, happened after 3 weeks of incubation when a loopful of a colony grown after several successive subculturing on YWIA was transferred to PDA. The growth on PDA has been empirically proven due to the inoculum size effect, and the cultural and microscopical features were more resemble *Aspergillus* spp. Two fungal-specific primer sets (EF4F/ITS4r and EF60F/ITS4r) were used to amplify partial sequences of fungal rRNA gene included ITS sequences. The two partial sequences of the two isolates were aligned through the BLASTN phylogenetic analysis against NCBI database which revealed higher identities with *Aspergillus terreus*. The blast tree showed that both isolates are neighbor joined to *Aspergillus* spp. These results led us to consider the isolates as viable but non-culturable fungi (VBNC) like the common phenomenon found in some bacterial species.**

***Index Terms*—Non-culturable fungi, polymerase chain reaction, soil, viable but non-culturable fungi, yoghurt whey infusion agar**

I. Introduction

Non-sporulating fungi are being existed as a major challenge on studying biotrophic, endotrophic, and mycorrhizal fungal groups; thus, techniques are required when trying to detect fungi in the soil [1]. The gap between known microbial groups and their culturable representatives is now clearly obvious. Un or not cultured microbes are diverse in nature [2]. In fact, the cells in nature could be seen microscopically, most are viable, but more of them cannot form visible colonies when cultivated in plates [3,4]. In the past, it was reported that uncultivable microorganisms have been grown in pure culture when the media supplemented with component of their natural environments [5- 7]. These attempts are too little that if we know that a very few microorganisms are still cultivated in plates [8,9] and so there is no obvious success with this respect [9,10] whether we knew that the diversity of fungi has been estimated to be 1.5 million species [11,12] or may be up to 5.1 million [13], whereas only 5–10% of the first estimation have been mentioned to be described [13,14]. Soil is the major reservoir of fungi; it was studied extensively and led to some relatively comprehensive accounts of soil fungi [15]. The molecular methods widened the gap in fungal diversity and so it is unlikely to solve this problem in the nearest future [16]. Although culture dependent methods have been considered not the golden procedure to study diversity of fungi in comparison to culture independent molecular tools because of consuming time and biased toward easily and fast growing species [17] and because of the reports that mentioned to that even the cultivation in combination with molecular methods leads to fail in detection of a majority of the fungi [18], but others suggested the using of culture dependent but by using more media [19]. Whey agar was firstly used for growing fungi in 1923 [20] but the whey that was extracted from cheese was sterilized by autoclaving. Autoclaving has an inhibitory effect on the nutritional value of whey when it used in culture media [21]. Yoghurt whey was used as selective medium for isolation of *Saccharomyces cerevisiae* from food but whey was autoclavized with medium too [22] so that there is a need to sterilize whey without autoclaving, for example, it could be used as infusion by sterilizing with filtration to preserve its nutritional composition.

This study aims to cultivate those fungi that do not form obvious colonies on ordinary growth media used in isolation of fungi from soil but may remain viable or to cultivate microcolonies that may appeared after long times of incubation by replicating the primary master plates on an modified yoghurt whey infusion agar (YWIA). Then, when the potential viable or microcolony forming fungi grow well on YWIA, they

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Corresponding author's e-mail: bahrouz.jaff@univsul.edu.iq

regrown on the primary ordinary media and identified through amplification of partial rRNA gene sequences including internal transcribed spacers (ITS), and then, their sequences used as queries in BLASTN search to test their identity with those sequences in database and their potential affiliation to fungal groups to ascertain the validity of the method.

II. Materials and Methods

A. Preparation of Media

To prepare yoghurt whey infusion new full fat industrialized cow yoghurt obtained from local market was used as a source of whey. The yoghurt was centrifuged in 15 ml centrifugal tubes at 1000 rpm for 10 min. The whey filtrate was then filtered aseptically through Whatman No.4 filter paper and collected as infusion in test tubes and preserved in refrigerator for not more than 2 days. The medium YWIA was prepared by adding 20 g agar to 250 ml of distilled water, agar dissolved by boiling with shaking, the volume completed to 1 L with distilled water, and autoclaved at 121°C at 15 pounds/in^2 for 15 min. The medium maintained melted at 44°C in shaking water bath, and then, 5 mg of streptomycin was added to prevent bacterial growth with existent shaking. Concentrations of whey in medium were prepared by completing 5, 10, 20, 40, 80, 160, and 320 ml of whey infusion to 100 ml with melted medium and poured into Petri dishes to prepare 0.5, 1, 2, 4, 8, 16, and 32% YWIA. Concentrations higher than 4% were omitted from isolation process because of interruption in results. Potato dextrose agar (PDA), malt extract agar (MEA), Sabouraud dextrose agar (SDA), and Czapek Dox Agar (CDA) were prepared according to manufacturer (Lab. M Ltd, UK).

B. Soil

Agricultural soil samples were collected from a garden in the University of Sulaimani. Samples were taken from the superficial layer within a depth not exceeded 30 cm, mixed thoroughly and collected in sterile containers, sealed and carefully placed in a sterile polyethylene bag, and brought to the laboratory. Samples were mixed and sieved twice to remove large stones and debris and tested immediately.

C. Isolation of Fungi

Serial dilutions till 10^{-6} were done from 1 g of soil in 90 ml of sterilized distilled water [23]. Aliquot of 0.1 ml from the dilutions was spread on YWIA plates containing different concentrations of whey. Cultures were incubated at 28°C for 1–3 months and checked every 5 days. The medium become selective for fungi because of the antibiotic enrichment and the selective activity of whey which inhibits bacterial growth [22]. The plates used as master plates and replicated on Potato PDA, MEA, SDA, and CDA using an inoculation needle. The replicated plates were incubated again as that of master plates. When tiny colonies of 1 mm in diameter on master plates failed to grow on the replicated plates, incubation of the master plates was extended for 1 week more with adding drops of normal saline to the medium to prevent dryness of the medium. The colonies that failed to grow on replication were purified and subcultured for several times till the size of the colonies reaches an enough size to be succeeded in growth when inoculated on ordinary media. A loopful from the colonies subcultured on the YWIA was inoculated on PDA to examine their growth with larger inoculums' size. Several other subcultures on PDA slants were preserved in refrigerator and repeated every 1 month as working preserved cultures and to obtain vigorous growth to be used for cultural, morphological, and molecular identification. Along with inoculation soil samples on YWIA, they were inoculated by the same manner on PDA, MEA, SDA, and CDA as positive control and on agar plates without whey as negative control.

D. Identification of the Isolates

Taxonomic guides and standard procedures [24,25] were used to identify the isolates culturally and microscopically.

E. Inoculum size effect

The method of Choi, *et al.* [26] was used in repetition to obtain colonies originated from single spores. The growth of germinated single spores was tested on YWIA and the four ordinary media at 28°C for 3 weeks. The ability of the single spore to grow was interpreted as the ability of the germinated single spore to form apparent colony on the agar medium. The isolates that fail to form colonies from single spores were considered as viable but non-culturable (VBNC) because of insufficient inoculum size.

F. Polymerase Chain Reaction (PCR)

Genomic DNA was extracted depending on the protocol provided by the manufacturer of the extraction kit (Quick-DNA™ fungal/Bacterial Miniprep kit) and purified using Zymo-Spin™ technology of the same company. The amplification of partial genomic sequences of rRNA genes between 18SrRNA and 28SrRNA included ITS region's sequences was done using Maxime PreMix Kit (i-Taq). Two pairs of fungal-specific primers that are shown in Table I were used. Run was optimized as came in the literatures that used the primers (Table I). PCR products were visualized on 1% agarose gel electrophoresis in 1× TBE buffer (9 mM Tris-borate, 0.2 mM EDTA) and staining with ethidium bromide.

G. Sequencing and Alignment of Amplicons

PCR products (amplicons) were sequenced at Macrogen Company (Korea) using Applied Biosystems 3730 mXL

TABLE I
Primers Used in PCR Analyses

primers	Sequence	Target	References
EF4F/ITS4r	Forward: 5′-GGAAGGGATGTATTTATTAG-3′	18S rRNA	[27]
	Reverse: 5′-TCCTCCGCTTATTGATATGC-3′	28S rRNA	[28]
EF60F/ ITS4r	Forward: 5′-TGTCTAAGTATAAGCAATT-3′	18S rRNA	[29]
	Reverse: 5′-TCCTCCGCTTATTGATATGC-3′	28S rRNA	[28]

PCR: Polymerase chain reaction

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automated DNA sequencer. Then, the sequences were submitted to BLASTN for pairwise alignment against sequences available at GenBank database (https://www.ncbi.nlm.nih.gov/) and for phylogenetic analyses. Alignment between the sequences of the isolates obtained in this study was also achieved as multiple queries against each other to identify the phylogenetic relatedness between them.

III. Results

A few tiny pale white colonies were grown on 1% YWIA from the 10^5 dilution and from 10^3 of 2% YWIA (Fig. 1). The purification of the colonies on 2% YWIA and several subculturing on the same medium has led to enlarge the size of colonies. Two colonies were succeeded in growth when replicated on PDA, they symbolized W7 and W8. The positive control plates showed vigorous fungal growth while negative ones showed no growth.

The cultural characteristics on PDA and microscopic examinations revealed that both isolates are *Aspergillus* spp. They have typical conidiophores and conidial heads (Fig. 2). The effect of inoculum size on growth test revealed that the germinated single spores grow only on PDA plates after 14 days of incubation in a mean diameter of colonies of 2.2 mm, but growth was failed on MEA, SDA, and CDA.

The PCR targeted the sequences between 18SrRNA and 28SrRNA gene sequences as expected. The bands of both isolates W7 and W8 revealed length more than 1000 bp; the sequencing of the amplicons revealed that the amplicon of W7 that amplified with EF4F/ITS4r is 1280 bp and that with EF60F/ITS4r is 1264 bp. The amplicon of W8 that amplified with EF4F/ITS4r is 1191 bp and that with EF60F/ITS4r is 1276 bp (Fig. 3).

When the sequences of the four amplicons of the two isolates were used as BLASTN queries against the GenBank database, the EF4F/ITS4r amplicon of W7 showed higher identity (97%) with the partial rRNA sequence of *Aspergillus terreus* strain VV08 (unpublished) (acc. No. KT031990.1) followed by 96% with 13 other *A. terreus* and one partial rRNA sequence of *Penicillium* sp. (acc. No. DQ810191.1). The amplicon of EF60F/ ITS4r of W7 showed higher identity (98%) with *A. terreus* (acc. No. KT031990.1) and with more other *A. terreus* strains. The EF4F/ ITS4r amplicon of W8 showed higher identity (97%) with the partial rRNA sequence of *Aspergillus* sp. (acc. No. KX186570) (unpublished) and *A. terreus* (acc. No. MG271961), whereas the amplicon of EF60F/ITS4r of W8 showed identical results to that of the amplicon of EF4F/ITS4r of the isolate W7 (Table II). The multiple alignments between the two sequences of the two isolates revealed that EF4F/ITS4r amplicon of W7 was in 96% identity with its analog of W8, whereas the EF60F/ITS4r amplicon of W7 was in 94% identity with its analog of W8 (Results are not showed in tables or figures).

The phylogenetic tree of the EF4F/ITS4r amplicon RNA gene sequence of the isolate W7 revealed closely neighbor joining to *A. terreus* (acc. No. KT031990.1). The isolate W8 was closely neighbor joining to *Aspergillus* sp. (acc. No. KT031990.1) (Fig. 4).

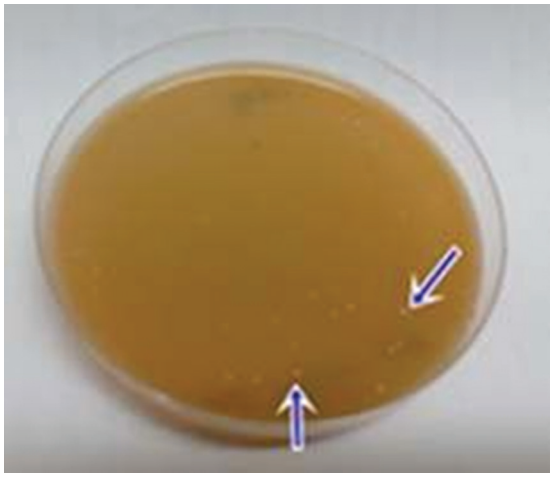

Fig. 1. The growth of tiny colonies on 2% yoghurt whey infusion agar medium.

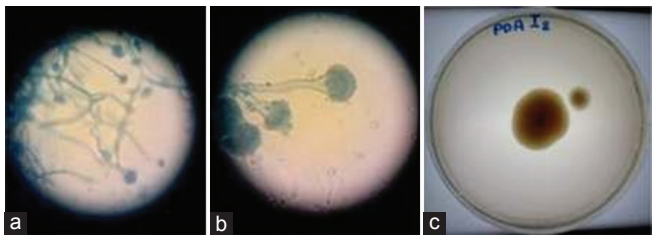

Fig. 2. The microscopic features of W7 (a) and W8 (b) isolates showed *Aspergillus* conidiophores, and the PDA plate of W8 (c) showed an *Aspergillus* isolate after 3 weeks of incubation.

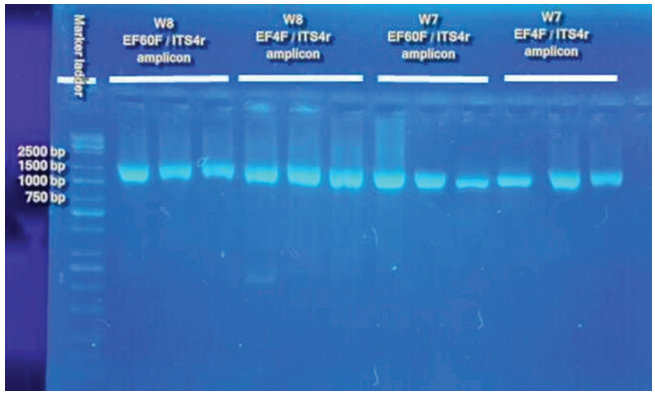

Fig. 3. Amplification products of rRNA gene fragments for the two fungal isolates W7 and W8 using two primer pairs for each isolate.

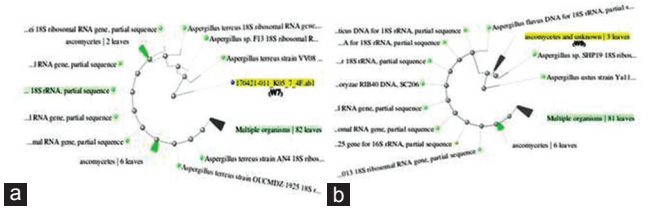

Fig. 4. The phylogenetic tree of the two isolates W7 (a) and W8 (b) Both isolates are closely neighbor joined to *Aspergillus* sp., namely *Aspergillus terreus*.

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TABLE II
THE BLASTN MATCHING RESULTS OF RRNA FRAGMENT SEQUENCES OF TWO FUNGAL ISOLATES AGAINST SEQUENCES AVAILABLE IN GENBANK DATABASE

Isolate	Source	Query (amplicon of)	Cultural and microscopic identification	BLASTN match		
				Species	Acc. No	Identity (%)
W7	Soil	EF4F/ITS4r	*Aspergillus* spp.	*Aspergillus terreus*	KT031990.1	97
				Aspergillus terreus	KX889082	96
				Penicillium	DQ810191.1	96
		EF60F/ITS4r		*Aspergillus terreus*	KT031990.1	98
			Aspergillus spp.	*Aspergillus terreus*	MG228391.1	95
W8	Soil	EF4F/ITS4r		*Aspergillus* sp.	KX186570	97
				Aspergillus terreus	MG271961	97

IV. Discussion

There were signs for occurrence of VBNC fungi [30-33], but there are no serious attempts to cultivate these fungi. However, another work did not detract objective of detecting VBNC fungi. There is a need for cultivating VBNC fungi because that PCR cannot differentiate between VBNC and dead fungi [34] so that this attempt is a good step toward starting cultivating the viable fungi that survive in the culture media but lack the ability to grow. The relative enlargement of colonies on repeating subculturing on YWIA may return to a state of adaptation on the medium which is natural medium. In the state of VBNC in bacteria, the subculturing of some pathogenic species may retard the growth by subculturing rather than enhance it [35,36]; however, those pathogens could be cultivated even in synthetic media. In fungi, it is difficult to cultivate unculturable fungi even that most are saprophytic. It seems that the use of natural medium is suitable for growing of VBNC fungi, but the low concentrations of the supplement and long period of incubation are needed even if producing tiny colonies, but the tiny colonies become active by subculturing and can grow on PDA easily when inoculated by a large inoculum size or by a germinated single spore as this study revealed. Whey contains components with significant nutritive elements [37].

Whey proteins are simple protein supplements and novel healthy ingredients in the food industry, they provide health-promoting functions which promote health conditions and prevent diseases [38]. Cheese whey was used in the past as a substrate for the growth of fungi [39- 42], whereas the antibacterial activity of whey was described extensively [22- 38,43] and so it becomes good medium as selective for fungi. The effect of size of the inoculum on growth and production of biomass is studied in the use of fungi in biotechnology when cheese whey used as medium, it is clear that a large inoculum size is needed to grow morel mushroom; the minimum size was 8 mg mycelium/ml [42].

rRNA gene sequences are used frequently in phylogenetic studies as a part of environmental biodiversity screening [44,45]. The current study revealed that only *Aspergillus* spp. were isolated as the isolation, alignment, and the phylogenetic tree ensured. In fact, *Aspergillus* spp. are common fungal flora in soil [46]. The two isolates were with higher identity with *A. terreus,* but they not originated from a same individual strain because that their sequences that were amplified using the same primers were not fully identical. The cultural and microscopical characteristics of the two isolates ensure their return to *A. terreus* in the view of the molecular identification. The potential question here is why this species was only isolated by YWIA? To answer this question, future studies are needed to ascertain whether this case is frequent or other species of *Aspergillus*, or other genera, can be isolated also. It was mentioned that the yeasts most commonly used for fermentation *Kluyveromyces fragilis* and *Saccharomyces cerevisiae* are unable to ferment lactose so that whey that used as substrate must be supplemented with nitrogenous compounds, usually ammonia, as well as with vitamins and minerals [47]. In a study, whey fermented with kefir grains, a symbiotic bacterial and yeast culture reduced the conidial germination of more *Aspergillus*, *Trichoderma*, and *Rhizopus* spp. so that fermented whey suggested to be used as a supplement for poultry feed [48]. These observations suggested that whey is a selective substrate for isolation of VBNC fungi when it used in low concentrations which involve the potentiating whey to select those non-culturable fungi that benefit from whey but not inhibited by it. This may be discriminate *A. terreus* from other fungal species with this respect.

V. Conclusion

This study reports the possibility of using low concentrations of yoghurt whey in a culture medium (YWIA) to isolate VBNC *A. terreus* strains within 1–3 months of incubation at 28°C that increases the hopes to characterize fungi in screening of fungi in biodiversity studies as well as it opens up new avenues for research in the development of cultural methods for the diagnosis of unculturable fungi.

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