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Possibility of use the recycled fungal media

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Abstract

In this work the ability of some food, industrial and medical fungal strains to grow on fungal recycled media was investigated. The growth ability of Aspergillus flavus, Penicillium sp., Aspergillus niger, and Fusarium equiseti was tested using two specific types of media namely Potato Dextrose Agar and Sabourod Dextrose Agar. The fungi were able to grow on the fungal recycled media for two times. The diameter of the colonies was decreased by about 15% in the recycled media in the first time and decreased by 45% in the recycled media for the second time, only in the three strains: Penicillium sp., Aspergillus niger and Fusarium equiseti. However, Aspergillus flavus had different behavior as the decrease in the diameter of the colonies reached 6% in the recycled media in both the first and second time of recycling, which means that Aspergillus flavus can grow better in recycled media than the other three strains. The work contributes to find safe and clean laboratory methods to dispose of the biological wastes, to preserve the environment and to reduce media costs.

Keywords: *Aspergillus flavus*; *Aspergillus niger*; *Fusarium equiseti*; *Penicillium sp.*; Media; Recycling

1. Introduction

In the field of microbiology (bacteria, fungi), it has been widely reported that solid media consisting of a mixture of agar, salts, vitamins, colorings and some texture enhancers [1,2] are used to identify and isolate the microorganisms and these media, on the other hand, produce biological waste. The biological wastes management becomes more important, especially with the growing factories and population; and in the developing countries the media could be expensive [3,4]. Limited studies have referred to the reuse of media [2,5]; re-dissolve the media by heating in autoclaves after take out the bacterial colonies from the media, after transforming the small pieces of used media to new liquid to be poured directly into the Petri dishes [2], whom dissolved only the bacterial medium, without converting it to a dry storable material, and therefore it could be used immediately after recycling for only one time**.** While other researchers [5] were able to recycle the used bacterial media and plant tissue culture into new agar in a form of a dry substance after treatment with some chemicals, but without providing data on recycling fungal media or testing the ability of fungi to grow on the recycled media. Recently, Abboudi [4] also addressed the possibility of recycling bacterial culture media, but not the fungal media.

Some reports have attempted to replace the basic traditional culture media (NA: Nutrient agar, PDA: Potato Dextrose Agar, SDA: Sabourod Dextrose Agar) with cheaper natural materials such as grains, vegetables and fruit peels for the development of fungi [3,6-7], to be used for economical and ecological reasons. Algae culture medium can be recycled up to four times only by centrifugation after growth and with additives (50% of nutrients of urea media) during anaerobic fermentation processes [8-11]. There is no indication about the recycling of fungal culture media or the extraction of agar from these media. The media can have different impacts on the quality of fungal during the growth [12].

Some studies have addressed the effect of environment on the fungal growth through the measurement of mycelial mat and the change in the color of fungi. The effect of growth temperature, medium composition and pH of media could affect

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the products of fungal growth [13], and the modification of the normally media -such as Sabouraud agar- could be technically useful in simultaneous fungal enumeration and determination of the presence of some product without prior isolation [14]. Benefiting from different culture media is of scientific and technical work that deserves attention for two reasons. Firstly, its importance in the recycling of biological waste and thus recycling the plastic Petri dishes used in food, industry and health laboratories as well. Secondly, obtaining of biological materials recycled for several times in the form of a powder that can be stored for long periods, and then used again in all biological and bacteriological experiments, and additional fund is needed to purchase new media, which is very expensive in developing countries. As far as we know from literature, there is no technique to safely dispose of used Petri dishes that contain fungal colonies, except using high humid heat (autoclave), which forms large quantities of biological waste. The aim of the present work was to look for environmental and economical solutions for the big quantities of biological wastes caused by fungal culture in food factories and scientific laboratories.

2. Materials and Method

2.1. Fungal strains

Four fungal strains were tested: *Aspergillus niger* and *Fusarium equiseti* where Potato Dextrose Agar (PDA) medium (HIMEDIA-India) was used to cultivate these fungi, and *Aspergillus flavus*, *Penicillium sp.* were cultivated on Sabourod Dextrose Agar (SDA) medium (Techno pharmachem-India) [15]. In order to monitor the size and the morphology of the fungal colonies during the growth on the original and the recycled media, a special original method was used to inoculate the same number of Colony Forming Unit: CFU (7 CFU in our study) on the Petri dishes; depending on the fungal suspension from each pure fungal strain. This solution was used in a way that ensured that 7 CFU were obtained on each plate from each fungus separately. All Petri dishes were incubated at 25°C (room temperature); this temperature was appropriate for the growth of fungal strains for 5 days [16]. The aim of using two different media was to apply the same protocol of recycling whatever the fungal medium was.

2.2. Recycling process

After obtaining the fungi (first culture, which can be called the control: R0), only the fungal colonies were eliminated by mechanical removal, being superficial gametocytes, while preserving the solid material. The solid gels were processed in several ways (filtration - laying - drying) after heating with an appropriate temperature in order to complete the recycle process. The used media were cut into small pieces, then they were placed in large flasks for sterilization with the autoclave at 121°C/15 min/1 Bar (Autoclave- Italian-PBI). The melted medium was filtered smoothly by force of gravity, using a glass funnel containing pieces of sterile medical gauze. The dissolved medium was obtained in its liquid state and it was dried by placing it in shallow plastic trays (2 cm) using an oven (Memmert-looding Model 100-800. Germany) at 30°C for one night (10 hours), until weight stability. All produced dry materials were crushed (Starway, SW-012-SS, 150W, China) and transformed into powder. This powder was reused to form a new solid media (R1: recycled once) to test the fungal growth. This process was repeated several times (ex: R2 recycled twice) to determine how many times the recycled media could be used in fungal growth experiments. It was crucial to test the growth of each fungus on its own medium and recycled media separately from the other fungus.

2.3. Physical and chemical measurements

The pH of the fungal culture solutions was measured during the experiment by pH-meter (HANNA, HI8314, Romania). The diameters of the formed fungal colonies were measured after each growth on an original and recycled fungal medium in order to compare the different effects of recycling on colony morphology and the capacity of each fungus to grow on the new media. Attempting to investigate the possibility of a change in the composition of the recycled culture media through implementing thermal measurements using differential thermal scanning technology (DSC) (SETRAM- DSC131) [17-18] were conducted.

2.4. Statistical analysis

Data were subjected to the analysis of variance test (ANOVA) using the SUPERANOVA computer package (Abacus Concepts Inc, Berkeley, CA, USA; 1998). A separation test on treatment means was conducted using Fisher's least significant differences (LSD) methods at 95% confidence level.

3. Results and Discussion

The large number of Petri dishes of bio-experiments in the different laboratories around the world, constitutes the impetus to find new and innovative ways to recycle the ingredients of fungal culture. In the case of autoclavage of Petri dishes that contain fungal colonies which form large quantities of biological waste, no study has investigated the environmental and economical solutions for the big quantities of fungal biological waste, where other work focused on bacterial recycled media without getting a new dry material [2] or without recycling the fungal used media [4-5]. From environmental and economical points of view, we have therefore attempted to fill this gap by providing novel information on the ability of some fungal strains to grow on different qualitative and recycled media by thermal dissolution and to make new dry media able to be stored for long time until needed.

For the first time, the developmental capacity of several fungi: *Aspergillus flavus*, *Penicillium sp.*, Aspergillus *niger*, and *Fusarium equiseti* were tested using two recycled used media: PDA and SDA, and the findings of this study have special importance when compared to previous reports, that investigated only the recycling of bacterial and algae media [2,4,5,11]. In the current investigation, all fungal colonies were grown normally, noting the difference in external appearance according to each fungus (Figure 1); *F. equiseti* and *A. niger* filled Petri dishes more than the other two strains. Our results showed the possibility to recycle the used fungal media. Figure 2 explained the first step in the processes of preparing the used fungal media for heat melting by reducing the size into small pieces to facilitate the process, then the sterilizing was carried out by wet heat using autoclave. Using a glass funnel containing pieces of sterile medical gauze contributed effectively to melt media free as much as possible from fungal mycelium. Figure 3 shows the step of drying the melted used media, the difference in the color of the dissolved reused media could be due to the presence of a different pigment for each fungus. After drying and grinding the used media, a new powder media were obtained. The process was consecutively repeated to determine the number of times through which the fungal culture media can be recycled and to compere our finding with the bacterial and algal recycled media [4,11]. Then the recycled media were treated as new media in terms of solubility with water, sterilization using an autoclave, then pouring into Petri dishes, and inoculating with the four studied fungal strains. It is important to clarify that, the same basic and recycled medium was used for each fungus separately without mixing (each fungus was grown on its own medium and repeated each time with the same recycled medium). It was a crucial point in our strategy to observe the different effects of the consecutive culture of the same fungus on the recycled media as mentioned previously.

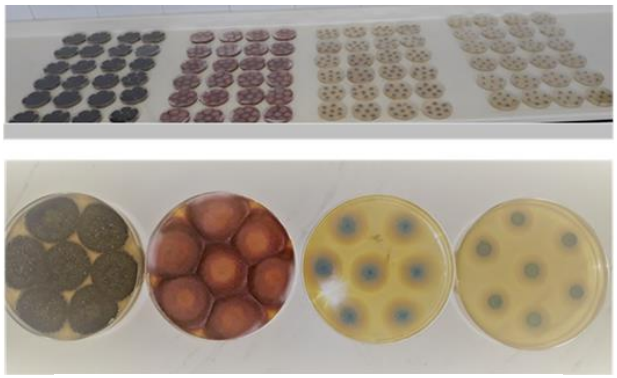

Figure 1. Different fungal cultures; from left to right *Penicillium sp.*, *Aspergillus flavus*, cultivated on Sabourod Dextrose Agar medium (SDA) and *Fusarium equiseti* and *Aspergillus niger* cultivated on Potato Dextrose Agar medium (PDA) in one experiment with a large quantity of replicates

Figure 2. Preparation step of recycling process by the mechanical removal of different fungi with media and the transformation to small fragments before melting by moist heat. From left to right: *Aspergillus flavus*, *Penicillium sp.*, *Aspergillus niger* and *Fusarium equiseti*

The present work shows the possibility of growing *A. niger*, *F. equiseti*, *A. flavus* and *Penicillium* sp. on the recycled media for several times (Figure 4). The diameter of the colonies gradually decreased each time in which the medium was recycled and used it in fungal cultures. The colony diameter on the Petri dish was decreased by approximately 15% ($p < 0.05$) in the first time of recycled media and by 45% ($p < 0.05$) in the recycled media in the second

time only for the three fungi (*Penicillium sp.*, *A. niger* and *F. equiseti*). *A. flavus* showed a different behavior, the decrease in the diameter of the colonies was 6% ($p < 0.05$) in the media recycled in the first time, also, the same percentage in the recycled media in the second time, which gives this fungus (*A. flavus*) the advantage to grow normally on the recycled media compared with the other fungi (Figure 4), with slight change in the colony diameter. The decrease in diameter of the colonies could be explained by the decrease of concentration of nutrients in the medium during the recycling stages; however, the quantities of nutrients were enough to allow the colonies to grow [2]. Another work [11] proved the possibility of recycling the bacteria media twice and maintaining the bacterial population viability, but the growth force represented by the diameter of the growing colonies in the Petri dish was reduced, and it corresponds to our results in a certain way.

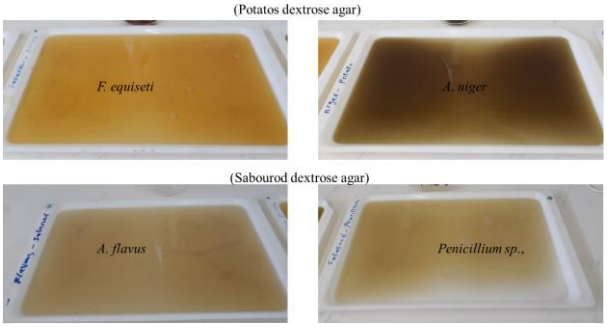

Figure 3. Preparation step of placing the melted fungal used media in shallow plastic trays (2 cm) before drying in the oven at 30°C.The medium Potatose Dextrose Agar used in the fungal cultures of *Fusarium equiseti* and *Aspergillus. niger*, and the medium Sabourod Dextrose Agar used in the fungal cultures of *Penicillium* sp., and *Aspergillus. Flavus*

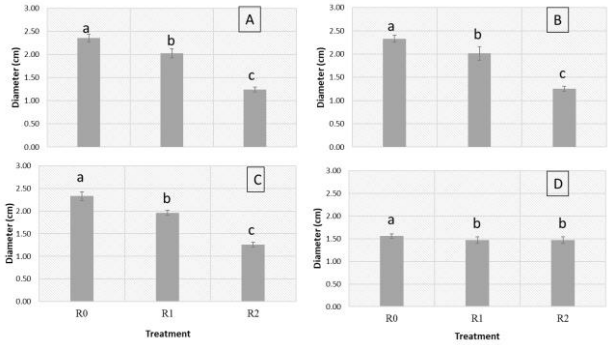

Figure 4. The change in the diameter of fungal colonies during the growth on fungal culture media used in the control (R0: non recycled), in media recycled once (R1) and recycled twice (R2); (A) *Aspergillus niger*, (B) *Penicillium sp.*, (C) *Fusarium equiseti*, (D) *Aspergillus flavus* at the same condition of growth. Columns with different letters (a-c) between treatments denote significant difference at ($p < 0.05$)

A change occurred in the shape and color of the four fungal strains in general, as the color turned to lighter (Figure, 5A), especially in the case of *A. niger* (Figure, 5B), where the difference appeared clear in color and this was associated with the decrease in the diameter of the colonies (Figure, 5B). In the recycling stages, the pH was measured in the liquid media before solidification in the Petri diches, and the results showed that the pH was not affected by most of the strains used, with the exception of the case of *A.niger*, where the pH decreased by approximately 40% compared to the control (Table 1). This decrease in pH could be related to producing citric acid in the media as noted by some researchers [19]. The change in the pH could affect the color and size of the colonies growing on the recycled medium [12], and the results also correspond to VanderMolen [12] as the media can affect the fungal growth.

Table 1. Changes in pH of fungal media during growth of *Aspergillus niger*, *Fusarium equiseti* on Potatose Dextrose Agar medium and *Aspergillus flavus*, *Penicillium sp.*, on Sabaroud Dextrose Agar medium in the control media (R0: non recycled), in media recycled once (R1) and in recycled twice (R2)

Fungi	Control (R0)	R1	R2
A. niger	6 ± 0.04^{a}	5 ± 0.01^{b}	3.8 ± 0^{c}
F. equiseti	6 ± 0.04^{a}	5.3 ± 0.02^{b}	5.2 ± 0.02^{c}
Penicillium sp.	$5.7\pm0.\ 16^{a}$	5.6 ± 0.05^{a}	5.4 ± 0.04^{a}
A. flavus	5.7 ± 0.016^{a}	5.4 ± 0.02^{ab}	5.1 ± 0.03^{b}

Means in the same row followed by different letters (a-c) are significantly different at ($p < 0.05$)

The analysis preliminary of DSC technique for one medium (Sabourod dextrose Agar) in order to obtain some data that may explain the changes in the thermal properties of the recycled culture medium was conducted (Figure 6). The curves of the thermal behavior in the figure 6 show no clear differences between the unused medium (R), used-non recycled (R0), and recycled once (R1). This means that the recycling process almost did not thermally affect the composition of this medium. Whereas, we observed a change in the thermal behavior curve starting in the recycled twice (R2), indicating the changes in the medium structure observed in the decrease in diameters of the fungal colonies. The DSC gives indications about the changes in the structure of powdered substance [18]. This change indicated that the media structure was likely to be destroyed or modified; the same behavior was observed in a previous work [4] where the structure of the recycled culture medium

cracked the third time of recycling and lost its properties.

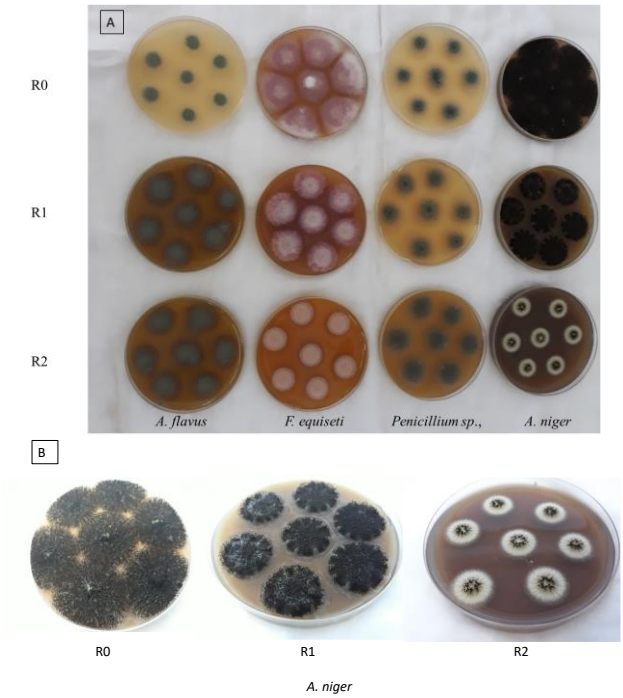

Figure 5. (A) presents the morphological change in the color and diameter of the fungal colonies during growth on the used media in the control (R0: non recycled), in media recycled once (R1) and recycled twice (R2) for *Aspergillus niger*, *Penicillium sp.*, *Fusarium equiseti* and *Aspergillus flavus*; (B) presents only the growth of *Aspergillus niger* on the Potatose Dextrose Agar in control medium (R0) and in recycled medium once (R1) and twice (R2)

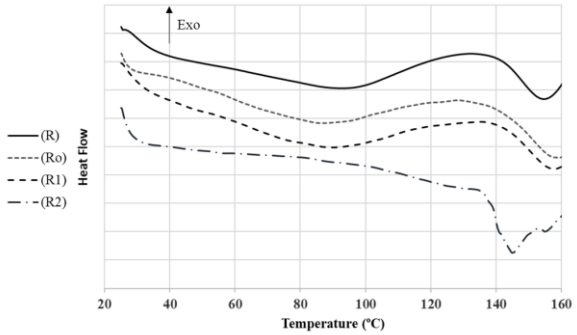

Figure 6. Thermogram changes of differential thermal scanning technology (DSC) of the fungal Sabourod Dextrose Agar medium (SDA) in different treatment; unused medium (R), used-non recycled (R0), recycled once (R1) and recycled twice (R2)

Additional experiments in thermal analysis are needed to obtain interpretations of the results and more precise chemical analyzes are needed. Some fungi (*A. niger* and *penicillium sp.*,) have effects in the field of industry, biology and medicine through the byproducts such as the extracellular enzymes, organic acids, antibiotic and also different toxins [20-24]. Therefore, it is necessary to conduct some experiments from an industrial and pharmaceutical point of view to find out the effect of the growth of fungi in recycled media on the ability of these fungi to produce pharmaceutically effective compounds. Aflatoxins were produced by *Aspergillus* sp., and ochratoxins were made by *Aspergillus* and *penicillium* [25]. *Fusarium* fungi are common to the soil and has a strong capacity to produce a range of different toxins, including trichothecenes, as well as zearalenone and fumonisins that could be produced in a high amount under the laboratory conditions [26]. In this work, the possibility of the presence of toxins due to the growth of these fungi was not examined. Each fungus was grown on the same recycled medium to eliminate the possibility of foreign products being affected- if found in the middle- on fungal growth. Future work will investigate the possibility of the presence of toxins and their impact on the growth of other fungi, and find safe methods to remove them for the best use of recycled media. Some fungi are able to grow in extreme environments, in acidic, metal-rich environments, and this leads us to search for the factors that are responsible for tolerance [27]. It is possible to add some components to compensate the losses or to support the existing components or to add some new characteristics to the recycled culture media [14]. The present work differs from other scientific studies in the recycling of the used fungal culture media two times and the converting them into a powder that can be stored for long periods, with results presented for different fungal strains (growth and morphology). This procedure is easily applicable in all microbiology laboratories.

4. Conclusion

The results presented in this work give for the first time data on recycle the used fungal culture media (PDA and SDA) which are used in microbiological laboratories two times and to transform it to new dry substance able to be stored for a longer time. We relied on a precise methodology using specific fungi during the stages of growth and recycling separately which is consider an original experiment, in order to obtain accurate results and prevent complications in the recycled medium due to possible and different secretions from one fungus to another. *Aspergillus niger* showed different behavior through a clear change in shape and a noticeable decrease in the diameter of the colony when it was cultivated on the different recycled media, it could be related to the change in pH and/or

change in the composition of the medium, especially at the second recycling process. Further experiments are recommended to investigate the possibility of toxic products from some fungi into the recycled media, and to test the recycling of medium that contains fungi mixed together. Certain chemical analysis is also recommended to investigate the changes in the composition of the media.

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Conflict of Interest. Author has declared that no competing interests exist.

Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human / or animal subjects (if exist) respect the specific regulation and standards.

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