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JSuNR

Journal homepage: http://publisher.uthm.edu.my/jsunr
e-ISSN : 2716-7143

Journal of Sustainable Natural Resources

Entrapment of *Volvariella Volvacea* Spores in Electrospun Nanofibers

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DOI: https://doi.org/10.30880/jsunr.2020.01.01.002
Received 25 November 2019; Accepted 25 March 2020; Available online 26 June 2020

Abstract: Nanofibers containing *Volariella volvacea* spores were prepared by electrospinning technique. It has been one of the promising and alternative techniques in immobilizing microorganism cells including bacteria, virus and fungi through entrapment process. The nanosize electrospun nanofibers exhibit outstanding properties including high porosity and reusability which make them an ideal candidates for various types of potential application especially in bioprocess development. In the present study, the spores of *Volvariella volvacea* were suspended in a series of polymer solutions (poly (vinyl alcohol) and polyvinyl pyrrolidone) and subjected to an electrostatic field supplied by the power voltage. The viability of the entrapped spores was estimated through microorganism cells test by culturing the obtained nanofiber mats on potato dextrose agar (PDA) and studying their growth rate. The *Volvariella volvacea* spores electrospun in nanofibers remained viable for 12 days at room temperature (30˚C) with PVP electrospun nanofiber chosen as the best carrier matrix for entrapment of *Volvariella volvacea*. The present result demonstrated that electrospinning process possess high potential in entrapping and immobilizing of microorganism cells.

Keywords: Entrapment, electrospinning, *Volvariella volvacea*, viability

1. Introduction

Entrapment of microorganism cells on a dry carrier material while preserving its bioavailability makes them vitally important in various applications. Their functions in releasing extracellular enzymes, organic acid, therapeutic proteins and biomolecules to a desired destination increased the demand of entrapment process [1]. Recently, there is a surging interest in using fungal cells for potential applications in the field of agriculture, environmental protection and textile industry [2]. However, certain entrapment process such as spray drying technique may not be compatible for fungal cells entrapment due to high working temperature which may lead to disruption of the fungal cells. Hence, it is crucial to have a competent delivery approach and development of means for effective entrapment that can enhance the fungal cells bioavailability and to perform at their optimum activity. By considering effective entrapment on a dry material, electrospinning is one of the promising technologies and a possible method due to its numerous advantages such as

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high surface area with nano-level diameter, light-weight, possessing nano-porous structure, high flexibility and reusability which enables the nanofibers to be a suitable candidate to act as a carrier or matrix [3]. Our aim was to elucidate the best type of polymer that allows the entrapment of fungal cell at high efficiency and the conditions of electrospinning process. In addition, the viability of fungal cell after storing the material was examined.

Electrospinning is an alternative approach in producing nanofibers in the range of 100nm in diameter. Electrospinning refers to the technology of fiber formation which is based on the electrostatic attraction of charges [4]. The instrument comprises of three main parts; the high voltage power supply, the syringe pump and the collector as shown in Fig 1 [5]. The solution contained in the syringe usually has its own surface tension which can supply and charge out by applying high voltage of power supply. In order to collect the ejected fibers from the syringe, the collector was designed in oppositely charged. As the solution emerges from the syringe by the help of voltage supply, it will produce a cone shape called 'Taylore cone" [6]. It can be extended by ensuring existence of high charge density on the collector. Basically, electrospinning process is conducted at room temperature with atmospheric condition. Electrospin instrument can be classified into many classes, and the major classes are the vertical and horizontal setup. In general, horizontal type refers to the charged force received by applying the voltage supply and opposite attractive charges on the collector. Meanwhile, vertical type is when two forces are used to draw the fibers in which the common one is collector, and the other one is the gravitational pull [7].

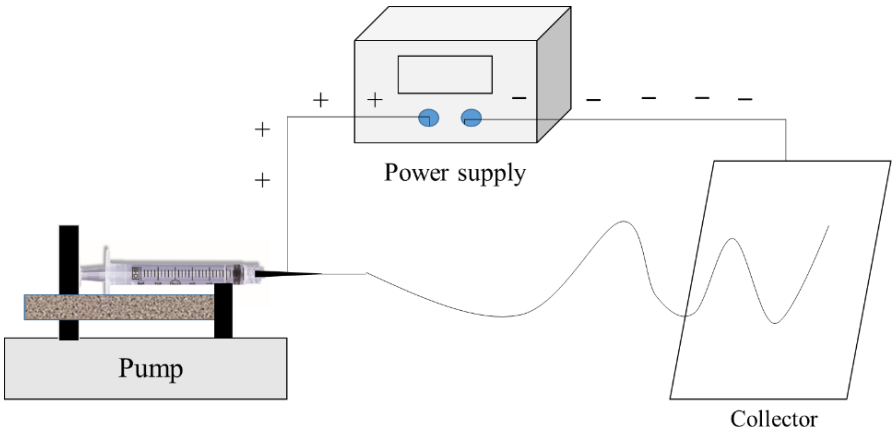

Fig. 1 - Electrospinning machine setup

The feasibility of microorganism cell entrapment in electrospun nanofibers mat has been demonstrated by previous researchers. For example, virus M13 has been successfully entrapped in electrospun nanofibers for biomedical applications and biosensors production [8]. It was found that intact viral structure of M13 virus in polyvinyl pyrrolidone (PVP) electrospun nanofibers possess highly sensitive catalytic and biological functions. Meanwhile, *Trichoderma viride* spores, a type of fungi that can be found in soil has been successfully entrapped in electrospun nanofibers for plant biocontrol against pathogenic fungi [9]. It was proven that *Trichoderma* strains which possess an ability to exhibit antifungal activity inhibit the growth of pathogenic fungi such as *Fusarium* and *Alternaria* when inoculating the nanofibers along with these pathogenic fungi in a petri dish. The filamentous *Trichoderma* spores which were entrapped in the electrospun nanofiber remained viable for a week when stored at room condition. Meanwhile, bacteria such as *Escherichia coli* and *Staphylococcus albus* has been successfully entrapped in polyvinyl alcohol (PVA) electrospun nanofibers for utilisation in biomedical area to treat wounds and cutaneous fungal infection [10].

In this work the efficiency of fungi entrapment by using *Volvariella volvacea* spores in PVA and PVP electrospun nanofibers was demonstrated through electrospinning process. Here we used an edible mushroom spores which is *V. volvacea* under fungi kingdom because of their short growing time, low input requirement and involving simple technologies to grow. These PVA and PVP nanofibers were studied to see whether they can be efficient and feasible mean to preserve the *V.volvacea* spores and it was found that such cell can retain its viability for 8 days at room temperature (30˚C).

2. Materials and methods

2.1 *Volvariella volvacea* spores

The fungus used in this work was *V. volvacea* which is classified under the genus of *Volvariella* or commonly known as straw mushroom. It is one of the edible mushrooms that are mostly cultivated throughout East and Southeast

Asia. The growth of mushroom normally comes in four stages starting with the spawning of spore on a medium substrate which provides nutritional value for the growth of *V. volvacea*. As for the condition required for growth of *V.volvariella*, the temperature must be above 30˚C as it is cultivated from paddy area surroundings. However the optimum temperature that can produce high volume of spores is 37˚C [11]. In this study, the mushroom was obtained from a local mushroom grower in Mersing. It was inoculated on potato dextrose agar (PDA) in 9cm petri dish and subculture to keep the culture stock. According to Ma et al. (2016), the culture medium consists of the following components such as potato (20%), glucose (2%) and fish peptone (0.3%) which are essential as nutritions for *V. volvacea* growth.

2.2 Electrospinning of *V. volvacea*

Electrospinning process was carried out by making a suspension of *V. volvacea* spores in a series of polymer solution. About 0.2 g of *V. volvacea* spores was dispersed in two types of polymer solutions with an equal volume of 8% w/v aqueous solution of polyvinyl alcohol (PVA, MW = 146,000 g mol^{-1}, Aldrich) and polyvinyl pyrrolidone (PVP, MW = 1,300,000 g mol^{-1}, Aldrich) respectively. These polymers were dissolved in distilled water as their solvent to acquire 5 ml of solution. The solution was brought up to a temperature of 80˚C and stirred to expedite the dissolution as the solvent used was only distilled water. The spinning solution was then placed in 5 mL syringe which was used to eject the polymer solution at a regulated flow rate. DC power supply (Ormond Beach, PL) was used to supply high voltage current in order to create the electric field needed for the electrospinning process. The syringe needle was attached with positive electrode of high voltage supply via alligator clip and the flat collector wrapped with aluminium foil was connected with grounding electrode. Throughout the electrospinning process, parameters such as applied voltage, spinning distance and volume flowrate were fixed at 15 kV, 12 cm and 1 mL/h respectively [9]. Once the nanofibers mats had been formed, aluminium foil was removed from the collector and nanofibers mats were collected. The best type of polymer will be chosen by evaluating *V. volvacae* mycelia diameter over times on PDA agar. All the experiments were performed at room temperature.

2.3 Viability and growth rate determination

To observe the number of living *V. volvacea* spores in the nanofibers sample, a piece of electrospun nanofibrous mat (1.5 cm x 1.5 cm) was placed on the agar surface for 7 days to inoculate the *V. volvacae* mycelium and stored in darkness at room temperature. The extension of mycelium in PDA was measured at day 2, 4, 6 and 8 after inoculation [9]. In order to determine the growth rate of *V. volvacae* mycelium, an equation was used. The mycelial diameter collected every two days was inserted into Equation 1 in order to analyse the growth rate of the *V. volvacea*.

$$GR = (Fd - Id)/(Ft - It) \quad (1)$$

where “Fd” is the final diameter growth, “Id” the initial diameter of growth and “Ft It”= the days of mycelial growth [12].

3. Results and Discussion

3.1 Electrospinning of *V. volvacea* in PVA and PVP Nanofibers mats

This study presents an entrapment of fungal cell by means of electrospinning technique. The removal of solvent by rapid evaporation during the electrospinning process is thought to lead a major change in the fungal osmotic environment and conditions, and hence, a strain that is considered resistant to adverse environmental conditions, *V. volvacea* was chosen for this study. The PVA and PVP matrix have been chosen because they are water-soluble material which is not expected to affect the bioactivity of the cell, possesses good biocompatibility and economical. The parameters such as polymer concentration, spinning distance, volume flowrate and voltage supply were fixed for both entrapments of *V. volvacea* in PVA and PVP electrospun nanofibers. These conditions have been chosen from a screening of literature of previous works producing high growth rate and bioavailability up to 4 months in storing condition [9].

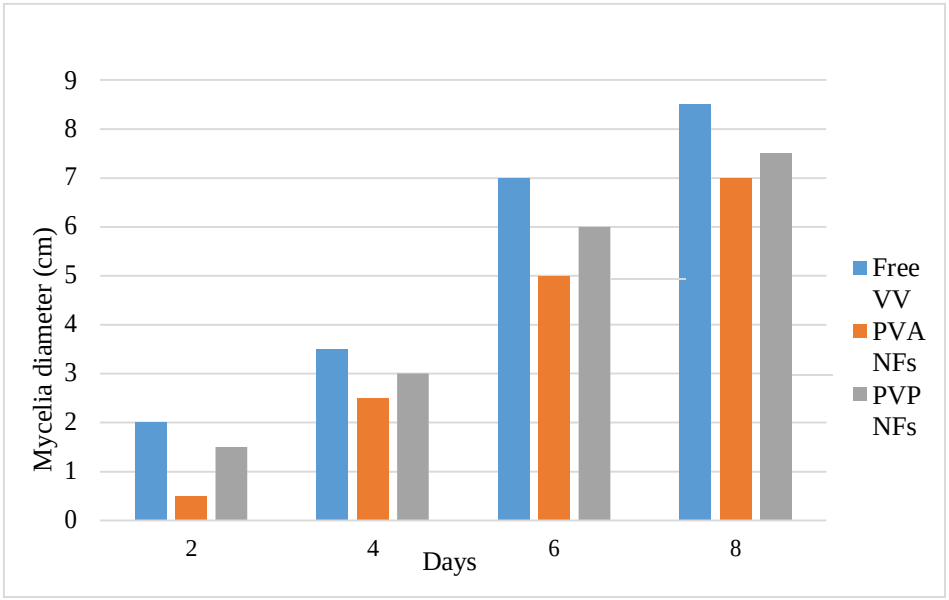

Fig.2 - *V. volvacea* mycelia diameter in PDA agar from different sources

Free cell of *V. volvacea* was also inoculated in PDA agar as a control to observe the mycelia diameter along the *V. volvacea* elctrospun in PVA and PVP nanofibers. As observed from Fig 2, the control shows the fastest and highest number in mycelia diameter measurement over time with maximum diameter of 9 cm reached in approximately 8 to 9 days. On the other hand, PVP nanofibers shows good result of entrapment where the mycelium diameter presented significant differences in growth ($p \leq 0.006$) between *V. volvacea* electrospun in PVP nanofibers and as a free cell. As for PVA nanofiber, the extension of mycelia diameter was relatively slower than PVP nanofiber after inoculation which shows that PVP acts as a better carrier in entrapping the *V. volvacea* spores. This is due to the structure of PVP ring which contains a proton accepting carbonyl group from the lignocellulose in *V. volvacea* where hydrogen bonds are expected to form. Among all the samples, mycelia had developed on the second day of inoculation. On the fourth day, after placing all the samples on agar, in all cases, the fungal colonies had proliferated to such a great extent that the entire petri dishes surface was colonized all the way to the dish wall.

3.2 Viability and growth rate determination

To determine the viability of *V. volvacea* spores electrospun in both PVA and PVP nanofibers, the mats were placed in agar. Microscopically, the *V. volvacea* strains developed branched, septate, smooth hyphae with elongated hyphal termination, frequent and abundant clamps with white in color as shown in Fig 3.

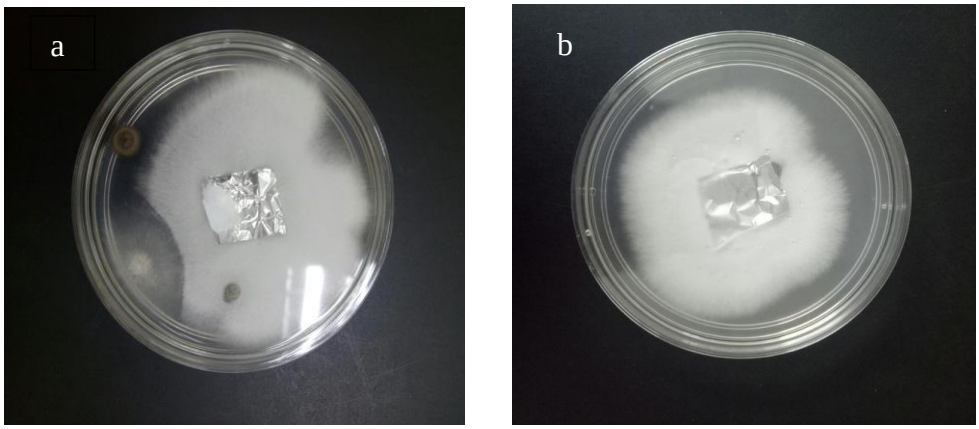

Fig.3 - The growth of *V. volvacea* mycelia after sixth days of inoculation from: (a) PVA nanofibers mat (total polymer concentration 8% w/v); (b) PVP nanofibers mat (total polymer concentration 8% w/v)

As observed from Fig 3 (a), there was a cross contamination occurred during inoculation of *V. volvacae* electrospun with PVA nanofibers where presence of unknown fungi was detected. This phenomenon might happen due to low sterility aspect in preparing the PDA itself. Besides, there is also high possibilities of cross contamination to occur due to improper way in handling the *V. volvacea* electrospun nanofibers during inoculation process. However, the growth of *V. volvacea* in PDA did not affect by the contamination and it can still grew abundantly. As for Fig 3 (b), mycelia of *V. volvacea* grew smoothly and free from ay cross contamination. The development of mycelia was seen for the different electrospun nanofiber mats on the second day after placing the samples on PDA at room temperature. In order to measure the growth rate of *V. volvacea*, equation by [13] was applied as it is difficult to monitor the growth of viable *V. volvacea* that remained in the electrospun nanofibers without proper quantitative analysis.

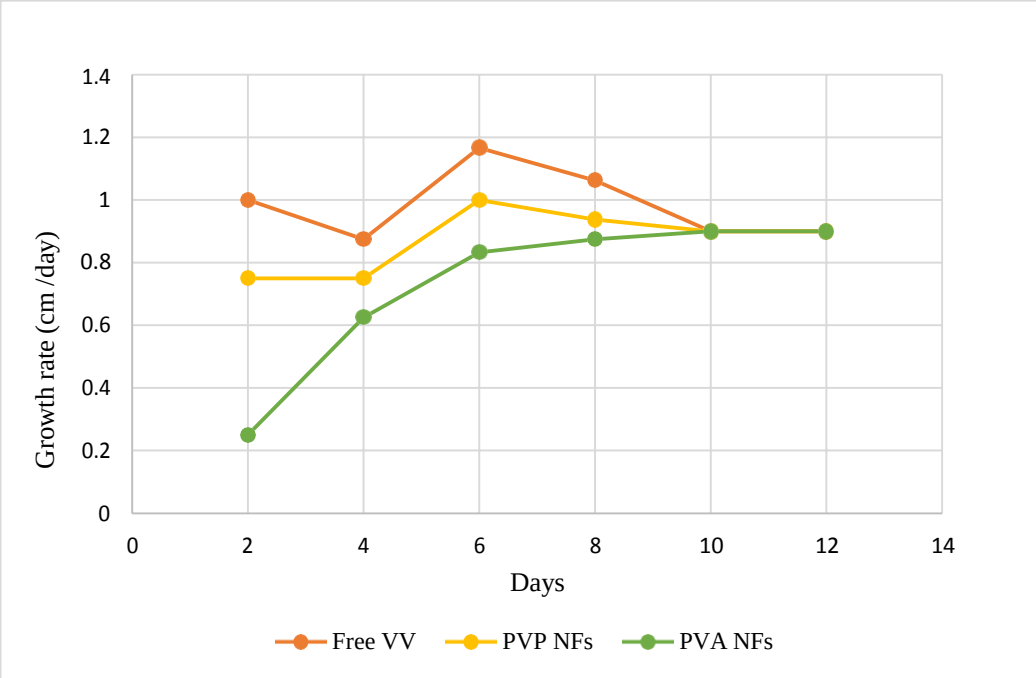

Fig.4 - Growth rate of *V. volvacea* from three different sources

The survival of the entrapped *V. volvacea* in both electrospun nanofibers were evaluated every two days (Fig 4). There was no decrease in the growth rate of *V. volvacea* in electrospun PVA nanofiber over time and it can reach up to 0.9 cm dia^{-1}. Meanwhile, electrospun PVP nanofiber showed better performance in entrapping *V. volvacea*, where the growth rate was higher than the PVA nanofiber. From day four until day six, all of the samples underwent exponential phase or known as log phase where cell divides as fast as possible according to the growth medium and the environmental conditions. During this phase, the nutrients served by the PDA has been taken and absorbed by *V. volvacea* optimally especially on day six. After the sixth day, all of the samples showed similar pattern where all of them started to approach the same growth rate on day 10 with 0.9 cm dia^{-1}. This phase is called as stationary phase where the mycelia growth stops and resulting in spore formation. During this process, nutrients are transferred from vegetative mycelium to the developing spores and followed with the death phase [14].

4. Conclusions

This pioneering study shows that fungal cell of *V. volvacea* can be successfully entrapped in dry carrier material which is nanofibers by means of electrospinning. The cells survived the electrospinning process in spite of drastic osmotic change due to electrostatic force generated from the high voltage supply. Moreover, *V. volvacea* remained viable at room temperature (30˚C) up to 12 days before entering the cycle of death phase for both types of the polymer used. However, PVP electrospun nanofibers was chosen as the best type of polymer in entrapping the *V. volvacea* due to the high growth rate as the structure of polymer itself is more immobilizing friendly compared to PVA electrospun nanofibers. These initial results show the potential of electrospinning technique as a facile and viable means for entrapment of fungal cells.

Acknowledgement

The authors are thankful to the Universiti Teknologi Malaysia for the High Impact Research Grant (Cost center no. Q.J130000.2446.04G22) for supporting this research work.

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