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Bio inactivation of furazolidone by the novel soil fungal strain *Aspergillus tamarii*

Nurul Izzati Abdul Rahman[1], Shahrul Hisham Zainal Ariffin[2], Muhd Fauzi Safian[1], Zaidah Zainal Ariffin[1]*

[1]Faculty of Applied Sciences, Universiti Teknologi MARA, Shah Alam, 40450, Selangor, Malaysia
2Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600, Bangi, Selangor
**drzaidah@uitm.edu.my*

Abstract: The present study used laboratory-scale experiments to develop methods for the bio inactivation of furazolidone from the environment. A fungus was identified as Aspergillus tamarii by morphological observation and sequencing the PCR-amplified ITS fragments of its rRNA-coding genes, it has the potential to bio inactivate furazolidone via submerged fermentation. Aspergillus tamarii was isolated from poultry farm soil with the GPS coordinates of N3.093219 E101.40269. This study observed that the best conditions for the Aspergillus tamarii to bio inactivate furazolidone are at 30°C, 150 rpm, pH 6 and 96 hours. The bio inactivation efficiency of furazolidone exceeded 97% by day 4 under optimized culture conditions which indicates its potential for future use in large-scale bio inactivation of furazolidone.

Keywords: *furazolidone, bioinactivation, Aspergillus tamarii*

Introduction

Furazolidone is a nitrofuran synthetic antimicrobial compound. It is used to treat diarrhea and enteritis caused by bacterial or protozoan infections in humans [1]. It is a stable, yellow and crystalline compound. Furazolidone, (N-(5-nitro-2-furfurylidodene)-3-2- oxazolidone) is an example of an antibiotic drug that has been used for years in the treatment of bacterial and protozoal infections [2]. According to [3], furazolidone has been widely used in aquaculture for the treatment of fish diseases or other diseases caused by bacteria. It has also been widely used in the form of premixed food additives for the treatment of gastrointestinal infections in cattle, pigs and other poultry. As it reaches toxic concentrations, it will cause harm to the environment. In order to reduce pollutants in the environment, the bio inactivation process is used to detoxify the hazardous or toxic compounds in nature. Previous research showed that eukaryotic organisms such as fungi have potential to be involved in the bio inactivation by using their own metabolic systems. The *Aspergillus* species have been proven to have the ability to degrade chemical substance pollutants by using a self-enzyme degrading system. The ability of fungi to transform or metabolize chemical pollutant is one of the ways to reduce environmental persistence and toxicity [4]. Research by [5] showed that the fungal biochemical activity is able to enhance adverse environmental damage in nuclear waste disposal units. Another research by [6] also showed *Aspergillus terreus* has an ability to degrade contaminants of endosulfan, which is an insecticide. Thus, the use of fungi as a method of pollutants bio inactivation is a potential option to clean up environmental pollutants.

Methodology

Chemicals

Furazolidone (FZD) (purity, 99.999%) was obtained from Sigma. A stock solution of 5 mg/mL furazolidone was prepared with DMSO and stored at 4°C. HPLC solvent, acetonitrile and water were purchased from Merck. All other chemicals that were used were of analytical grade.

Isolation of the Furazolidone-removing Fungi

The fungal strains were isolated from the soil of a poultry farm with GPS coordinates N3.093219 E101.40269. The diluted soil samples were plated on Potato Dextrose Agar (PDA). The plates were incubated at 25°C for two to five days depending on the visible growth of fungi. Single spores were isolated by streaking on a new streak plate PDA to acquire the pure cultures. Each pure fungal isolate was then inoculated in the Potato Dextrose Broth (PDB) and a volume of 10 mL of furazolidone was added to the culture broth after the fungal exponential phase to give a final concentration of 500 ug/ml. The flasks were then incubated for another 96 hours with 120 rpm at 25°C. Every 24h, 6 mL of medium were withdrawn as bio inactivation products. All the bio inactivation products were stored at 4°C before the extraction of nitrofuran antibiotic residual.

Corresponding Author: Zaidah Zainal Ariffin, Faculty of Applied Sciences, Universiti Teknologi MARA, Shah Alam, 40450, Selangor, Malaysia. Email: drzaidah@uitm.edu.my

Molecular Identification of F3

The isolated fungus strain that showed the best furazolidone bio inactivation was designated as F3 and grown on PDA medium for 3 days at 25°C. Genomic DNA was obtained using a kit from QIAGEN (DNeasy plant mini) and Analytic Jena (Innuprep plant mini). DNA fragments covering partial 18S rRNA, ITS 1 (5'- TCC GTA GGT GAA CCT GCG G3') forward primer and 0.2 µM ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') reverse primer were amplified and sequenced. The sequences were compared with known sequences in the database from the National Center for Biotechnology (NCBI) using BLAST search. In addition, F3 was also morphologically analyzed and stored under the Genebank accession number of KX610719.1.

Optimization of Fermentation Parameters

Single factor tests were applied to determine the best culture conditions for *Aspergillus tamarii* to bio inactivate FZD. The parameters tested included shaking frequency (90-210 rpm), initial pH (4.0-10.0), temperature (30-40°C) and incubation time (24-96 hours). The value of the parameter that gave the highest rate of FZD bio inactivation was used in subsequent steps of the study. The test of initial pH was conducted at the optimized medium, while other parameters were maintained at the same values as the previous experiment. Other parameters were similarly optimized. Each treatment was performed in triplicate.

Antimicrobial Activity of Furazolidone Residual

The soil bacteria strain of Gram-positive bacteria, *Bacillus subtilis* ATCC6633 was used. The pure cultures were grown overnight in a Muller Hinton broth and the turbidity of the suspensions were adjusted with a spectrophotometer at 600 nm to match 0.5 McFarland standard. The cells were lawned on Muller Hinton agar evenly using a sterile cotton swab. Equidistant wells were bored into the solidified agar using a sterilized cork borer of diameter 8 mm. Each plate was divided into five equal portions along the diameter. In the central hole, 100 µl of the distilled water was loaded. In one hole, 100 µl of prepared antibiotic furazolidone was loaded while in the remaining holes, 100 µl of extracted antibiotic from different flasks were loaded. The Petri dishes were incubated at 37°C for 18 hours. The diameter of the zone of inhibition was measured and the average diameter for each sample was calculated.

Quantification and Identification of Furazolidone Residual using Reversed Phase High - Performance Liquid Chromatography (HPLC)

Reversed phase analysis was performed with the use of water and acetonitrile) mixture as mobile phase [7]. Isocratic elution was used with a ratio of 40 % water and 60 % acetonitrile with a flow rate of 1.2 ml/min, C18 reversed phase column with diode array detector. In this study, HPLC grade solvents were used throughout the chromatographic analysis of the investigated antibiotic.

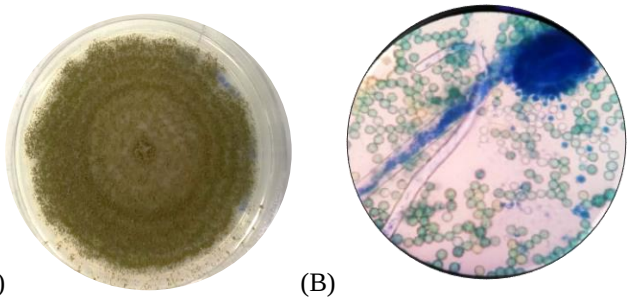
Results and Discussion

Isolation and Screening of Furazolidone Removing Fungi

A total of five species of *Aspergillus* fungi were isolated from antibiotic overexposed soil. These fungi grew on PDA and had FZD removal rates ranging in between 4.2 -29.8 % with the highest removal rate found in fungal strain F3 [8]. Strain F3 was therefore used in all subsequent experiments.

Molecular Identification of F3

The sequence of F3 was compared to other fungal sequences in the National Center for Biotechnology (NCBI) using BLAST search. The sequence alignment results showed that F3 had a 100 % similarity with *Aspergillus tamarii*. The fungal strain showed a yellow to green colored colony. It was observed to be in circular and powdery forms. The colony diameter was 8.4 cm after four days of incubation. No colour change was observed after 14 days of incubation. Microscopic observation showed long conidiophores with vesicles attached to phialides and the end of it was conidiophores (Fig 2). Based on the morphological observation and molecular identification conducted, it can be concluded that F3 was *Aspergillus tamarii*.



(A) (B)

Fig 2 Morphological observation of *Aspergillus tamarii*: Colonies of *Aspergillus tamarii* incubated at 25°C for 4 days (A). Conidia and conidiophores as observed through a microscope (Olympus CX12) under 1000 X total magnification (B).

Optimization of Fermentation Parameters

In order to improve FZD bio inactivation by *Aspergillus tamarii* and develop its potential for use in large-scale applications, the fermentation parameters including the initial pH, the agitation frequency, the incubation hours and the temperature were optimized in the laboratory.

Initial pH

Initial pH plays a big role in the bio inactivation n of FZD. In this study, *Aspergillus tamarii* showed optimum initial pH at pH 6 with 60 % percentage of inhibition against *Bacillus subtilis* ATCC6633 which contributed to 40% bio inactivation of FZD. Compared to alkaline conditions, an acidic initial pH in the culture broth had better effects on antibiotic removal. Furthermore, pH 4 also showed the same percentage of FZD bio inactivation which was 40 % after 96 hours of incubation time. However, at 24 hours incubation time, the rate of removal is lesser than the rate of removal in pH 6 (Fig 3). This finding is agreeable with [9] who also stated that the activity of fungal improved as the pH in the culture medium moved closer to a slightly acidic value. The finding also stated that this characteristic of fungal could make it widely applicable in the treatment of solid and liquid waste as the waste is usually acidic.

Agitation Speed (rpm)

Agitation speed was shown to be an important factor that affected the fungal biomass by changing the contact area between the microorganism and medium and increasing the dissolved oxygen concentration in submerged fermentation [10]. The antimicrobial activity of FZD residue against *B. subtilis* ATCC6633 showed a decrease of 47 % at 96 hours, 30 °C and 150. The rate of FZD bio inactivation at 90, 180 and 210 rpm fluctuated (Fig. 4). Thus, this shows that 150 rpm is the most effective condition for FZD bio inactivation.

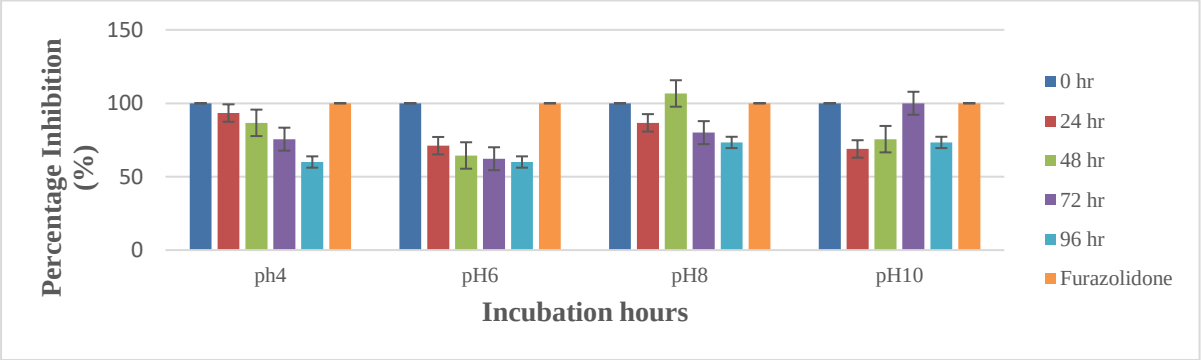


Figure 3 Percentage of inhibition of FZD residues *by Aspergillus tamarii* against *Bacillus subtilis* ATCC6633. The bar chart shows the effects of pH of the medium and incubation time on FZD removal at 150 rpm and 30°C.

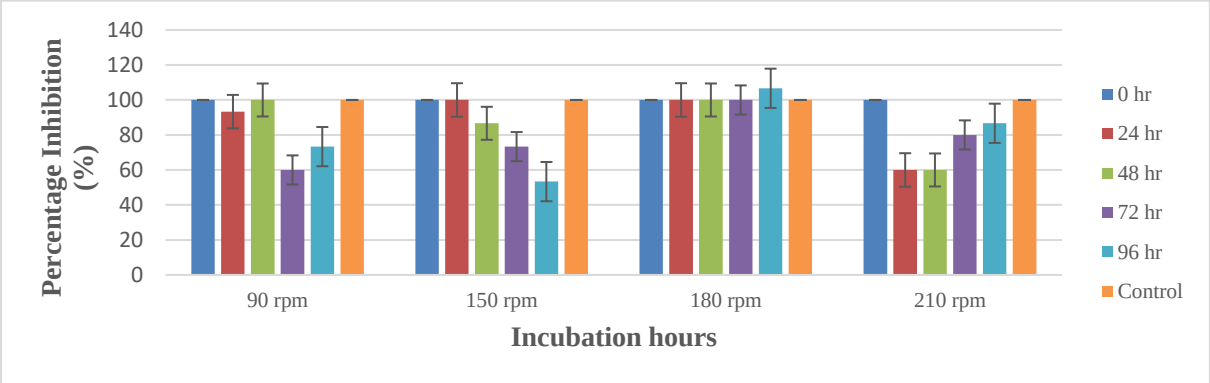


Figure 4 Percentage of inhibition of FZD residues by *Aspergillus tamarii* against *Bacillus subtilis* ATCC6633. The bar chart shows the effects of agitation speed of the medium and incubation time on the FZD removal at pH 6 and 30°C.

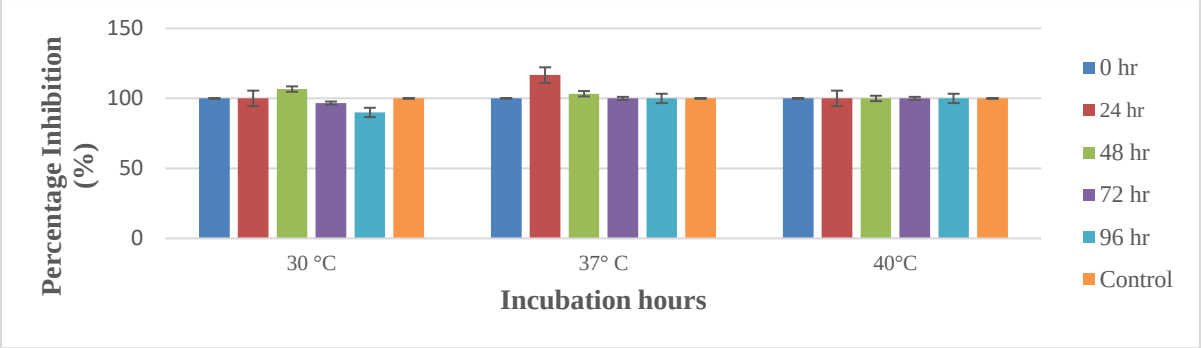


Figure 5 Percentage of inhibition of FZD residues by *Aspergillus tamarii* against *Bacillus subtilis* ATCC6633. The bar chart shows the effects of medium temperature and incubation time on the FZD removal at pH 6 and 150 rpm.

Optimization of Temperature (°C)

Temperature is usually regarded as the most important factor that affects the microbial growth of all physiological parameters. *Aspergillus tamarii* was grown at a constant initial pH 6 and 150 rpm at different temperatures of 30°C, 37°C and 40°C. Among the selected temperatures, the result which showed the optimum temperature was 30°C where the FZD bio inactivation was decreased to 80% after 96 hours of incubation time. In comparison to other temperatures, the temperature 30°C showed higher rates of FZD bio inactivation at about 20 % after 96 hours of incubation (Fig 5). Thus, this shows that 30°C is a more effective temperature to bio inactivate the FZD. This result is supported by the results of an experiment conducted by [11] using *Aspergillus carbonarius* which observed that the optimal temperature for growth was 30°C.

Optimization of Incubation time

In this study, five different incubation times were observed at 0 hour, 24 hours, 48 hours, 72 hours and 96 hours to determine the optimum time for FZD bio inactivation. Based on the results obtained, it can be concluded that 96 hours is the optimum time for the bio inactivation of FZD. According to [7], with prolonged incubation periods, nitrofurans antibiotics could be completely removed due to its fast-growing fungi with high production of mycelia mass that leads to high degradation efficiency.

Quantification of FZD Residual using HPLC

In this study, the percentage of FZD residual after 96 hours of incubation was further studied. Liquid-liquid extraction with solvent ethyl acetate as well as solid phase extraction with methanol and HPLC grade water was used to purify the FZD residuals. The FZD residuals were injected to HPLC with C18 column and the mobile phase was acetonitrile and water with a ratio of 60:40. A 20 µl of sample was injected to the HPLC. In the quantification of FZD residual using HPLC analysis, the optimal parameters used were 150 rpm, 30°C, pH 6 and 96 hours. Based on the results, the retention time for FZD standard in 50 ppm is 3.635. Based on the HPLC result, the percentage of FZD residue is 7 % which contributed to 93 % of degradation. Thus, the parameters of 150 rpm at 30 °C, pH 6 and 96 hours have been proven to be the most effective conditions to bio inactivate FZD.

Conclusion

The best conditions for the bio inactivation of FZD by *Aspergillus tamarii* is at 30°C, 150 rpm, pH 6 and 96 hours. The percentage of FZD removal in these optimal parameters of 150 rpm, 30°C, pH 6 and 96 hours is about 93 %.

Acknowledgments

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