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BIOTRANSFORMATION OF NITROFURANS ANTIBIOTICS BY *ASPERGILLUS* SPECIES – RESIDUAL ANTIBACTERIAL ACTIVITY

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Abstract

Nitrofurans antibiotics are banned drug. Nitrofurans antibiotics that administered to the animal will be excreted out in parent or metabolite through faeces. The excretion causes soil contamination and also the emerging of bacteria that resistant the nitrofurans antibiotics. The aim of this study is to isolate potential soil fungi species that able to degrade nitrofurans antibiotics and determine the antibacterial activity of the nitrofurans antibiotic residues. Nine different colonies were successfully identified using molecular tools, polymerase chain reactions. ITS 1 (forward) and ITS 4 (reverse) primer were used in this study. A total of six species from *Aspergillus* namely *Aspergillus sydowii* strain SCAU066, *Aspergillus tamarii* isolate TN-7, *Aspergillus candidus* strain KUFA 0062, *Aspergillus versicolor* isolate BAB-6580, *Aspergillus protuberus* strain KAS 6024 and the rest are *Cephaliophora tropica* strain xsd08001 and *Lichtheimia ramosa* strain R and two species from *Penicillium* which are *Penicillium citrinum* strain FIB SR4 and *Penicillium citrinum* isolate J33. Growth curve of *Aspergillus* species was plotted, most fungi showed exponential phase from day 2 to day 6. *Aspergillus* species were used in degrading nitrofurans antibiotics furaltadone, furazolidone and nitrofurazone. This study showed *Aspergillus tamarii* efficiently degraded 12-30 % of nitrofurans antibiotics after 96 h of incubation. Thus the isolated soil fungi, *Aspergillus tamarii* showed the ability to degrade nitrofurans antibiotics.

INTRODUCTION

The soil is an environment that is rich in major groups of microorganism such as bacteria, algae, and fungi [1]. It is a complex system that is an oligotrophic medium for the growth of fungi. According to [2], soil microorganisms play a significant role in soil organic matter and nutrient cycling. Species of mycoflora present in soil depend on the characteristics of the soil itself. Soil characteristics such as pH level, temperature, moisture, organic carbon and organic nitrogen, affect the growth population of soil fungi. Antibiotics are widely used to treat bacterial infections in both animals and humans. Antibiotics that used in animal husbandry to treat disease and act as growth promoter lead to soil contamination. Antibiotics that administered to the animal will be excreted out through faeces or urine in the form of parent drug or metabolites. Most administered veterinary antibiotics were unable to be absorbed by animals with a range of 20 to 90 % and excreted from the body in their original form via faeces or urine [3], which can lead to soil contamination and bacteria resistant to antibiotics.

Nitrofurans (NFs) are a group of synthetic broad antimicrobial spectrums agent. Four major nitrofurans (NFs) are nitrofurazone, nitrofurantoin, furaltadone and furazolidone [4]. Nitrofurans were mainly used for livestock in animals, to prevent bacterial infections and also commonly used as food additives to promote growth [5, 6]. Nitrofurans were banned in the European Union because it has carcinogenic effects on humans, [7]. As the presence nitrofurans residues in animal edible tissue become a crucial concern in health issue, the utilization of nitrofurans in animal food processing was the reason it was banned in European Union (EU). Nitrofurans can be introduced into the environment through the application of lagoon water and through manure from fertilizers [8]. Degradation or transformation of nitrofurans antibiotics using isolated soil fungi able to treat soil contamination and reduce antibiotic resistance.

MATERIALS AND METHODS

Soil sample collection

Soil sample was collected at a soil plot where veterinary antibiotics have been used extensively from Ayu Farm, Meru, Klang with a GPS coordinate of N3.093219 E101.40269. A weight of 500 g of soil was collected from a depth of 15 cm. Soil samples collected and preserved in sterile plastic bags and before being stored in an icebox to maintain the soil's natural properties. The soil was then taken to the laboratory and stored at 4 °C.

Isolation of soil fungi

The soil samples then underwent serial dilution. Soil with 3 and 4 dilution factors were plated on Potato Dextrose Agar (PDA). Plates were incubated at 25 °C for two to five days depending on the visible growth of the fungi. A single colony was streaked on PDA plate to obtain pure cultures. The plate was incubated for two to five days and kept at 4 °C in a refrigerator.

Identification of isolated soil fungi

DNA of the isolated soil fungi were extracted using two methods. DNA of *Aspergillus versicolor* isolate BAB-6580, *Penicillium citrinum*, *Aspergillus protuberus* strain KAS 6024 were extracted using a modified DNeasy Plant Mini Kit by QIAGEN with additional step prior using the kit. The additional steps involved Cetyltrimethyldibromide (CTAB) method. This method consists of, 2 % CTAB, 5 M Potassium acetate and 10 % SDS. Scraps of isolated fungi were taken and crushed using liquid nitrogen. 300 µL of 2 % CTAB was added and mixed using a vortex. This was then followed by the introduction of 120 µL of 10 % SDS into the tube and then a vortex process for a second round. The tube was incubated in water bath at 60 °C, for 30 minutes. 300 µL of 5M Potassium acetate was added and mixed. The tubes were allowed to cool before proceeding with the Dneasy Plant Mini Kit protocol. DNA of *Cephaliophora tropica* strain xsd08001, *Lichtheimia ramosa* strain R, *Aspergillus sydowii* strains SCAU066, *Aspergillus tamarii* isolate TN-7, *Aspergillus candidus* and *Penicillium citrinum* isolate J33 was extracted using innuPrep Plant DNA kit from Analytic Jena. *Cephaliophora tropica* strain xsd08001 was extracted using lysis buffer OPT, while *Lichtheimia ramosa* strain R, *Aspergillus versicolor* strain LSDSF0101, *Aspergillus tamarii* isolate TN-7, *Aspergillus candidus* and *Penicillium citrinum* isolate J33 involved a lysis buffer CBV. The extractions were followed the kit protocol. The DNA extracted was amplified using 0.2 µM 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. PCR master mix was prepared using Top Taq DNA polymerase by QIAGEN which contained 10x TopTaq PCR buffer, Coralload concentrate, Q-solution, dNTP mix and TopTaq DNA polymerase. PCR conditions were initial denaturation (94 °C for 3 minutes), 30 cycles of denaturation (94 °C for 30 seconds), annealing (61.4 °C, 30 seconds), extension (72 °C, 1 minute) and final extension (72 °C, 5 minutes)

Growth curve

Growth curve of the isolated soil fungi was determined based on [9] with some modifications. The growth curve was determined based on the dry weight of the fungus or the biomass production.
Two small cuts of the potato dextrose agar with soil fungus cultures were transferred into 250 mL Erlenmeyer flasks which contained 100 mL of potato dextrose broth. The fungal cultures will be incubated at 25 °C on an orbital shaker (160 rpm). 6 flasks were used for each culture. During the growth of strains in a liquid medium, replicate Erlenmeyer flasks were withdrawn from the orbital shaker one by one at 6 different times (1, 2, 3, 6, 8 and 10 days). The mycelia were filtered on a Buchner apparatus and were dried at 70 °C for 24 h. The growth curves of fungi were plotted of dry cell weights versus incubation time (day). The experiments were done in triplicates.

Fermentation of nitrofurans antibiotics

Fermentation was done according to [10] with some modifications using Potato Dextrose Broth (PDB). The PDB was distributed equally at 100 mL into 4 250 mL Erlenmeyer flasks. Under aseptic conditions, a scrap of fungi was inoculated into each culture flask accordingly. PDB with culture only was used as biotic control while PDB with nitrofuran only was used as abiotic control. All culture flasks were incubated with agitation fixed at 120 rpm and 25 °C until exponential or log phase was reached. All experiment were carried out in triplicates.

After the exponential or log phase, a volume of 10 mL of nitrofuran antibiotics such as furaltadone, furazolidone and nitrofurazone was added to each culture flask to give a final concentration of 500 µg/mL. The flasks were then incubated for another 96 h at 120 rpm and 25 °C. A volume of 6 mL of medium were withdrawn as biotransformation products every 24 h. All the biotransformation products were stored at 4 °C before the extraction of nitrofuran antibiotic residual.

Liquid-liquid extraction (LLE)

The biotransformation products were subjected to liquid-liquid extraction (LLE) every 24 h of incubation. This extraction technique was done according to [11] and was carried out in a separating funnel. All samples were mixed with an ethyl acetate (EtoAc) extraction solvent. A volume of 25 mL of EtOAc and 25 mL of distilled water were added to the separating funnel together with the 6 mL of biotransformation products. Extraction of nitrofuran antibiotic residual was carried out by shaking the sample with EtOAc and distilled water for two minutes and periodically venting air to allow phase separation process. After 10 to 15 minutes, the lower organic layer was repeated twice by adding the same volume of EtOAc for each process. All extractions collected were combined and concentrated by using a rotary evaporator. 500 µL of dimethyl sulfoxide (DMSO) was used to mix concentrated nitrofuran residual and stored in microcentrifuge tube at 4 °C.

Antibacterial of nitrofurans antibiotics residues

Antimicrobial testing was done using nitrofurans antibiotic residual such as furaltadone, furazolidone and nitrofurantoin. This test was conducted against selected bacteria species that are

susceptible to the respected nitrofurans antibiotics. *Escherichia coli*, *Salmonella typhi* and *Streptococcus pyogene* were selected for furaltadone residual, *Escherichia coli*, *Streptococcus pyogene* and *Streptococcus aureus* were selected for furazolidone antibiotic residues, and *Escherichia coli*, *Salmonella typhi*, and *Staphylococcus aureus* were selected for nitrofurazone residues.
The antimicrobial assay of the selected bacteria was carried out by using well diffusion techniques [12] with some modifications [13]. Overnight cultures were prepared in Mueller-Hinton broth and diluted to 10^8 CFU ml^{-1} based on 0.5 McFarland standards. Sterile cotton swabs were dipped into the culture and swabbed all over the surface of Mueller Hinton agar plates in triplicates and allowed to dry. Five wells were made on agar plates by using sterile cork borer. One well was reserved for sterile DMSO as a negative control and another one well was reserved for clindamycin antibiotic with a concentration of 40 µg/mL as positive control. The zones of inhibition produced by each well were measured in three directions around the disc and mean diameter was recorded.

RESULTS AND DISCUSSION

Identification of isolated fungus

Nine species of fungi were successfully isolated from the soil that had been exposed to antibiotics. The species that were identified using molecular tools were *Cephaliophora tropica* strain xsd08001, *Lichtheimia ramosa* strain R, *Aspergillus sydowii* strain SCAU066, *Aspergillus tamarii* isolate TN-7, *Aspergillus candidus* strain KUFA 0062, *Aspergillus versicolor* isolate BAB-6580, *Aspergillus protuberus* strain KAS 6024, *Penicillium citrninum* strain FIB SR4 and *Penicillium citrinum* isolate J33. The findings revealed that *Aspergillu*s species made up the majority of the isolated soil fungi. *Aspergillus* was the most common type found in the soil, followed by *Penicillium* species, *Cephaliophora tropica* and *Lichtheimia ramosa* [14]. The isolated soil fungi were isolated from soil with pH 6.8. According to [15], *Aspergillus* species tolerant more to alkaline pH rather than *Penicillium species.*

Growth curve

This research was focussed on Aspergillus species as their ability to biotransform xenobiotics. Growth curve of isolated *Aspergillus* species was plotted (**Figure 1**). Based on the graph plotted, most fungi showed exponential phase from day 3 to day 6. *Aspergillus tamarii* isolate TN-7 grews exponentially after 2 days of incubation and was in the stationary phase between day 3 and 6. The exponential phase of Aspergillus *candidus* strain KUFA 0062 was after 6 days of incubation. *Aspergillus versicolor* isolate BAB-6580 grews exponentially on day 3 and reached stationary phase after 4 days of incubation. *Aspergillus protuberus* strain KAS 6024 grews exponentially between day 2 and day 3. *Aspergillus candidus* strain KUFA 0062 showed slow growing fungi as the exponential phase started to occur on day 6 while *Aspergillus tamarii* isolate TN-7 showed fast growing fungi as the exponential phase range was between 2 – 3 days of incubation.

Antibacterial activity of nitrofurans residues

Five *Aspergillus* species such as *Aspergillus sydowii* strain SCAU066, *Aspergillus tamarii* isolate TN-7, *Aspergillus candidus* strain KUFA 0062, *Aspergillus versicolor* isolate BAB-6580 and

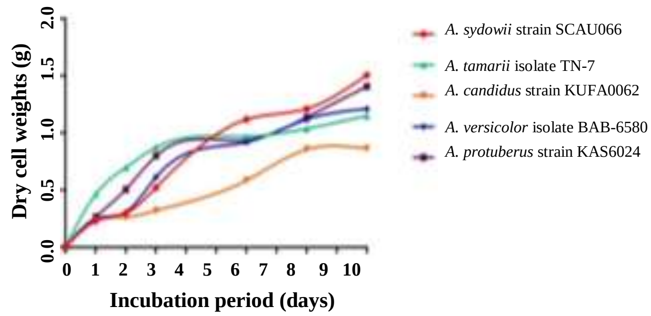

Figure 1. Growth curve of isolated *Aspergillus* species incubated for 10 days.

Aspergillus protuberus strain KAS 6024, were selected to undergo a degradation of nitrofurans antibiotics. This is because of the *Aspergillus* species have the ability to degrade antibiotics, dyes, steroids and chemical pollutant. Percentage of inhibition was calculated by the diameter of the nitrofurans residues inhibition zone over the diameter of the abiotic control inhibition zone. The diameter of abiotic control served as a control, where no degradation of nitrofurans antibiotic occurred. Degraded or transformed antibiotic would lose its function as antibacterial as the chemical structure of the antibiotic was modified or degraded. Nitrofurans antibiotic residues from 96 h of incubation showed a decrease in the percentage of inhibition towards selected bacteria (**Table 1**). All *Aspergillus* species that were used showed a good degradation activity as the inhibition zone of the nitrofurans residues were decreased when compared to the abiotic control. 96 h of nitrofurans residues showed all the *Aspergillus* species degradation were able to degrade the antibiotics. *A. sydowii* strain SCAU066 led to a 6-16 % degradation of nitrofurans antibiotics. *A. sydowii* showed its potential ability in degrading organopollutant in the environment. According to[16], *A. sydowii* that was isolated from marine environment degraded methyl parathion (MP) efficiently in 30 days. *A. sydowii* phosphatase efficiently hydrolyzed pesticides at 300 to 100 ppm in soil. Meanwhile, nitrofurans antibiotics residues of 96 h incubation by *A. versicolor* isolate BAB-6580 showed a 2-23 %. *A. versicolor* after 3 days of cultivation [17] demonstrated that *A. versicolor* is a PBSA-degrading microorganism. According to [18], *A. versicolor* was able to degrade low-density polyethylene (LDPE) waste disposal by 40 % after 90 days of incubation. Both *Aspergillus sydowii* and *Aspergillus versicolor* which are closely related fungus showed the ability to degrade formaldehyde and other polymer materials [19]. *A. candidus* strain KUFA 0062 showed a slow reduction of nitrofurans antibiotics as only 2-13% of nitrofurans were reduced after 96h of incubation. Due to its slow-growing fungi, the reduction of nitrofurans antibiotics was equally slow. Other than that, [20] reported that *A. candidus* was able to remove arsenic in the marine environment. It was recorded that the highest concentration removal of arsenic occurred after 3 days. No research has been conducted studying degradation activity of *A. protuberus*. However, in this study, *Aspergillus protuberus* showed its potential ability to degrade antibiotics. From the *Aspergillus* species used, *A. tamarii* showed good degrading fungi because it showed a decrease in percentage of inhibition compared to abiotic control after 24 h of incubation (**Figure 2**). After 96 h of incubation, nitrofurans were reduced to

Table 1. Percentage of inhibition zone of nitrofurans antibiotic residues.

24 h residues	**Culture**	***A. tamarii***	***A.versicolor***	***A. protuberus***	***A.sydowii***	***A. candidus***
Furaltadone	*E. coli*	94.05	100.00	100	98.81	100.00
	S. typhi	97.44	98.15	100	98.72	100.00
	S. pyogene	95.24	100.00	90.48	98.67	98.413
Furazolidone	*E. coli*	100.00	100.00	100.00	98.85	100.00
	S.pyogene	84.52	100.00	96.55	100.00	100.00
	S. aureus	91.95	96.55	95.46	100.00	100.00
Nitrofurazone	*E. coli*	101.33	100.00	100.00	100.00	100.00
	S. typhi	87.65	88.89	100.00	95.062	100.00
	S. aureus	92.47	95.70	97.37	100.00	90.32
48 h residues	**Culture**	***A. tamarii***	***A.versicolor***	***A. protuberus***	***A.sydowii***	***A. candidus***
Furaltadone	*E. coli*	94.04	89.29	98.15	96.44	97.62
	S. typhi	93.59	97.44	90.39	97.44	100.00
	S. pyogene	90.91	64.00	78.57	85.33	96.83
Furazolidone	*E. coli*	96.55	100.00	94.12	97.70	96.55
	S.pyogene	76.19	96.43	96.55	97.62	98.81
	S. aureus	90.80	95.40	95.46	97.70	97.70
Nitrofurazone	*E. coli*	85.71	100.00	93.55	97.70	100.00
	S. typhi	81.48	88.89	94.83	93.83	100.00
	S. aureus	76.34	95.70	94.83	98.923	91.40

72 h residues	**Culture**	***A. tamarii***	***A.versicolor***	***A. protuberus***	***A.sydowii***	***A. candidus***
Furaltadone	*E. coli*	86.91	88.10	96.30	100.00	96.43
	S. typhi	85.90	94.87	88.46	96.15	98.72
	S. pyogene	89.74	60.00	78.57	77.33	92.06
Furazolidone	*E. coli*	86.21	98.85	94.12	97.70	95.40
	S.pyogene	70.24	95.24	94.823	96.43	95.24
	S. aureus	80.46	95.40	93.94	98.85	93.10
Nitrofurazone	*E. coli*	85.71	96.55	92.12	93.10	98.85
	S. typhi	81.48	85.19	93.10	92.59	96.30
	S. aureus	75.27	90.32	94.74	97.85	88.17

96 h residues	**Culture**	***A. tamarii***	***A. sydowii***	***A. protuberus***	***A. versicolor***	***A. candidus***
Furaltadone	*E. coli*	88.36	84.52	92.58	100	95.24
	S. typhi	84.62	94.23	86.54	98.87	98.72
	S. pyogene	85.71	94.11	76.19	77.33	90.48
Furazolidone	*E. coli*	80.46	94.25	91.18	97.70	94.83
	S.pyogene	70.24	91.67	94.83	95.24	92.86
	S. aureus	75.86	91.95	84.85	88.51	93.10
Nitrofurazone	*E. coli*	77.01	93.10	86.l8	90.81	97.70
	S. typhi	77.78	81.48	86.21	90.12	96.30
	S. aureus	70.97	85.48	92.11	87.10	87.10

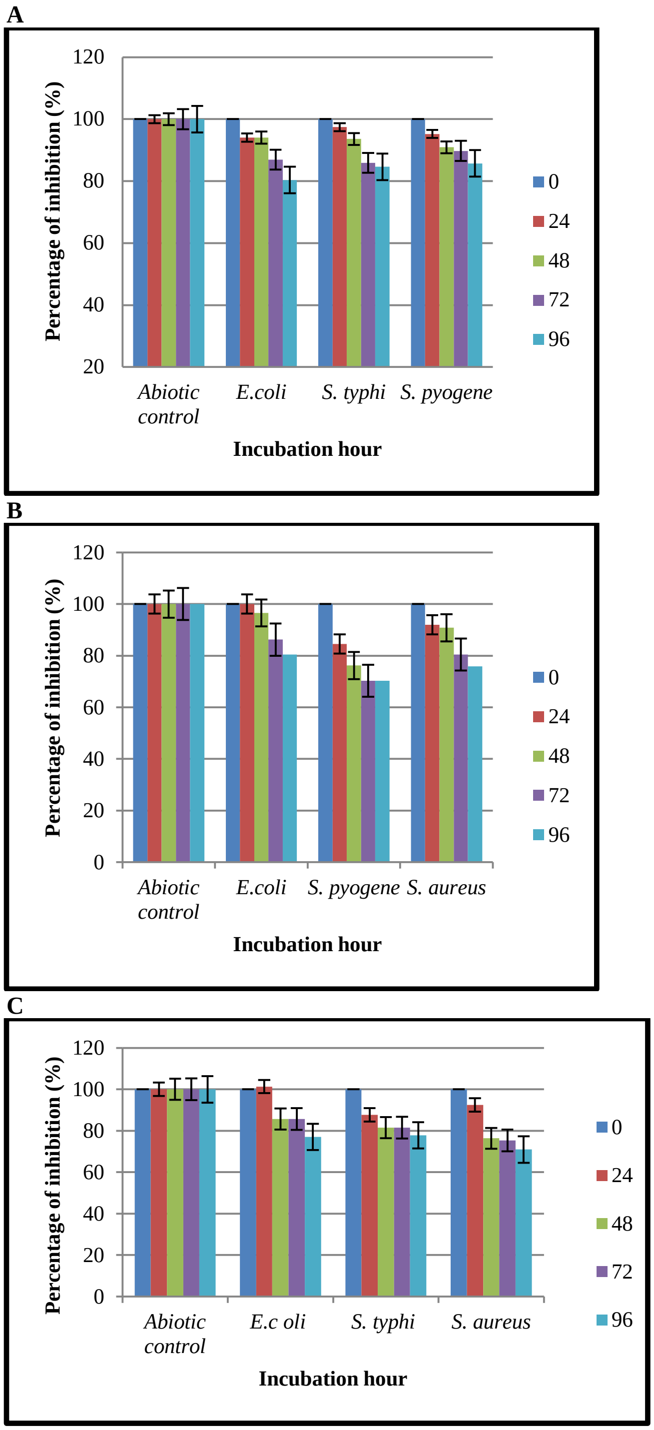

Figure 2. Percentage of inhibition zone of nitrofurans residues by *Aspergillus tamarii* isolate TN-7. A) Furaltadone; B) Furazolidone; C) Nitrofurazone. The error bars represent the mean ± SE of triplicates.

12-30 % by *A. tamarii*. A fast growing fungus with high production of mycelia mass will lead to high degradation efficiency. With prolong incubation period, nitrofurans antibiotics could be totally removed due to its fast-growing fungi with high production of mycelia mass that leads to high degradation efficiency. Studied done by [21] reported that, *A. tamarii* was capable of degrading endosulfan sulfate. Also, laccase extracted from *Aspergillus tamarii* had the ability to degrade almost 90% of recalcitrant dyes in textile effluent after 13 days of incubation. Other than that, [22] reported that *A. tamarii* was able to remove chromium synthetic dyes in batch and continuous bioreactors. To date, no research has been done in studying degradation or transformation of nitrofurans antibiotics using *A. sydowii*, *A. tamarii*, *A. candidus*, *A. versicolor* and *A. protuberus*.

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