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Article

Kinetics of Deoxynivalenol Degradation by *Aspergillus oryzae* and *Rhizopus oryzae* in Submerged Fermentation

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O objetivo foi avaliar a capacidade das espécies fúngicas *Aspergillus oryzae* e *Rhizopus oryzae* em degradar desoxinivalenol (DON) em fermentação submersa. O meio submerso utilizado foi água destilada estéril contaminada com 1μg mL^{-1} DON, inoculado com 4×10^6 esporos mL^{-1} das espécies fúngicas. A amostragem foi realizada a cada 48 h até 240 h de processo, determinando a massa residual de DON, percentual e velocidade de degradação. A massa residual de DON no meio coletado foi extraída por partição líquido-líquido e quantificada por cromatografia gasosa através de derivação com anidrido trifluoroacético. O tempo requerido para a maior degradação de DON foi 96 e 240 h para *Aspergillus oryzae* e *Rhizopus* oryzae, respectivamente, e velocidade de degradação de 0,62 e 0,54 μg h^{-1}, respectivamente. *Rhizopus oryzae* ocasionou a maior diminuição na concentração de DON, aproximadamente 90% em 240 h, enquanto *Aspergillus oryzae* ocasionou com maior rapidez, 74% de redução em 96 h.

The objective was to evaluate the capacity of *Aspergillus oryzae* and *Rhizopus oryzae* to degrade deoxynivalenol (DON) during submerged fermentation. The submerged medium utilized was sterile distilled water with 1μg mL^{-1} DON, added and inoculated with 4×10^6 spores mL^{-1} of the fungal species. Sampling was performed every 48 h to 240 h. DON analyses included residual mass, percentage and velocity of degradation. Residual mass of DON in the collected medium was extracted by liquid-liquid partition and quantified by gas chromatography through derivation with trifluoroacetic anhydride. The time required for the largest DON degradation was 96 h and 240 h by *Aspergillus oryzae* and *Rhizopus oryzae* respectively, and degradation rate were 0.62 and 0.54 μg h^{-1}, respectively. *Rhizopus oryzae* caused the largest decrease in DON at around 90% in 240 h, while *Aspergillus oryzae* caused the most rapid degradation with a 74% reduction of DON at 96 h.

Keywords: biodegradation, mycotoxin, submerged fermentation

Introduction

Fungi produce a wide variety of secondary metabolites called mycotoxins with various structures,[1-4] which occur in the filamentous fungi, usually after a balanced growth phase followed by stressful conditions.[1,5] *Aspergillus*, *Penicillium*, *Claviceps* and *Fusarium* are among the fungal genera which possess toxigenic species and most frequently appear in contaminated raw materials, food and feed.[6,7]

The presence of toxigenic fungi in food does not mean that mycotoxins are present, but indicates the possibility of their production in response to environmental conditions. On the other hand, the absence of these fungi in food does not correspond to the lack of these toxic compounds, considering that mycotoxins may persist for long periods in adverse conditions even if the microorganisms have lost their viability.[8]

Commonly detected fungal toxins are aflatoxins, ochratoxin A (OTA), trichothecenes (deoxynivalenol-DON and T-2 toxin), zearalenone, fumonisins and some alkaloids. Trichothecenes are considered one of the most important mycotoxin groups, with over 100 detected natural structures, which contaminated about 35% of agricultural products produced worldwide.[2,6,9,10] Deoxynivalenol (vomitoxin, DON) is the most frequently detected trichothecene, belonging to trichothecene group B, in food and feed. The molecule is a tetracyclic sesquiterpenoid with seven stereo centers, registration number 51481-10-8; empirical formula $C_{15}H_{20}O_6$, name trichothec-9-en-8-one,12,13-epoxy-3,7,15-trihydroxy- (3α,7α)-(9CI).[6,11]

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Generally, DON is resistant to environmental and food processing degradation. It is a non-volatile compound and may be deactivated by the destruction of the epoxy ring under drastic acid or alkaline conditions, in the presence of aluminum or lithium hydrates or peroxides and hydration in autoclave. Physical and chemical treatments cause variations in the properties of the compound and its matrix, making it difficult to determine the presence or absence of the compound in decontaminated materials.[4,12]

DON may have its chemical structure altered by microbial metabolism and, in some cases, this causes detoxification.[19] In the literature, there are no studies that demonstrate DON degradation by filamentous fungi; however, acid, lactic and propionic bacterium *Agrobacterium*, *Rhizobium*, *Eubacterium*, as well as a yeast, have been shown to have such potential.[14-22] In some cases, total degradation occurs, resulting in an efficient decontamination process. Some species of the genera *Rhizopus* and *Aspergillus* have been cited as capable of degrading other mycotoxins, such as ocratoxin A, zearalenone, patulin and aflatoxins. They may, therefore, be promising for use in degradation of DON with appropriate fermentative processes.[14-17,20,21,23-25] The objective of this work was to evaluate the capacity of the fungal species *Aspergillus oryzae* and *Rhizopus oryzae* to degrade deoxynivalenol in submerged fermentation.

Experimental

Mycotoxin

Mycotoxin standard was acquired from the Sigma Chemical Company (EUA). DON stock solution was prepared by dissolving the toxin in benzene:acetonitrile (95:5), resulting in a 100 μg mL^{-1} concentration, according to Shepherd and Gilbert.[26] The work solution was 50 μg mL^{-1} as estimated by the (m/v) relationship in the preparation of solution, and confirmed by the procedure described of Bennett and Shotwell,[27] utilizing the molar absorptivity of the standard (5913 at 219 nm). Mycotoxin handling followed all the safety procedures like the use of suitable equipment, besides no dispersion of mycotoxin in the preparation local.

Fungal species

The fungal species *A. oryzae* CCT 3940 and *R. oryzae* were donated by the Microbiology Laboratory (Laboratório de Microbiologia) of Universidade Federal do Rio Grande, and were maintained on potato dextrose agar for 7 days. After this period, the agar surface was washed with sterile 0.1% Tween 80 for spore removal, and spore concentration estimated by direct microscopic counting utilizing a Neubauer chamber.

Degradation study in submerged fermentation

The culture medium was prepared containing 1 μg mL^{-1} DON in sterile distilled water. It was inoculated separately with 4×10^6 spores mL^{-1} of *A. oryzae* or *R. oryzae* in a 150 mL flask. In parallel, non-contaminated and inoculated media (control 1) and contaminated but not inoculated media (control 2), were prepared. In all cases, triplicates were tested.

Flasks containing the fermentative medium were inoculed for 240 h, at 30 °C under orbital agitation (200 rpm). A sample of fermented media (5 mL) was removed for analysis every 48 h and the residual DON was quantified. The sampling procedure was performed asseptically.

Quantification of residual DON

Quantification of the mycotoxin residual level in fermented medium was performed by liquid-liquid partition utilizing methylene chloride at a 5:3 (medium:solvent) according to Garda *et al.*[18] DON was derivatized with trifluoroacetic anhydride reagent and quantified by gas chromatography employing the method described in Garda and Badiale-Furlong.[28]

The gas chromatograph utilized was a Varian model 3400 equipped with a split/splitless injector and flame ionization detector (Varian-USA) 30 meter DB-17 column (J&W Scientific-USA) with 0.25 mm internal diameter and 0.25 μm 50% phenyl methylpolysiloxane film. The equipment was monitored by Star Chromatography Workstation software, version 4.1, Varian. The chromatographic conditions employed were: injector temperature 250 ºC, valve opening 0.75 min, injector cleaning flow 75 mL min^{-1}, detector 300 ºC, attenuation of 16×10^{12}. The chromatographic column program was 100 ºC for 1 min increasing at 50 ºC min^{-1} to 200 ºC holding for 2 min, followed by a 4 ºC min^{-1} increase to 250 ºC, and a final hold time of 11.5 min, completing 29 min of chromatographic run.

The detection limit was determined by successive dilutions of a 10 ng μL^{-1} solution, until generating a detection signal three times above the standard deviation at the same DON retention time when injecting the derived control. The relative time and area were evaluated considering the arachidonic acid methyl ester as internal standard (Sigma - USA) added to the sample at 0.01 μg μL^{-1}. Recovery was established through addition of DON ranging from 10 to 50 μg

to 50 mL of medium. Extraction and quantification steps were executed according to method described by Garda and Badiale-Furlong.[28] The experiments were done in triplicate.

Degradation rate

Percentage of degradation was estimated by determining the residual DON over the course of the fermentation using equation 1. The degraded DON mass was correlated with time and DON residual resulting in a degradation rate, equation 2.

$$\text{Degradation } \% = (\Delta m_{DON} \times 100) / m_{initial} \quad (1)$$

$$v = \Delta m_{DON} / \Delta t \quad (2)$$

Statistical treatment

Significant differences among degradation data (%) and specific rate (equation 2) were determined through analysis of variance (ANOVA) and comparison of means by Tukey Tests. Differences were considered significant when $p < 0.05$.

Results and Discussions

Relative DON retention time in the chromatographic system employed was 0.62, as presented in Figure 1. Method performance had merit, as indicated by a 0.28 μg mL^{-1} fermented medium detection limit and 96% recovery, presenting an 8% variation coefficient among the different tested levels (1 to 10 μg mL^{-1}).

DON degradation by *A. oryzae* and *R. oryzae* was calculated based on DON mass remaining in the medium fermented during 240 h. Table 1 shows the results for residual DON mass (μg) contained in 50 mL of fermented medium, graphed in Figure 2; and percentage and rate of degradation as mean of three experiments. The initial degradation for control 2 was around 11.1% due to contamination carried out 24 h

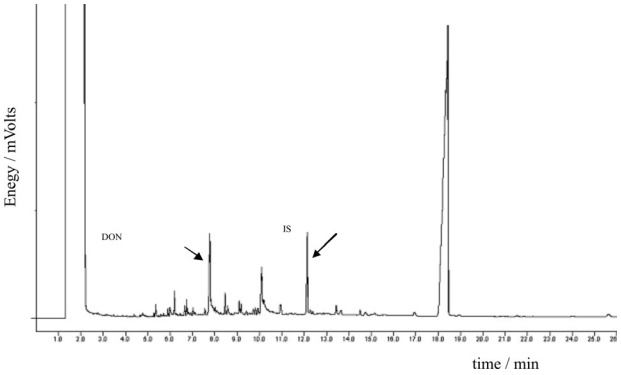

Figure 1. Chromatogram of the fermented medium contaminated with DON (IS: internal standart).

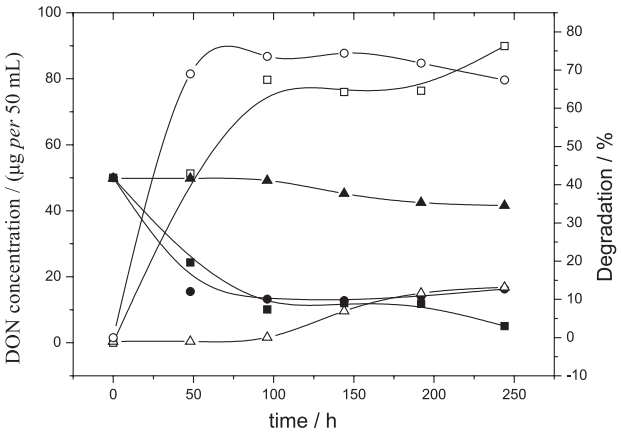

Figure 2. Kinetics of DON degradation during 240 h of submerged fermentation by fungal species *A. oryzae* and *R. oryzae* (□) degradation % in extract fermented with *R. oryzae*; (○) degradation % in extracted fermented with *A. oryzae*; (■) DON concentration in extract fermented with *R. oryzae*; (●) DON concentration in extract fermented with *A. oryzae*; (△) degradation % in experiment without inoculum-control; (▲) DON concentration in experiment without inoculum-control.

Table 1. Residual DON content, degradation percentage and rate of DON degradation during submerged fermentation

time / h	*A. oryzae*				*R. oryzae*			
	DON residual / μg (SD)	Degradation / % (SD)	Rate / (μg h^{-1})	Protein soluble / (mg mL^{-1})	DON residual / μg (SD)	Degradation / % (SD)	Rate / (μg h^{-1})	Protein soluble / (mg mL^{-1})
0	44.4 (2.0)	11.14 (0.07)	0^{c}	32.7	49.7 (0.37)	0.7 (0.01)	0^{a}	32.0
48	15.6 (6.1)	69^{a} (1.00)	0.62^{c}	32.7	24.4 (3.4)	51 (0.17)	0.54^{d}	32.8
96	13.2(4.0)	$73.6^{b,c}$ (1.03)	0.05^{d}	41.1	10.1 (0.8)	80 (0.51)	0.3^{c}	40.5
144	12.8 (0. 9)	74.4^{c} (1.06)	0.01^{b}	42.1	12 (1.9)	76^{1} (0.58)	0.0^{a}	41.8
192	14.1 (2.11)	71.8^{b} (1,01)	0.0^{b}	42.5	11.8 (0.9)	76^{1} (0.26)	0.0^{a}	43.2
240	16.3 (3.4)	67.4^{a} (0.23)	0.0^{a}	42.6	5.0 (0.5)	90 (0.26)	0.14^{b}	44.1
Mean			0.12	39.0			0.19	39.0

SD: Standard deviation. $^{a-d}$ Different letters in the same column indicate that means differ significantly among them at a 95% level ($p < 0.05$) according to Tukey Test. Rate: DON degradation rate by fungal species.

before inoculum addition, when was started the time of fermentation.

The monitoring of percentage of DON degradation showed that the time necessary for occurrence of the highest decrease in contamination was 48 h by *A. oryzae*, with approximately 74% of degradation. This effect may be caused by the high fungal activity indicated by an increase in soluble protein level at this fermentation time and decrease in DON detected in the culture media (Table 1), suggesting the use of mycotoxin as carbon source. At 144 h of fermentation, a degradation of 74% occurred too (Table 1). This aspect is important when considering the maintenance cost of fermentative conditions for long periods, besides the contamination risk.

Young *et al.*[22] showed that a process utilizing chicken intestinal microorganisms in an anaerobic system after 72 h resulted in rupture of the epoxide of DON. The genus *Aspergillus*, according to Varga *et al*.,[20] 96 h were also efficient for ochratoxin A degradation. In this work, *R. oryzae* presented the highest degradation percent (90%) after 244 h.

The highest degradation rate was verified for both fungal species at 48 h after the experiment beginning. During this time, *A. oryzae* degraded DON with a higher rate (0.62 μg DON h^{-1}) than *R. oryzae* (0.54 μg DON h^{-1}): the degradation rates are presented in Table 1. After this time, the velocity decreased for both fungal species and it remained almost constant until the end of the studied time intervals. The degradation rate suggests that it was a reaction of pseudo first and zero order registered after 48 h when the highest degradation rates were verified for both fungal species.

The mean rate of degradation was 0.12 and 0.19 μg DON h^{-1} for *A. oryzae* and *R. oryzae* respectively, suggesting that *R. oryzae* possessed higher rate for toxin degradation when considering 240 fermentation hours. However, when considering the time interval of 48 h of the submerged fermentation, the highest rates occurred for both fungal species. During this time, *A. oryzae* degraded DON with a higher rate (0.62 μg h^{-1}) when compared to *R. oryzae* (0.54 μg h^{-1}).

It is notable that despite the fact that *R. oryzae* reached the end of the process with the highest degradation percentage, *A. oryzae* degraded DON with the highest rate. This behavior suggests that the system degradation of *R. oryzae* was saturated after 48 h of the process. Therefore, comparison of mean rate at the studied time is not the best indicative of adequacy of the microorganism for decontamination. When the intention is reaching decontamination levels superior to 50%, both microorganisms are similar at 48 h of the process. In situations where a fast decontamination of raw material is intended, *A. oryzae* is the indicated microorganism, due to the fact that it presents higher rates and degradation percentages, also after 48 h of the process. Contrarily, *R. oryzae* is indicated when the intention is reaching higher decontamination rates over longer terms, with evidenced applicability if stocking is required.

The presence of interferents, possibly produced during fermentation when utilizing *A. oryzae* inoculum for control group experiments (experiment without contamination DON) were detected in the chromatogram. These compounds may also be from the secondary metabolism of the utilized microorganism, provoking an insignificant effect for the degradation kinetics study. For *R. oryzae*, values were 0.29 with a 0.023 deviation, with the same effect being observed.

Degradation kinetics reinforces the hypothesis that the degradation mechanism occurring throughout the fermentation process was toxin adsorption as the main degradation pathway up to the 144 h interval (74% degradation). After this time, the conditions present in the medium alter the adsorption process, liberating once again portions of mycotoxins and resulting in a lower rate in 240 h, 67% DON degradation.

Conclusions

Fungal species *A. oryzae* and *R. oryzae* during submerged fermentation process showed that the time required for attaining the highest degradation was 96 and 240 h; the mean degradation rate was 0.12 and 0.19 μg h^{-1}; and the maximum degradation velocity was 0.62 and 0.54 μg h^{-1}, respectively.

These data demonstrated that fungal species *R. oryzae* presented the highest capacity of utilizing DON as a carbon source and *A. oryzae* the highest velocity use of this source.

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