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Original

Deoxynivalenol (DON) degradation and peroxidase enzyme activity in submerged fermentation

Degradação de deoxinivalenol (DON) e a atividade da enzima peroxidase durante fermentação submersa

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Abstract

This work aims to evaluate deoxynivalenol degradation by *Aspergillus oryzae* and *Rhizopus oryzae* in a submerged fermentation system and to correlate it to the activity of oxydo-reductase enzymes. The submerged medium consisted of sterile distilled water contaminated with 50 μg of DON and 4×10^6 spore.mL^{-1} inoculum of *Aspergillus oryzae* and *Rhizopus oryzae* species, respectively in each experiment. Sampling was performed every 24 hours for monitoring the peroxidase specific activity, and every 48 hours for determining mycotoxin levels. Results showed that the fungi species were able to decrease DON levels as the peroxidase activity increased. The 48 hours fermentation interval presented the highest peroxidase specific activity (ΔABS/minute.μg.protein^{-1}), 800 and 198, while the highest DON degradation velocity was 10.8 and 12.4 ppb/hour, respectively in both cases for *Rhizopus oryzae* and *Aspergillus oryzae*.

Keywords: *mycotoxin; peroxidase enzyme; degradation; fermentation; fungal species.*

Resumo

Este trabalho teve por objetivo avaliar a degradação de deoxinivalenol por *Aspergillus oryzae* e *Rhizopus oryzae* durante fermentação submersa e correlacioná-la com a atividade de enzimas oxidoredutases. O meio submerso foi constituído por água destilada estéril contaminada com 50 μg de DON e inóculo de 4×10^6 esporos.mL^{-1} de meio das espécies fúngicas *Aspergillus oryzae* e *Rhizopus oryzae*, separadamente em cada experimento. A amostragem foi realizada a cada 24 horas de processo para medida da atividade específica da enzima peroxidase, e a cada 48 horas para a determinação dos níveis de DON. Os resultados mostraram que as espécies fúngicas avaliadas possuem capacidade de metabolizar DON, acompanhada de aumento na atividade da enzima oxidativa PO. No intervalo de 48 horas de fermentação, ocorreu a maior atividade específica da enzima peroxidase (ΔABS/minute.μg.protein^{-1}), 800 e 198, correspondendo a maior velocidade de degradação de DON de 10.8 e 12.4 ppb/hour, respectivamente para *Rhizopus oryzae* e *Aspergillus oryzae*.

Palavras-chave: *micotoxina; enzima peroxidase; degradação; fermentação; espécies fúngicas.*

1 Introduction

Deoxynivalenol (vomitoxin, DON) is a trichothecene characterized by the presence of a tetracyclic sesquiterpenoid, belonging to the trichothecene B group, named 12,13-epoxy-3,4,15-trihydroxytrichothec-9-en-8-one, (3α,7α)-(9CI) which is frequently detected in food and feed (ATROSHI et al., 2002; SCIENTIFIC COMMITTEE ON FOOD, 2006) and possesses seven stereo centers. DON is synthesized mainly by the toxigenic fungi of *Fusarium* genus, present in food products, especially grains and cereals (MELLO; MACDONALD, 1997; RICHARD, 2007).

This mycotoxin remains stable for many years when stocked at room temperature or even when heated at 135 °C (PRONYK; CENKOWSKI; ABRANSOM, 2006). Its deactivation occurs through the destruction of the epoxide ring under drastic acid or alkaline conditions, reactions with aluminum and lithium hydrates or peroxides and hydration in autoclave. The change of sesquiterpenoid and matrix structure by these conditions makes its detection and use of decontaminated raw material difficult (UENO, 1983; PRONYK; CENKOWSKI; ABRANSOM, 2006). DON, as well as other trichothecenes, may have its chemical structure altered by bacteria or fungi, which utilize their enzymatic systems as a carbon source (SUDAKIN, 2003).

Fermentative processes have been studied in several scientific and technological areas, and they are still scientifically interesting due to their high potential of decontamination, motivating the search for deeper knowledge on the mechanisms through which the microorganisms act as decontamination agents (WESTBY; REILLY; BAINBRIDGE, 1997; BATA; LASZATITY, 1999; NIDERKORN; BOUDRA; MORGAVI, 2006; CACCIAMANI et al., 2007; GARDA et al., 2005). It has been demonstrated that some microorganisms produce enzymes that could alter the structure of mycotoxins and/or proteins that can conjugate these compounds, making them less active as pathogenic agents (PASTER et al., 1992; WESTBY; REILLY; BAINBRIDGE, 1997). The destruction (the opening of the epoxide grouping - the main cause for DON's toxicity) may be

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**A quem a correspondência deve ser enviada*

correlated to the action of oxidative enzymes that would catalyze the opening of the epoxide ring (SWEENEY; DOBSON, 1998; MOSS; THRANE, 2004).

The purpose of this work was to evaluate the degradation of deoxynivalenol by *Aspergillus oryzae* and *Rhizopus oryzae* in a submerged fermentation system, and to correlate it to the activity of oxydo-reductase enzymes.

2 Materials and methods

2.1 Mycotoxin

The DON standard was acquired from Sigma Chemical Company (USA). DON stock solution was prepared with benzene:acetonitrile (95:5) at 100 μg.mL^{-1}, according to Shepherd and Gilbert (1988). The solution was obtained by dilution, 50 μg.mL^{-1} concentration, estimated by the w/v relation and confirmed through the procedure described by Bennett and Shotwell (1990), utilizing molar absorptivity of the standard.

2.2 Experiment 1: evaluation of peroxidase enzyme activity (PO) in submerged fermentation

Experiments

The following sets of assays were prepared for each microorganism (*Aspergillus oryzae* – CCT 3940 and *Rhizopus oryzae* – isolated from rice, identified, with culture collection performed at the Microbiology Laboratory of Federal University of Rio Grande/Brazil) inoculated at a 4.10^{6} spore.mL^{-1} concentration under the following conditions:

- 50 mL sterile distilled water contaminated with 50 μg of DON.
- 50 mL sterile distilled water not contaminated with inoculum.
- 50 mL sterile distilled water containing 50 μg of DON with viable inoculum.
- 50 mL sterile distilled water containing 50 μg of DON with unviable inoculum (inactivated in autoclave at 120 °C and 1 kgf.cm^{-2}).

Assays were performed in triplicate for each microorganism. The media remained at a temperature of 30 °C under 200 rpm orbital agitation for 240 hours. 1 mL analytical samples of the fermented medium were removed every 24 hours, utilizing sterile material and suction valve, for quantification of specific enzymatic activity of peroxidase. Every 48 hours, 5 mL aliquots were removed, in sterile conditions, for DON quantification.

DON quantification

Quantification of the mycotoxin present in the fermentation medium was performed by three liquid-liquid partition utilizing 10 mL of methylene chloride, according to Garda, Macedo and Badiale-Furlong (2004). The derivation occurred with the addition of 18 mg of sodium bicarbonate and 200 μL of trifluoroacetic anhydride reagent (TFAA), reacting for six minutes at 74 °C, procedure described by Garda-Buffon and Badiale-Furlong (2008), and the quantification was measured through gas chromatography and flame ionization detector employing an internal standard arachidonic acid methyl esther.

The utilized GC was a Varian, 3400 model, equipped with a split/splitless injector and flame ionization detector, 30 m DB-17 column (J&W Scientific), 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.

Employed chromatographic conditions were: injector temperature of 250 °C, valve opening at 0.75 minutes, injector cleaning flow of 75 mL/minute, detector 300 °C, and attenuation of 16 × 10^{12}. The program for the chromatographic column was 100 °C for 1 minute, 50 °C/minute to 200 °C, maintained for 2 minutes, 4 °C/minute to 250 °C, maintained for 11.5 minutes, in a total of a 29 minutes chromatographic run.

The standard curve was established by deriving solutions from 1.4 to 21 μg DON. The detection limit was determined by successive dilutions of a 10 ng.μL^{-1} solution, until the generation of a detection signal three times greater than the standard deviation of the control signal derivation (GARDA-BUFFON; BADIALE-FURLONG, 2008). The quantification, time and relative area according to aracdonic acid methyl esther internal standard (Sigma Chemical Company – USA), were employed utilizing a mass of 0.01μg/chromatographic injection (BADIALE-FURLONG; SOARES, 1995).

The recovery was established through the contamination of 50 mL of medium with DON from 10 to 50 μg (10, 30 and 50), in triplicate for each level. Extraction and quantification steps were executed according to method described for DON residual level determination in fermented medium.

Enzymatic activity: peroxidase (PO)

Determination of peroxidase activity was performed obtaining 1mL of diluted fermented medium, 1.5 mL phosphate buffer pH 6.0 (100 mM), 0.5 mL guaiacol (1%), and 1 mL hydrogen peroxide (0.08%). The mixture was incubated for 10 minutes at 30 °C, and absorbance was determined at 470 nm at a Cary 100 Varian (USA) spectrophotometer. Initial rate of increase at ΔA_{470} was used to measure enzyme activity. One unit of enzyme activity was defined as variation in absorbance of minute.mL^{-1} enzyme extract (KHAN; ROBINSON, 1994). PO activity in the media was evaluated every 24 for 240 hours.

The fermentation interval that presented the highest specific activity was at 48 hours, indicating that this interval was the best for enzyme extraction and characterization of fermentative experiment in order to evaluate the relation of PO activity and DON presence. In experiment #1, contaminated and non-contaminated media were prepared, with the second being the control one.

After 48 hours, each recipient containing fermented medium, denominated fraction 1, was filtered on qualitative filter paper for the removal of fungal spores. $(NH_4)_2SO_4$ was added to the filtered fraction (fraction 2) with agitation

until 80% saturation (LIMA; PASTORE; LIMA, 2001). After 24 hours of refrigeration (4 °C), the solution was centrifuged at 7000 rpm for 30 minutes. The precipitated protein was dissolved with phosphate buffer at pH 5.2 (5 mM), (fraction 3) (ERAT; SAKIROGLU; KUFREVIOGLU, 2006) and submitted to dialysis for 48 hours at 4 °C against distilled water in an acetate membrane (fraction 4). The dialyzed fraction was precipitated with 70% ethylic alcohol under refrigeration for 24 hours, followed by a centrifugation at 14000 rpm for 15 minutes at 4 °C (fraction 5) (LIMA; PASTORE; LIMA, 2001). PO specific activity was evaluated and soluble protein and DON mass were quantified in each fraction.

The determination of soluble proteins was carried out according to Bradford (1976), employing in a Cary 100 Varian (USA) spectrophotometer, with an albumin standard curve from 10 to 100 $\mu g.mL^{-1}$.

2.3 Statistical treatment

DON degradation was expressed as percentage of degraded mass, estimated according to Equation 1.

$$\%\ of\ degradation = \frac{(\Delta\ DON\ mass \times 100)}{DON\ mass\ initial} \quad (1)$$

Degradation velocity was estimated based on degraded DON mass and fermentation time, expressed according to Equation 2.

$$v = \frac{\Delta\ DON\ mass(\mu g)}{\Delta t\ (h)} \quad (2)$$

The estimated unit of oxidative peroxidase enzyme activity (U) was expressed as $\Delta ABS/minute.\mu g\ of\ protein^{-1}$.

Statistical differences between responses were verified through Analysis of Variance (ANOVA) and by comparing the means utilizing the Tukey test, with differences being considered significant when $p < 0.05$.

3 Results and discussion

The limit quantification was 0,28 $\mu g.mL^{-1}$, the mean recovery under the tested DON range was 96% and the variation coefficient was 8%, as presented in Figure 1.

Decrease in the analytical toxin signal in the chromatographic method was considered as consequence of the alteration in the mycotoxin structure due to peroxidase action on the DON epoxide group, which is the most susceptible structure point to enzymatic action. In experiment #3, the carbon source was only DON and the results confirmed the mechanism proposed above. DON may be epoxidate before the use as carbon source. A similar behavior was reported by Abrunhosa and Venâncio (2007), where hydrolyse obtained from *Aspergillus niger* degraded ochratoxin A to compounds not detected in the analytical system.

DON degradation by *Aspergillus oryzae* and *Rhizopus oryzae* was estimated considering the DON mass reminiscent in the medium after 240 hours of the fermentation. The results are present as means of experiments and statistical significance in the studied intervals.

Experiment #1 (the control) was carried out without DON and a shorter signal value corresponding to 3% of the highest DON concentration was detected after 96 hours of fermentation. In control 2, constituted by the submerged medium without addition of fungi inoculum, the degradation value was 8.4%; also verified after 96 hours of fermentation, standing until 240 hours.

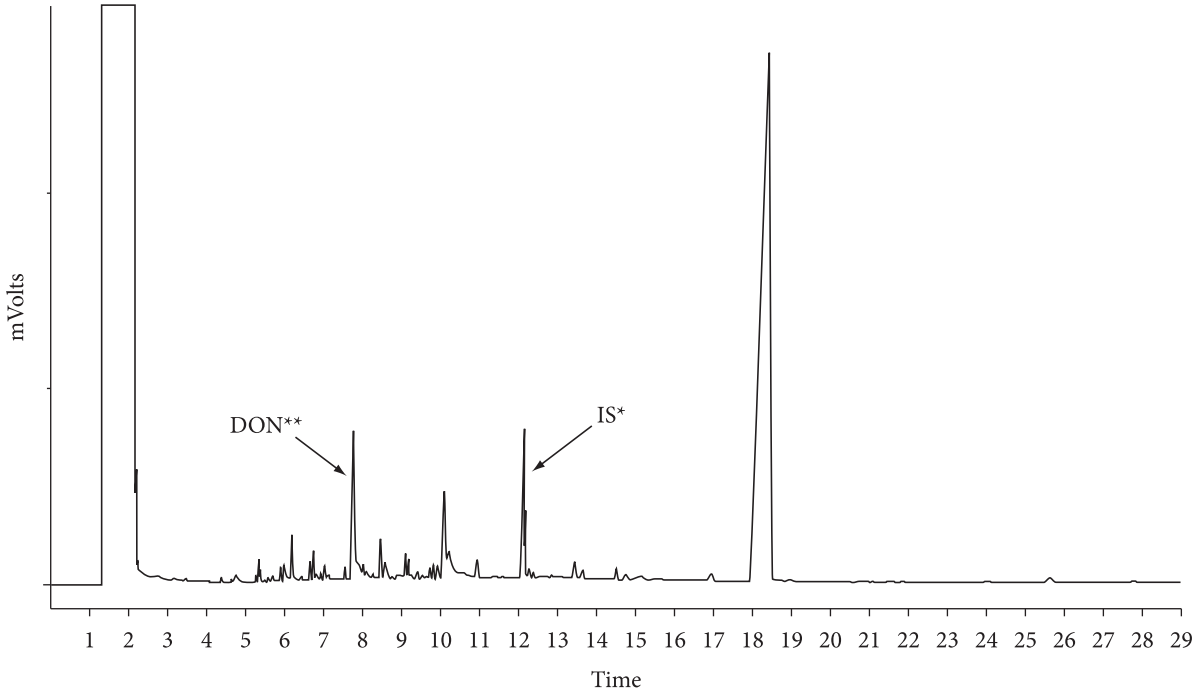

Figure 1. Chromatogram of fermented medium contaminated with DON.
*Arachidonic acid methyl esther internal standard; ** Deoxynivalenol.

Experiments performed with the inactivated spores of the microorganism, 15.9 and 16.5 µg of DON, were encountered; they corresponded to degradation percentages of 89 and 67% at the end of the studied interval, in media fermented by *Aspergillus oryzae* and *Rhizopus oryzae*, respectively. These results suggest that the mycotoxin was adsorbed on the cell surface (BAJAQUI et al., 2005).

DON degradation percentage and degradation velocity allowed the evaluation of the highest degradation indexes for each microorganism, *Aspergillus oryzae* degraded 74% of initial DON mass in 96 hours at medium degradation velocity of 0.12 µg/hour. *Rhizopus oryzae* presented the highest degradation in 240 hours, reducing by 90% the initial DON level at a medium velocity of 0.19 µg/hour. At the 48 hour period, the highest degradation velocities were verified by both fungal species, *Aspergillus oryzae* showed the highest DON degradation velocity, 0.62 µg/hour, comparing to *Rhizopus oryzae*, 0.54 µg/hour (Table 1). These velocities also permitted estimating degradation at 12.4 and 10.8 ppb/hour for *Aspergillus oryzae* and *Rhizopus oryzae*, respectively.

The ANOVA results demonstrated that there were no similarities in the enzymatic activity of the extracts fermented by the two microorganisms during the fermentation process, as it can be verified in Table 2. Oxidative enzyme activity media were 198 and 800 U for *A. oryzae* and *Rhizopus oryzae*, respectively. The highest activity of peroxidase and percentage of DON degradation were verified at 240 hours in the *Rhizopus oryzae*. However, the control group (without DON) fermented by this fungus presented the highest peroxidase activity of all times, when compared to the control group fermented by *Aspergillus oryzae*. It was interesting to observe that from 24 to 48 hours of fermentation, there was no difference between the specific peroxidase activities for both microorganisms (Figure 2).

The results of the experiments carried out for PO enzyme purification from the fermented media, with and without DON, are shown in Table 3, where the protein content of each fraction and the estimated enzymatic activity are also presented.

The specific activity of the PO enzyme in fraction 1, contaminated medium, was similar to the values determined in experiment #1, confirming the significance of the data. However, for the remaining fractions, after the purification steps, there was no detection of PO activity as a result of biomass removal.

The experiments permitted to verify that higher enzymatic activity was accompanied by higher degradation velocities, especially in the time interval between 0 and 96 hours, suggesting that *Rhizopus oryzae* and *Aspergillus oryzae* may

Table 1. DON residual mass, degradation percentage and degradation velocity during fermentation process.

Time (hour)	Control 2 (µg DON)	Aspergillus oryzae			Rhizopus oryzae		
		$DON_{RESIDUAL}$ (µg (SD)*)	Degradation (%)	DV** (µg DON/hour)***	$DON_{RESIDUAL}$ (µg (SD))	Degradation (%)	DV (µg DON/hour)
0	49.8	44.4 (2.0)	11.1[a]	0	49.7 (0.4)	0.7[1]	0
48	49.8	15.6 (6.1)	69[b]	0.62	24.4 (3.4)	51.3[2]	0.54
96	49.2	13.2(4.0)	73.6[c, d]	0.05	10.1 (0.8)	79.7[4]	0.3
144	45.2	12.8 (0. 9)	74.4[d]	0.008	12 (1.9)	76[2]	0.0
192	42.5	14.1 (2.1)	71.8[c]	0.0	11.8 (0.9)	76.4[3]	0.004
240	41.6	16.3 (3.4)	67.4[b]	0.0	5.0 (0.5)	89.9[5]	0.14
Means	46.4	19.4	61.2	0.12	18.8	62.3	0.19

* SD – Standard deviation; ** Degradation velocity; ***µg DON/hour – DON mass degraded by hour of process.

Table 2. Specific activity of PO enzyme during submerged fermentation with *Aspergillus oryzae* and *Rhizopus oryzae*.

Time (hour)	*Aspergillus oryzae*		*Rhizopus oryzae*	
	Control* ($U.mg^{-1}$/minute)	Contaminated** ($U.mg^{-1}$/minute)	Control* ($U.mg^{-1}$/minute)	Contaminated** ($U.mg^{-1}$/minute)
0	0.00	173.5[i]	422.7	697.4[s]
24	277.9	287.5[i]	798.2	2380.7[v]
48	11.4	356.7[l]	753.0	1136.7[t]
72	35.1	325.5[j]	930.3	1579.1[u]
96	16.4	156.9[d]	184.0	585.2[r]
120	18.7	147.7[c]	259.5	382.1[m]
144	112.3	215.0[h]	250.9	454.1[q]
168	0.0	107.5[b]	238.3	387.5[n]
192	0.0	54.0[a]	264.4	345.5[k]
216	20.1	181.5[g]	261.6	432.9[p]
240	138.6	177.5[f]	244.9	424.0[o]
Means	57.3	198.5	418.89	800.5

* Experiment with non-contaminated medium inoculated with 4×10^6 spores.mL^{-1}; ** Experiment with medium contaminated with 50 µg DON inoculated with 4×10^6 spores.m^{-1}; Different letters on the same column or line indicate that media differed significantly at a 95% confidence level ($p < 0.05$) according to Tukey test.

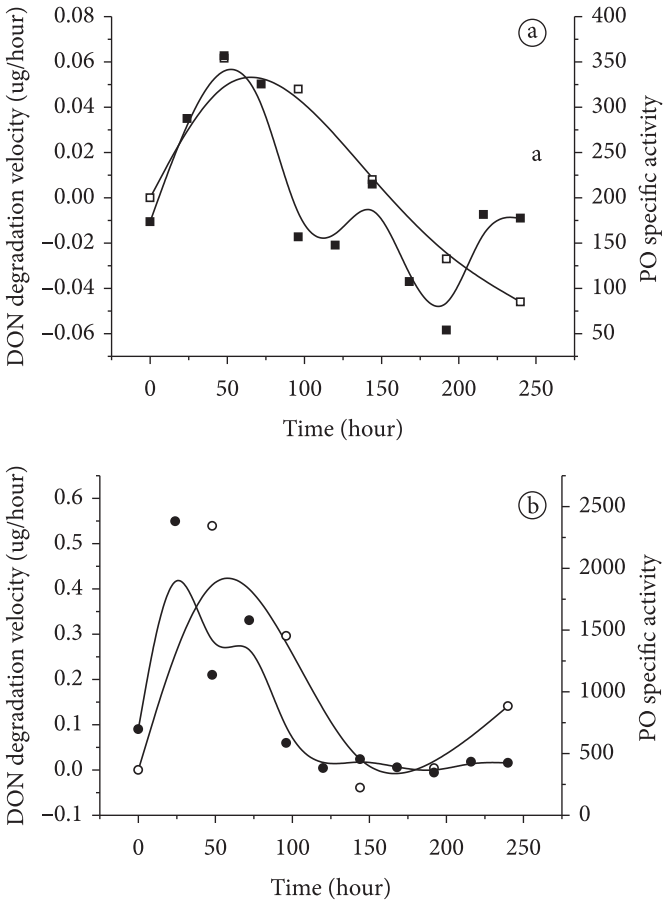

Figure 2. a) DON degradation velocity and oxidative enzyme activity during submerged fermentation with *Aspergillus oryzae*; and b) *Rhizopus oryzae*. (□) degradation velocity in extract fermented with *A. oryzae*; (■) oxidative enzyme activity in extracts fermented with *Aspergillus oryzae*; (○) degradation velocity in extract fermented with *Rhizopus oryzae*; (●) oxidative enzyme activity in extracts fermented with *Rhizopus oryzae*.

Table 3. Protein content and enzymatic activity during protein extraction.

Steps	Protein content (μg.mL^{-1})		Oxidative enzymes activity (U.mg^{-1}/minute)	
	A. oryzae	*R. oryzae*	*A. oryzae*	*R. oryzae*
Fraction 1	33.4	32.7	327	1332
Fraction 2	29.2	27.4	nd	nd
Fraction 3	28.0	26.5	nd	nd
Fraction 4	29.5	26.8	nd	nd

nd = not detected.

possess capacity for DON metabolization, indicated by the variation of PO enzyme specific activity according to the toxin degradation velocity.

The variability of specific activity of the PO enzyme in the time interval between 96 and 240 hours, or after its maximum speed, can be explained by the enzyme saturation with the substrate, which maintains the degradation velocity stable for a longer period of time. In the experiments performed utilizing inactivated *Aspergillus oryzae* spores, the degradation percentages were higher than in the experiments with viable spores, after 240 hours of fermentation; this fact may be a consequence of adsorbance of the mycotoxin by the biomass protein.

The not detected PO activity after the biomass removal suggested that the enzyme was endocellular, which was indicated by the observation that there was no significant decrease in the soluble protein level (Table 3). This fact suggested that the mycotoxin was adsorbed by the cell and, after that, it was metabolized. This behavior is illustrated in Figure 2, which shows DON degradation velocity and PO specific activity. For monitoring the action of PO produced by *Aspergillus oryzae* and *Rhizopus oryzae* over DON, it is necessary to open the spore wall before the purification step.

4 Conclusion

Rhizopus oryzae and *Aspergillus oryzae* possess DON metabolization capacity that increases at the same time interval of PO enzyme activity, where the highest toxin degradation velocities occurred. At the 48 hour fermentation interval, the highest PO specific activity and DON degradation velocity occurred for both microorganisms, 10.8 ppb/hour and 800 U.mg^{-1}/minute, for *Rhizopus oryzae*; and 12.4 ppb/hour and 198 U.mg^{-1}/minute, for *Aspergillus oryzae*.

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