# Evaluation of zearalenone *in vitro* removal by *Saccharomyces cerevisiae* strains isolated from bovine forage

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## Abstract

Zearalenone (ZEN) is a mycotoxin that has relatively low acute toxicity. However, it is a potent oestrogen, interfering with the reproductive tract of animals. Among other effects, ZEN decreases animals fertility, and induces fibrosis in the uterus, breast cancer and endometrial carcinoma (Zinedine et al., 2007). Anti-mycotoxin additives (AMA) are defined as a group of products that, when added to animal feed, are capable of adsorbing, inactivating, or neutralizing mycotoxins in the gastrointestinal tract of animals. One example of these products are adsorbents based on yeast cell walls, a safe and beneficial animal feed additive (Abreu et al., 2008). When based on active cells, yeast based products also act as a probiotic, contributing to improve the general animal health because it stimulates their immune system and promotes the integrity of intestinal mucosa (Albino et al., 2006).

Strains of *Saccharomyces cerevisiae* isolated from silage were tested for their ZEN removal capability. Their effect on α- and β-zearalenol (α-ZOL and β-ZOL) was also tested. Strains were grown on YPD separately supplemented with ZEN, α-ZOL and β-ZOL, and their elimination from culture media was quantified over time by HPLC-FL.

## Results and Conclusions

### RESULTS

- The most active strains (*S. cerevisiae* Procreatin 7, LL 74 and LL 83) were able to eliminate more than 90% of ZEN present in the culture media in one day (Fig. 1.). The time required by those strains to eliminate 50% and 90% of ZEN was estimated as 9 and 18 h, respectively (Table 1.). For the same level of elimination, the less active strain (*S. cerevisiae* LL Silg.) required 20 and 54 h, respectively. An example of chromatograms obtained for strain LL 08 is depicted in Figure 2.

- The concentration of ZEN in the cell fractions was also investigated. Up to 26% of ZEN was found in the cells after one day of incubation (Fig. 3.). Nevertheless, after the second day, ZEN was no longer detected in cells. This results may indicate that some ZEN adsorbed to cells walls and, subsequently, was released or, in alternative, that ZEN was uptaked into cells cytoplasm to be further metabolized.

- The metabolization of ZEN into α-ZOL and β-ZOL was also investigated because it has been advocated by Böswald et al. (1995). Standards were used to identify those metabolites in chromatograms by comparing retention times. The retention times obtained for β-ZOL, α-ZOL and ZEN were 12.5, 18.5 and 21.0 min, respectively. α-ZOL and β-ZOL were detected in chromatograms (Fig. 2.). In average, at the end of the incubation period, α-ZOL represented 8% of the initial amount of ZEN added to culture media and β-ZOL represented 53%, which means that 39% of the ZEN added to culture media has been metabolized in a different way. With the current methodology no additional compounds from ZEN metabolization could be identified.

- To evaluate if yeasts were able to subsequently metabolize α-ZOL and β-ZOL, experiments with YPD supplemented with those metabolites were also performed. None of the yeast strains was able to eliminate α-ZOL from culture media. Nevertheless, in average, 17% of the β-ZOL added to culture media was eliminate by the strains.
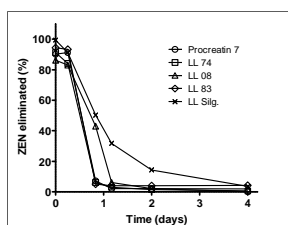


FIG. 1. Elimination of ZEN in culture media over time. Values expressed in % of initial amount.
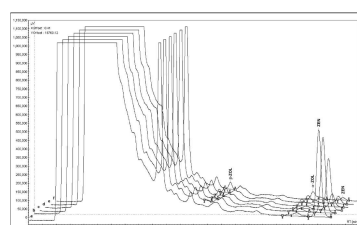


FIG. 2. Chromatograms showing the elimination of ZEN from YPD by strain LL 08 (a to f: 0, 6, 20, 28, 48 and 96 hours).
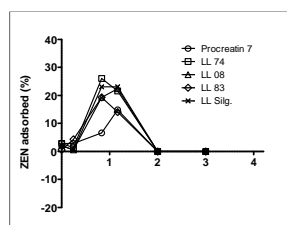


FIG. 3. Percentage of ZEN detected in the yeast cell fractions over time. Values expressed in % of initial amount.

TABLE 1. ZEN concentration in culture broth after day 1 and time taken to eliminate 50 and 90% of ZEN (DT50 and DT90, respectively)

| Strains | ZEN (µg/mL) | Logistic model DT50 (h) | Logistic model DT90 (h) | Curve Fitted Errors $R^2$ | Curve Fitted Errors Sy.x (%) |
|---|---|---|---|---|---|
| Controls | 1.95 ± 0.08 | - | - | - | - |
| Procreatin 7 | 0.04 ± 0.01 | 9 | 18 | 0.9996 | 1.2 |
| LL 74 | 0.06 ± 0.02 | 9 | 18 | 0.9995 | 1.3 |
| LL 08 | 0.12 ± 0.05 | 17 | 28 | 0.9925 | 4.9 |
| LL 83 | 0.08 ± 0.02 | 9 | 18 | 0.9961 | 3.8 |
| LL Silg. | 0.62 ± 0.12 | 20 | 54 | 0.9986 | 1.9 |

### CONCLUSIONS

- The elimination of ZEN by *S. cerevisiae* is associated to a biodegradation pathway that involves its conversion into minor quantities of α-ZOL and major quantities of β-ZOL.

- β-ZOL seems to be subsequently converted to another compound that was not detected, nor identified.

## Materials and Methods

- **Biological material:** Four strains of *S. cerevisiae* isolated from bovine forage coded as *S. cerevisiae* LL 74, LL 08, LL 83 and LL Silg. were used. Strains are deposited in the culture collection of the Research Center for Mycology and Mycotoxicology from the Federal Rural University of Rio de Janeiro (NPMM - UFRRJ). The *S. cerevisiae* strain that constitutes the commercial product Procreatin 7® (Lessafre, Brazil) was also used. Throughout the study, strains were maintained active in Yeast Peptone Dextrose (YPD) broth and preserved at 4 °C.

- **Inoculum preparation:** Yeasts were propagated at 30 ºC for 2 days in falcon tubes containing 10 mL of YPD broth. The D.O. was determined at 600 nm and adjusted to 2.0 with sterile distilled water.

- **ZEN Biodegration assays:** Strains were tested in YPD broth supplemented with 2 µg/mL of ZEN (Sigma-Aldrich). α-ZEN and β-ZEN were also tested separately. Falcon tubes with 5 ml of YPD+ZEN were inoculated in triplicate with 0.1 mL of inoculum. Negative controls were also prepared using 0.1 mL of sterile distilled water. Incubation was performed at 30 °C for 4 days. Yeast cells and culture media were extracted together.

- **ZEN adsorption assays:** Experiments were conducted as described in previous section. At the end of the incubation period, cells were centrifuged (9000 x *g* for 20 min) and supernatants transferred to new tubes. Yeast pellets and culture media were extracted separately.

- **Extraction of samples:** Culture media with or without yeast cells were extracted with an equivalent volume of acetonitrile/acetic acid (98:2, v/v) using vortex agitation for 1 min. Extraction of cell pellets was performed with 2 mL of acetonitrile/water/acetic acid (99:99:2, v/v/v) using an agitation period of 1 hour. Two mL samples were collected and filtered into clean amber borosilicate glass vials using syringe-fitted 0.45 µm PP filters and analysed by HPLC-FL. Retention times for ZEN, α-ZEN and β-ZEN were 21, 12.5 and 18.5 min, respectively.

- **HPLC-FL analysis:** Column $C_{18}$ reversed-phase YMC-Pack ODS-AQ analytical column (250 x 4.6 mm I.D., 5 µm) fitted with a pre-column with the same stationary phase. Flow rate of 1.0 mL/min for a 23 min isocratic run with methanol/water/acetic acid (65:35:1, v/v). Injection volume of 50 µL, fluorescence detection at $\lambda_{exc}$=280 nm, $\lambda_{em}$=460 nm and gain=1000.

- **DT50 and DT90:** The time required for 50% and 90% of ZEN to be degraded (DT50 and DT90, respectively) was determined by fitting concentration of ZEN over time to the logistic model according to FOCUS (2006) and using the equations:

$$ S = S_0 * \left[ \frac{a_{max}}{a_{max} - a_0 + a_0 * e^{(r*t)}} \right]^{\frac{a_{max}}{r}} \qquad DT50 = \frac{1}{r} * \ln\left[ 1 - \frac{a_{max}}{a_0} * \left( 1 - 2^{\frac{r}{a_{max}}} \right) \right] \qquad DT90 = \frac{1}{r} * \ln\left[ 1 - \frac{a_{max}}{a_0} * \left( 1 - 10^{\frac{r}{a_{max}}} \right) \right] $$

**References:** Zinedine, A. et al. (2007) Food & Chemical Toxicology 45, 1-18; Abreu, A.P.N. et al. (2008) Veterinária & Zootecnia 15, 551-560; Albino, L.F.T. (2006) Revista Brasileira de Zootecnia 35, 742-749; Böswald, C. et al.,(1995) Natural Toxins 3, 138-144; FOCUS (2006) available at: http://focus.jrc.ec.europa.eu/dk/docs/finalreportFOCDegKin04June06linked.pdf