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Saccharomyces cerevisiae strain associated to a fish culture additive: antifungal activity and AFB1 adsorption potential

Saccharomyces cerevisiae associada a um aditivo de piscicultura: atividade antifúngica e potencial de adsorção de AFB1

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ABSTRACT: *Saccharomyces cerevisiae* is widely applied as a probiotic in aquaculture activities, due to its ability to decontaminate the mycotoxin Aflatoxin B_1 (AFB_1). From this point, the present study aimed to evaluate the antifungal and anti-aflatoxigenic activities of inactivated *Saccharomyces cerevisiae* mixed with a commercial product (CP) of animal feed to assess its influence on the *Aspergillus flavus* and *A. parasiticus* fungi growth on the AFB_1 production. Besides, AFB_1 adsorption potential of *S. cerevisiae* and the commercial product was also investigated. Different concentrations of the commercial product alone and in the presence of inactivated yeast were analyzed by *Aspergillus* growth inhibition test, aflatoxin production by *Aspergillus* species, and AFB_1 adsorption capacity. AFB_1 detection and quantification were carried out by High-Performance Liquid Chromatography. The inactivated yeast and commercial product combination were effective in reducing *A. flavus* and *A. parasiticus* growth. *A. flavus* produced less AFB_1 after the inactivated yeast treatment, whereas *A. parasiticus* produced significantly less AFB_1 under a combination of inactivated yeast and 50% CP. Regarding AFB_1 adsorption, 100% CP displayed the highest adsorption capacity at 10 ng mL^{-1} AFB_1. At 25 ng mL^{-1} AFB_1, only the treatment comprising inactivated yeast associated with 50% CP led to AFB_1 adsorption, albeit at low levels. It was concluded that the association between the commercial additive and inactivated *S. cerevisiae* was effective in reducing the growth of fungi and there was AFB_1 adsorption activity.

KEYWORDS: Inhibitory effect. Mycotoxin. *Aspergillus flavus. Aspergillus parasiticus.*

RESUMO: Saccharomyces cerevisae é uma levedura largamente utilizada como probiótico na aquicultura e que tem se destacado devido a capacidade para a descontaminação de AFB1. O objetivo desse trabalho foi avaliar a atividade antifúngica e anti-aflatoxigênica da Saccharomyces cerevisae inativada e misturada a um produto comercial, observando sua influência no crescimento dos fungos Aspergillus flavus e A. parasiticus e na produção destes da Aflatoxina B1 (AFB1), investigar o potencial de adsorção da levedura e do produto comercial sobre a AFB1. Foram realizadas analises de preparação de leveduras inativadas e das soluções com o produto comercial, Ensaio do efeito inibitório de espécies de Aspergillus in vitro, Teste in vitro sobre a produção de aflatoxinas, avaliação in vitro da capacidade de adsorção de AFB1, condições cromatográficas para detecção e quantificação de AFB1 por cromatografia Líquida de Alta Eficiência. A associação do aditivo comercial EPICIN – G2® e a levedura S. cerevisiae inativada foi eficaz na diminuição do crescimento fúngico dos A. flavus e A parasiticus. Quanto à produção de micotoxina, o A. flavus obteve menor produção com o tratamento composto por leveduras inativadas, enquanto, o A. parasiticus teve diminuição significativa na produção de AFB1 com a associação da levedura a uma concentração de 50% do produto. No referente a adsorção, em concentração de 10ng/mL, o tratamento que demonstrou melhor capacidade de adsorver AFB1 foi o 100% Produto (100%). Já na concentração 25 ng/mL, somente o tratamento que associava levedura e 50% do produto, apresentou uma pequena capacidade de adsorção.

PALAVRAS-CHAVE: efeito inibidor. Micotoxina. *Aspergillus flavus*. *Aspergillus parasiticus*.

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INTRODUCTION

Fish nutrition has advanced significantly with the expansion of aquaculture activities worldwide, aiming at improving performance indices to meet fish nutritional requirements. However, this must be carried out by providing all the nutrients and energy necessary to optimize fish development by combining both quality and safety (SANTOS et al., 2015).

The presence of fungi in animal feed leads to economic losses, as these organisms are associated with nutrient reduction and palatability, as well as the production of mycotoxins by some species under favorable conditions, i.e. metabolic substances toxic to both humans and animals (PRESTES et al., 2019).

Mycotoxins produced by the fungi, Aflatoxins are produced naturally by *Aspergillus flavus* and *Aspergillus parasiticus*, classified as aflatoxins B1, B2, G1, G2, M1, and M2. These metabolites present similar structures and toxic effects, including acute hepatotoxicity, teratogenesis, immunosuppression, anemia, and decreased fertility, among others (COPETTI et al, 2011; ZYCHOWSKI et al., 2013). Anti-mycotoxin Additives (AAM) adsorbents, after inclusion in animal diets, display the ability to adsorb, inactivate, biotransform or neutralize mycotoxins, thus reducing their bioavailability within the gastrointestinal tract, avoiding harmful effects, followed by toxin elimination via feces (KELLER et al., 2012).

The yeast cell wall is a physical barrier that protects cells from environmental factors and is also used as a biological mycotoxin adsorbent to reduce their gastrointestinal absorption during feeding (BOROVIKOVA et al., 2016; PEREYRA et al., 2018). In this scenario, *Saccharomyces cerevisiae* has been highlighted as a probiotic applied in fish farming, poultry farming, and pig farming, and its cell wall components have been shown to act as growth promoters and immune system stimulants, as well as mycotoxin adsorbents (ROSSI et al., 2010).

In this context, the present study aimed to evaluate the antifungal and anti-aflatoxigenic activities of inactivated *Saccharomyces cerevisiae*, both isolated and mixed with a commercial product, and determine its effects on *Aspergillus flavus* and *Aspergillus parasiticus* growth and Aflatoxin B1 (AFB_1) production. AFB_1 adsorption potential was also investigated.

MATERIAL AND METHODS

Saccharomyces cerevisiae (strain A8L1) belonging to the culture collection of the Microbiological Control Laboratory of the Nucleus of Studies, Research and Food Processing (NUEPPA), UFPI. The commercial product (CP) of animal feed containing *Bacillus subtilis, B. licheniformis, Lactobacillus acidophilus, B. pumilus, and Saccharomyces cerevisiae*. Aflatoxin-producing fungi species *Aspergillus flavus*, it also belongs to the laboratory collection, and *Aspergillus parasiticus* NRLL 2999 (USDA, Agricultural Research Service, Peoria, IL) were used for the inhibitory tests.

Saccharomyces cerevisiae was inoculated into 250 mL Erlenmeyer flasks in 100 mL (YPD) broth (comprising 5 g yeast extract, 5 g peptone, 40 g dextrose, 1000 mL distilled water) and incubated under stirring (150 rpm) at 25 °C for 24 hours. After incubation, serial dilutions were then prepared in Falcon tubes using PBS (phosphate buffer - pH 7.2), until obtaining 10^7 cells mL^{-1} concentration. The cell suspension concentration was determined using hemocytometer.

For inactivation of the yeast, Falcon tubes containing the yeast at concentrations of 10^7 cells mL^{-1} were centrifuged at 3500 rpm for five minutes, the supernatant was discarded and a further wash was performed with PBS, the resulting cell pellet was autoclaved at 120ºC for 15 minutes.

Later, in the tube containing the cell pellet inactivated was transferred 10 mL of product solution prepared with PBS in different concentrations (0%, 50%, 75%, and 100%), and mixed to obtain homogeneous treatments.

For the *in vitro* inhibitory assay using both *Aspergillus* species was performed according to Juri et al. (2015) with minor modifications, the fungi were first cultured in Malt Extract Agar (MEA) at 25°C for seven days until complete sporulation and then maintained at 4°C until use. The *in vitro* bioanalytical method was applied, where the development or inhibition of fungi growth in the presence of the four applied treatments was observed. A total of 1 mL of each yeast and product experimental solution was added to 15 mL of previously autoclaved MEA at 120 °C for 15 minutes.

After media solidification, a 7 mm center hole was drilled in the center of each plate and the spore solutions containing 4×10^6 spores mL^{-1} of each fungi species were added for each of the four treatments. Plates were then incubated at 25 °C. Orthogonal measurements of colony diameters were carried out on the 3rd and 7th incubation days, using the development of each species cultured in MEA without any treatment as reference. All experiments were performed in duplicate (MUÑOZ et al., 2010; JURI et al., 2015).

The *in vitro* test concerning *Aspergillus* aflatoxin production was carried out using fungal colonies grown in MEA plates at 25ºC for seven days in the inhibitory effect assay described above, followed by AFB_1 detection and quantification by High-Performance Liquid Chromatography (HPLC).

Quantification was performed by SHIMADZU® High-Performance Liquid Chromatograph (HPLC), PROMINENCE model, with a SUPER RF-10AXL model fluorescence detector, 20 µL loop, and excitation and emission of 360 nm and 460 nm, respectively (TRUCKSESS et al., 1994). It was equipped with a C18 silica gel reverse phase column (150 x 4.6 mm, 5.0 µm particle size, Phenomenex, Luna) connected to a Supelguard LC-ABZ precolumn 20 x 4.6 mm, 5.0 µm particle size, Supelco). The limit of detection of the analytical method was 0.4 $ng.g^{-1}$. AFB_1 was quantified by HPLC using 200 µL of each sample and 700 µL of the derivatizing solution, composed of glacial acetic acid: water (trifluoroacetic acid: 20:10:70 v/v). Experiments were carried out in duplicate.

The quantifications of AFB_1 adsorbed by yeast and commercial product mixtures will be performed by the following

equation, where: A = (B – C) x D/E. Where A represents the amount (ng.mL-1) of toxin by the yeast mixture and commercial product; B is the height of the chromatographic peak of the sample; C is the height of the chromatographic peak of the negative control; D is the concentration (ng.mL^{-1}) of the positive control; and E refers to the height of the chromatographic peak of the positive control (PINHEIRO et al., 2017).

The AFB_1 adsorption capacity assay was carried out using different concentrations of inactivated *S. cerevisiae* and CP, as follows: 100% Yeast, Yeast + 50% CP, Yeast + 75% CP, and 100% CP). The experiments were performed under simulated fish gastrointestinal conditions (Nile tilapia, pH 7). A phosphate-buffered saline (PBS) was prepared to add sodium hydroxide (0.1 N NaOH) until reaching pH 7 (ROTTA, 2003). The assay was performed using two aflatoxin B1 concentrations (10 and 25 ng mL^{-1}) at pH 7 for the evaluation of the adsorption capacity of each treatment (PINHEIRO et al., 2020).

Before the assay, 1 ml of each yeast and product experimental solutions were transferred to a microtube and centrifuged at 12000 rpm for five minutes. The supernatants were then discarded and the pellets were washed twice with a PBS solution (pH 2) and incubated for 60 minutes at 37 °C under constant agitation (150 rpm), to simulate stomach conditions and promote contact between the toxin and the experimental solutions. After the incubation period, second centrifugation was carried under the same conditions as above, the supernatants were discarded and 1 mL of AFB_1 was added at pH 7. The incubation period was then repeated, followed by another centrifugation step and the supernatants were then transferred to other microtubes for quantification.

An analysis of variance (ANOVA) test was performed to verify the significance of the differences between treatments. The level of significance was set at 0.05 for multiple comparisons, and the Student Newman Keuls test was used when ANOVA pointed to the rejection of the hypothesis of equality between the treatment means. The Assistat version 7.7 software was used for all statistical analyses.

RESULTS AND DISCUSSION

Inactivated *S. cerevisiae* (A8L1)**,** in association with the commercial additive**,** decreased the growth of *A. flavus* (A615) and *A. parasiticus* (NRLL 2999). However, no difference (p<0, 05) was observed between the control sample and the inactivated yeast treatment (Table 1), indicating that inactive yeast alone is not effective in inhibiting *Aspergillus* growth.

Regarding *A. flavus* (A615), the control treatment presented a growth diameter means of 68 mm, lower than that observed in the 100% yeast treatment, which on the 7th day reached a means of 79.20 mm.

The mixture of commercial additive and inactivated yeast had a similar effect on *A. flavus* growth inhibition than that of the commercial product alone (p<0.05). Both results remained below the minimum of 50mm compared to fungi growth at the 7th day on the control treatment dishes, demonstrating potentiation of the effect of the mixed treatment on *A. flavus* growth inhibition (Figure 1).

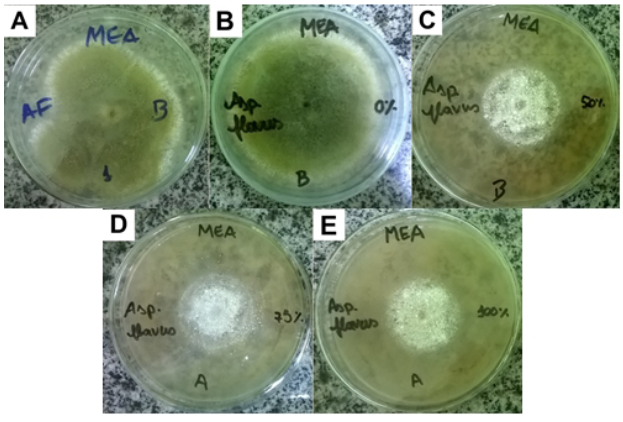
Concerning *A. parasiticus* (NRLL 2999), growth means ranged from 61,30 mm to 29.80 mm, with no significant difference between the control and 100% *S.cerevisiae* treatments, similarly to *A. flavus*. According to Pitt and Hocking (2009), *A. parasiticus* in MEA for seven days presents a growth diameter ranging from 50 to 65 mm. Therefore, the 100% yeast treatment presenting 60.50 mm was not effective in inhibiting fungal growth. The inactivated yeast associated with commercial additive, on the other hand, succeeded in inhibiting *A. parasiticus* growth (Figure 2). The most efficient treatment for both species was the association of Yeast + 75% commercial additive, with inhibited growth means of 39 mm (*A. flavus*) and 29.80 mm (*A. parasiticus*).

Table 2 displays the results for fungal AFB_1 production. The addition of inactivated yeast decreased *A. flavus* mycotoxin

Table 1. Inactivated *S. cerevisiae*, CP and their interactions on the growth parameters of *A. flavus* and *A. parasiticus* strains on the 7th day of the experiment.

Treatments	Growth rate (mm) ± Standard deviation	
	***Aspergillus flavus* A615**	***Aspergillus parasiticus* NRLL 2999**
Control	68,0^{a}± 16,90	61,3^{a}± 14,00
100% *S.cerevisiae*	79,2^{a}± 3,89	60,5^{a}± 13,10
S.cerevisiae +50% product	43,0^{b} ± 2,12	30,8^{b}± 3,00
S.cerevisiae +75% product	39,0^{b}± 2,12	29,8^{b}± 4,00
100% product	39,7^{b}± 0,35	32,6^{b}± 4,60

The averages followed by the same letter in the same column do not differ statistically from each other, by the Student Newman Keuls Test (p<0,05). Source: author's collection.

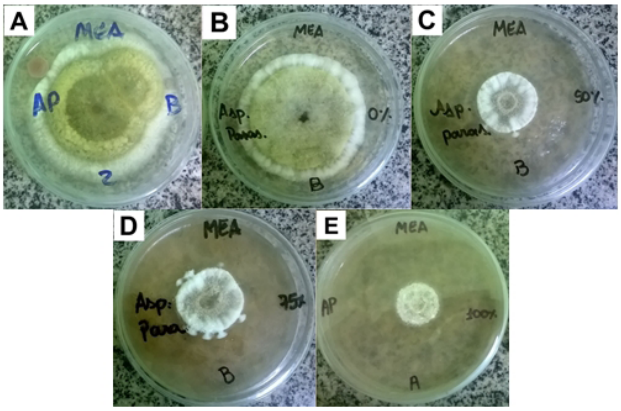


Source: author's collection.

Figure 1. *A. flavus* seeded in MEA under different treatments: A) Control, B) 100% *S.cerevisiae*, C) *S.cerevisiae* + 50% Product, D) *S.cerevisiae* + 75% Product E) 100% Product.

production, with a significant difference between the control treatment and the 100% *S.cerevisiae* treatment. *A. flavus* produced 2.77 µg AFB_1 in the latter; the lowest yield obtained in all experiments, thus demonstrating that inactivated yeast is effective in inhibiting AFB_1 production by *A. flavus*.

For *A. flavus* (A615), the lowest toxin synthesis was observed in the 100% *S.cerevisiae* treatment, where fungal growth exceeded the means established by Pitt and Hocking (2009) for this fungi species. This can be explained by a low aflatoxigenic capacity of the *A. flavus* strain and by the compounds present in the cell wall of *S. cerevisiae*, such as β-glucans and mannoproteins that are associated with the reduction of toxin production (ABDOLSHAHI et al., 2018).



Source: author's collection.

Figure 2. *A. parasiticus* seeded in MEA under different treatments. A) Control, B) 100% *S.cerevisiae*, C) *S.cerevisiae* + 50% Product, D) *S.cerevisiae* + 75% Product E) 100% Product.

Table 2. *A. flavus* and *A. parasiticus* AFB_1 production in the presence of inactivated *S. cerevisiae* and CP.

Treatments	AFB_1 (ug.g^{-1}) ± Standard deviation	
	Aspergillus flavus	***Aspergillus parasiticus***
Control	38,1^a±3,7	634,4^a±361,3
100% *S.cerevisiae*	2,7^b±3,7	503,0^a±65,1
S.cerevisiae +50% product	7,2^b±4,0	2,93^b±1,6
S.cerevisiae +75% product	5,2^b±3,9	41,6^b±21,5
100% product	8,4^b±0,9	4,5^b±4,0

The averages followed by the same letter in the same column do not differ statistically from each other, by the Normality Test (p<0,05). The mean comparison test was not applied because the interaction F was not significant.

For *A. parasiticus* (NRLL 2999), results were similar to those reported by Gerbaldo et al. (2012), as the lowest mycotoxin production was observed in the *S. cerevisiae* + 50% commercial additive, where the lowest growth means were observed.

Different commercial product concentrations led to good results, with a slightly higher AFB_1 production than 100% *S. cerevisiae* addition, with no difference between treatments. *A. parasiticus* in the treatment 100% *S.cerevisiae*, showed a high mycotoxin production, as well as the fungi, were grown in a petri dish containing MEA (Control).

A significant decrease in AFB_1 production for A. flavus was observed in the 100% yeast treatment. The *S. cerevisiae* + 75% product treatments led to increased AFB_1 synthesis for *A. parasiticus*.

S. cerevisiae has been reported as capable of inhibiting *A. flavus* and *A. parasiticus* AFB_1 production (ARMANDO et al. 2012; BOVO et al., 2010). In addition to being a well-known probiotic capable of preventing absorption in the gastrointestinal tract (AZEEM et al. 2019).

In another study, Abdolshahi et al. (2018), used *S. cerevisiae* mannoprotein to coat pistachio samples demonstrating that the presence of yeast caused a decrease and delay in sporulation in all pistachio samples inoculated with *A. flavus*, with the appearance of mycelia and conidia after 5 days incubation compared to the control group, that is, without mannoprotein. When evaluating the production of AFB_1, he also observed that there was less production of total aflatoxin and AFB_1 in samples containing yeast, the higher the concentration of yeast, the greater the decrease in the production of toxin.

Two AFB_1 concentrations, 10 and 25 ng mL^{-1}were used in the adsorption test. Variations between treatments were observed, where negative results were considered as no adsorption. At 10 ng/mL, two treatments demonstrated the ability to adsorb AFB_1, namely *S.cerevisae* + 75% Product and 100% Product, with adsorption rates of 12.93% and 21.08%, respectively (Table 3).

Table 3. AFB_1 adsorption percentages by inactivated *S. cerevisiae* and commercial additive.

Treatments	Aflatoxin B1			
	10 ng/mL		25 ng/mL	
	Mean	Deviation	Mean	Deviation
100% *S.cerevisae*	NA^b	±8,6	NA^a	±7,6
S.cerevisae +50% product	NA^b	±1,0	3,59^a	±12,6
S.cerevisae +75% product	12,9^a	±7,5	NA^a	±4,2
100% product	21,0^a	±8,4	NA^a	±27,4

The averages followed by the same letter do not differ statistically from each other, by the Normality Test (p<0,05). The mean comparison test was not applied because the interaction F was not significant. NA: No Adsorption.

The treatment composed solely of commercial additive was the most effective at adsorbing AFB_1 at 10 ng/mL. As demonstrated in other studies, the use of *S. cerevisiae* cell wall-based additives sterifies glucomannans capable of binding to AFB_1. The results reported by Pinheiro et. al. (2017) using a product anti mycotoxin additive from brewer's yeast, demonstrated that the highest amount of mycotoxin was adsorbed at 100% product concentration. Dawson, Evans, and Kudupoje (2006) reported up to 85% aflatoxin adsorption using only YCW-based products. In another study, Pinheiro et. al. (2015) when comparing the binding capacity of two additives against Ochratoxin A (OTA), one based on lactic acid bacteria associated with *Bacillus* sp. and another composed of dried *S. cerevisiae*, noted that the dry yeast commercial product was more efficient at adsorbing OTA (24.8 to 31.8%) than lactic acid bacteria associated with *Bacillus* sp. (1.2 to 7.7%), reporting the most efficient concentration as 100%. Concerning 25 ng/mL AFB_1, the only treatment with positive adsorption results was Yeast + 50% Product, leading to 3.59% adsorption.

The inactivated yeast treatment showed no adsorption capacity for both AFB_1 concentrations, indicating that inactivated *S. cerevisiae* (A8L1) displays no ability to bind to AFB_1. However, at 25 ng/mL, *S. cerevisiae* (A8L1) associated with commercial products demonstrated the ability to adsorb AFB_1, although this was not effective at the lowest AFB_1 concentration. Another factor that could justify the inability of the inactivated yeast to adsorb the investigated AFB_1 concentrations could be the experimental contact time, since Piotrowska and Zakowska (2005), when working with *Lactobacillus*, obtained 70% and 87% adsorption after five days of bacteria incubation at 37 °C with OTA, demonstrating that the contact period between the toxin and the biological product increases toxin removal rates. Another important aspect was studied by Bejaoui et al. (2004), who reported that the addition of acid and heat increased adsorption rates to 75%, hypothesizing that the formed complex is more stable under these conditions, thus indicating the physical nature of the bond.

CONCLUSIONS

It was concluded that the association between the commercial additive and inactivated *S. cerevisiae* was effective in reducing the growth of fungi. The production of mycotoxins by *A. flavus* and *A. parasiticus* showed a reduction for some treatments, and although low, there was also the AFB_1 adsorption activity.

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