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IMMOBILIZATION OF A FUMONISIN ESTERASE WITH DIFFERENT TECHNOLOGIES

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ABSTRACT

Toxicity of fumonisin B_1 (FB_1), the most common fumonisin mycotoxin, can be significantly decreased by enzymatic cleavage of its two tricarballylic acid groups, using fumonisin esterase. An enzyme product should be formulated in a solid form for use as a feed supplement, and have a long shelf-life and high heat stability. Therefore, a fumonisin esterase (further referred to as FE2) was expressed in Pichia pastoris and the enzyme was immobilized with different techniques. Covalent immobilization of FE2 on amine-functionalized zeolite, crosslinked with glutaraldehyde, resulted in low specific capacity (0.8 mg enzyme/g zeolite), and poor stability. Reversible immobilization of FE2 on different solid carriers resulted in even > 95% immobilization efficiency, and the enzyme could be recovered after resuspension in buffer without significant loss of activity. This immobilization method resulted in at least 3 years of shelf-life even at 50 °C. The thermal stability of these products significantly increased compared to the enzyme solution; the products retained even nearly 100% of their activity after incubating at 100 °C for 30 minutes. This increased heat stability makes the product compatible with feed processing techniques, such as pelleting.

ÖSSZEFOGLALÁS

A leggyakoribb fumonizin, a fumonizin B_1 (FB_1) toxicitása jelentősen csökkenthető a két trikarbalilsav csoport fumonizin észteráz segítségével történő enzimatikus hasításával. A takarmány-kiegészítőként történő felhasználáshoz az enzimterméket szilárd formába kell hozni, valamint annak hosszú eltarthatósági idővel és magas hőstabilitással kell rendelkeznie. Ezért egy fumonizin-észterázt (továbbiakban FE2) Pichia pastoris-ban expresszáltunk, és az enzimet különböző technikákkal immobilizáltuk. A FE2 kovalens immobilizálása amin-funkcionalizált zeoliton, glutáraldehiddel keresztkötve, alacsony fajlagos kapacitást eredményezett (0,8 mg enzim/g zeolit), gyenge stabilitás mellett. Az FE2 reverzibilis rögzítése különböző szilárd hordozókon akár > 95%-os immobilizálási hatékonyságot és 50 °C-os tárolási hőmérsékleten is legalább 3 év eltarthatóságot biztosított. Ezen termékek termikus stabilitása jelentősen megnőtt az enzimoldathoz képest; az oldott enzim 55 °C felett irreverzibilisen denaturálódik, míg a termékek 100 °C-on 30 perces inkubálás után is megőrzik aktivitásuk akár 100%-át. Ez a megnövekedett hőstabilitás lehetővé teszi, hogy a termék kompatibilis legyen a takarmányfeldolgozási technikákkal, mint például a pelletálás.

INTRODUCTION

Fumonisins – primarily produced by *Fusarium verticillioides* and *Fusarium proliferatum* – are among the most frequently occurring mycotoxins. The most toxic and prominent substance of this mycotoxin family is Fumonisin B_1 (FB_1), which causes 70% of fumonisin contaminations and can be associated with several diseases in livestock (Kamle et al., 2019). A comprehensive study between 2006 and 2016 on raw cereal grains worldwide showed detectable levels of FB_1 in 61% of the samples (Lee et al., 2017).

Due to the high contamination levels, and the serious health risks associated with its consumption, dealing with FB_1 contamination is a great challenge in the food industry. FB_1 is known to be very stable and altered only when heated above 150–200 °C or under strongly alkaline conditions. Physical (sorting, washing, irradiation, adsorbents) or chemical (alkaline- or ozone-) treatments are not effective enough, lower the nutritional value of the food, or are not applicable for food decontamination on an industrial scale (Liu et al., 2022). In contrast to the inefficiency of the physical and chemical techniques, enzymatic detoxification has become a promising approach for postharvest decontamination of FB_1.

Fumonisin esterases (FE) catalyze the consecutive de-esterification of FB_1 at the C-6 and C-7 positions, resulting in an aminopentol (hydrolyzed FB_1, HFB_1) and two tricarballylic acid (TCA) molecules as final products (Figure 1) (Incze et al., 2024).

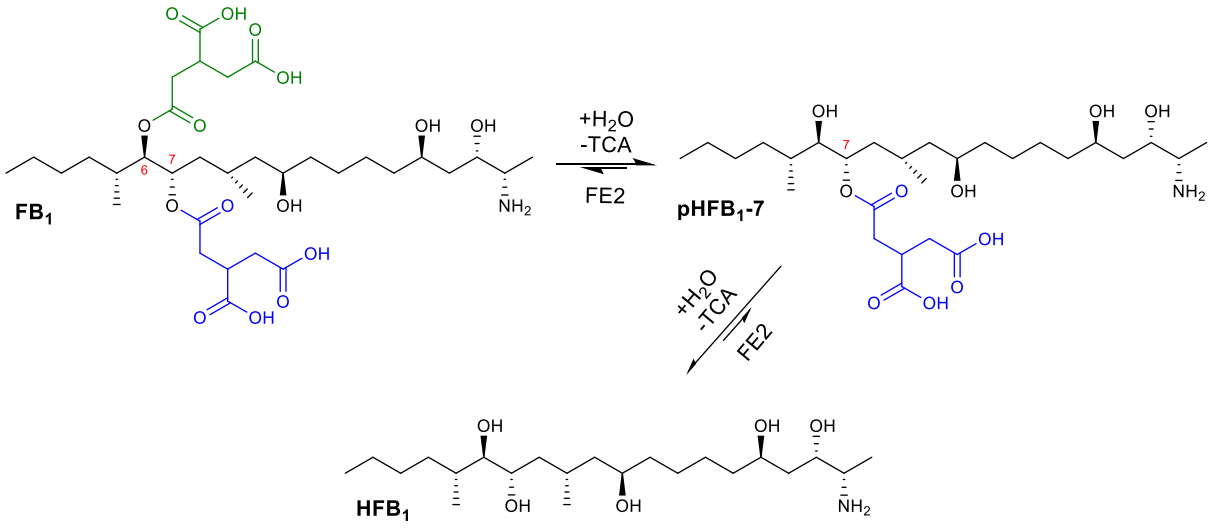

Figure 1 Consecutive de-esterification of FB_1 to $pHFB_1$_7 and HFB_1 catalyzed by FE2

According to Europen Food Safety Authority (EFSA), FB_1 hydrolysis should be considered a detoxification mechanism (Schrenk et al. 2022). The successful application of FE2 as a feed additive requires a solid enzyme formulation. The resulting product should retain the original enzyme activity, possess great storage stability, and tolerate the high temperatures of feed processing. Therefore, in this study, we investigated two different enzyme immobilization techniques.

MATERIALS AND METHODS

FE2 was produced by precision fermentation of *Pichia pastoris* pD912_FE2 (Incze et al., 2024). The FE2 solution was obtained by separating the cells by centrifugation at 7000 G and sterile filtration of the supernatant containing FE2 on a 0.2 µM filter. The reversibly immobilized products were prepared by mixing 100 g of the FE2 solution with 100 g of either ground corn cob, wheat bran, or shredded sugar beet pulp and drying them on a 4M8-Trix fluid bed dryer (PROCEPT, Zele, Belgium).

Small-sized zeolite (particle size <50 µm) was mixed with 50 ml of methanol (MeOH), while 1 ml of (3-aminopropyl) trimethoxysilane (APTMS) was added dropwise and the suspension was stirred overnight. The functionalized zeolite was consecutively washed with 50 ml MeOH and H_2O, resuspended in 50 ml of 5% glutaraldehyde in 50 mM K-phosphate buffer (pH 7.5) and stirred overnight. The amine- and glutaraldehyde-functionalized zeolite was consecutively washed with 40 ml MeOH and 40 ml 50 mM K-phosphate buffer (pH 6.5) and dried under vacuum. The irreversibly immobilized product was prepared by overnight stirring 7 g of the surface-modified zeolite with 42 ml of FE2 solution. The product was subsequently washed with 42 ml of 50 mM K-phosphate buffer (pH 6.5) and dried under vacuum. Enzyme load was estimated by comparing the protein concentration of the solution before and after the immobilization with the Bradford Protein Assay (Thermo Scientific, USA).

Portions of 1,00 g of the reversibly immobilized products and 100 mg of the irreversibly immobilized product were incubated at 20 °C, 80 °C, 100 °C and 120 °C for 30 minutes. Reversibly immobilized products of 1.00 g were shaken in 9 ml of 50 mM K-phosphate buffer (pH 6.0) for 15 minutes at 100 rpm. After a further 83.3-fold dilution of the extract, the residual activities were measured by mixing 20 µl of the diluted extract with 80 µl of FB1 solution in 50 mM K-phosphate buffer (pH 6.0) and incubating the reaction mixture at 37 °C for 15 min. The initial FB_1 concentration in the reactions was 100 µM. The residual activity of the immobilized enzyme product was measured similarly, except the suspension of the product in appropriate dilution was added to the reaction mixture. Relative activities were calculated as described by Incze et al. (2023).

RESULTS AND DISCUSSION

The enzyme FE2 was immobilized reversibly on ground corn cob, wheat bran and shredded sugar beet pulp; the residual enzyme activities after the immobilization were 45%, 107%, and 89% of the initial activity, respectively. The enzyme load of the FE2 irreversibly immobilized on surface-modified zeolite was 0.8 mg/g, and the residual enzyme activity was 95%.

The FE2 products were incubated at elevated temperatures to test their heat stability. FE2 immobilized on ground corn cob, wheat bran, and shredded sugar beet pulp showed exceptional heat stability, retaining more than 75% of their initial activities after a 30-min incubation at 100 °C, meanwhile, the covalently immobilized FE2 on zeolite lost nearly 90% of its activity under the same conditions (Figure 2). In solution, FE2 lost all its activity above 60°C, which corresponds to its melting temperature of 55°C (Incze et al., 2024). These results show that drying the solution of FE2 on agricultural byproducts is not only

a relatively straightforward and cost-effective method but also greatly protects the enzyme from heat denaturation. This immobilization technique could make these products compatible with standard feed pelleting, however, the moisture of the pelleted feed should be taken into consideration.

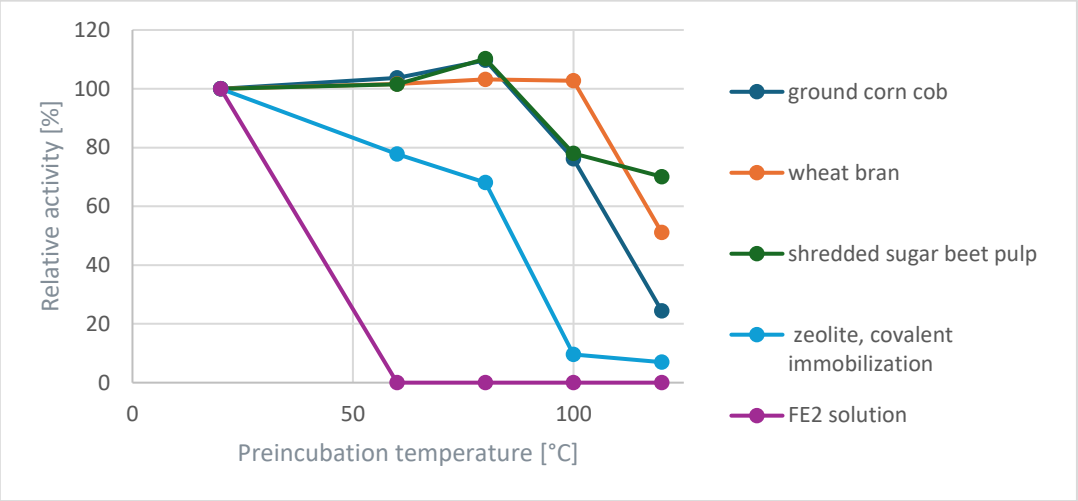

Figure 2 Heat stability of immobilized enzyme products. Relative activities were normalized to the activities of the products incubated at 20°C

FE2 immobilized on wheat bran – possessing the highest recoverable activity and the best heat stability among the tested products – was subjected to a 3-year shelf-life test, incubated at 20 °C and 50 °C in vacuum-sealed containers. Until 1 year of incubation at 20 °C, more than 90% of the activity was retained, and nearly 60% of the original activity was measured even after 3 years of storage (Figure 3). When stored at 50 °C, more than 80% and 50% of the initial activity was detected after 1 and 3 years, respectively (Figure 3). These results show that immobilization of FE2 on wheat bran is a promising technology to conserve the enzyme activity for an extended period.

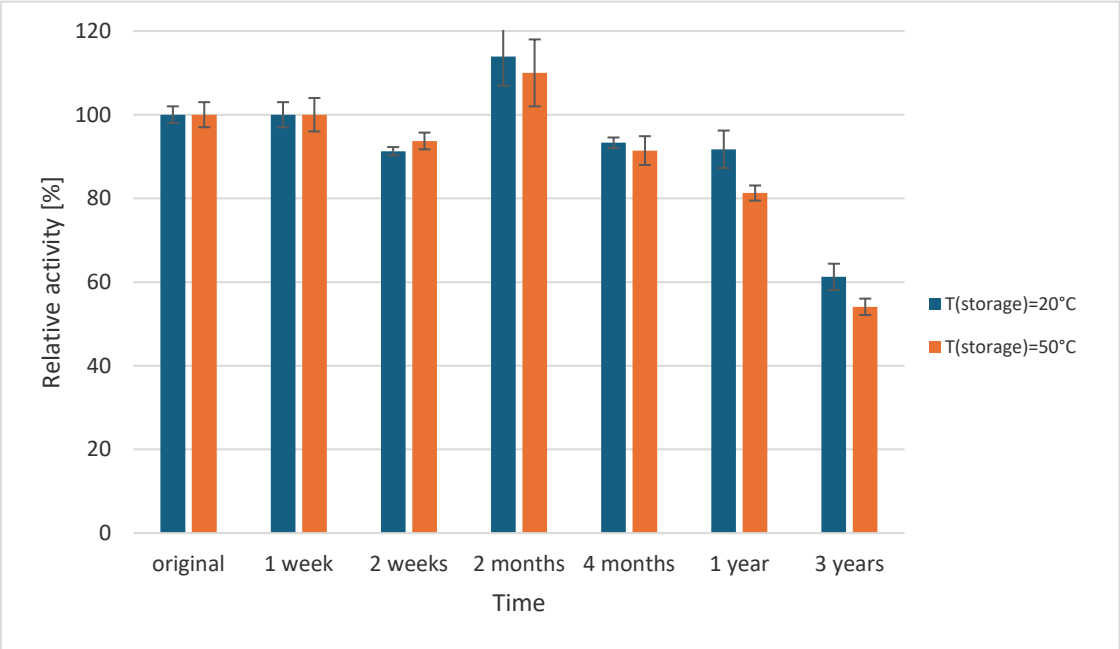

Figure 3 Storage stability of FE2 immobilized on wheat bran

CONCLUSIONS

Immobilization of the crude *Pichia pastoris* fermentation supernatant, containing recombinant FE2, directly to different agricultural byproducts showed great potential for increased heat stability and extended shelf-life stability of the enzyme while being an environmentally and economically feasible technology.

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