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(54) Title: CUTINASE FOR DETOXIFICATION OF FEED PRODUCTS

(57) Abstract: The present invention relates to a method comprising treatment with cutinase for detoxification of feed products contaminated by the mycotoxin zearalenone.

CUTINASE FOR DETOXIFICATION OF FEED PRODUCTS

REFERENCE TO A SEQUENCE LISTING

This application contains a Sequence Listing in computer readable form. The computer readable form is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to a method comprising treatment with cutinase for detoxification of feed products contaminated by the mycotoxin zearalenone.

BACKGROUND OF THE INVENTION

Any discussion of the prior art throughout the specification should in no way be considered as an admission that such prior art is widely known or forms part of common general knowledge in the field.

Several plant pathogenic and/or post-harvest *Fusarium* species on cereals produce toxic substances of considerable concern to livestock and poultry producers, e.g., deoxynivalenol, T-2 toxin, HT-2 toxin, diacetoxyscirpenol and zearalenone.

Zearalenone is found worldwide in a number of cereal crops, such as maize, barley, oats, wheat, rye, rice, millet and sorghum. Zearalenone production does not seem to occur in significant amounts prior to harvest, but under proper environmental conditions, it is readily produced on corn and small grains in storage.

When cereal grain is used in ethanol production and the starch is consumed the zearalenone is concentrated in the fermentation by-products, e.g., in the distiller's dried grain. The contents of zearalenone in the fermentation by-products may be increased three-fold relative to the cereal grain.

The toxin is heat-stable, and it is not destroyed by long storage, roasting, or by the addition of propionic acid or mold retardants.

Despite their structural dissimilarity to the steriodal estrogens, zearalenone and several of its derivatives possess estrogenic activity. Zearalenone undergoes a folding such that hydroxyl or potential hydroxyl groups become appropriately orientated to facilitate binding to tissue receptors that normally bind estrogens.

Zearalenone is the primary toxin causing infertility, abortion or other breeding problems, especially in swine. The symptoms are especially severe in prepubertal gilts including enlarged mammae, swelling of uterus and vulva, and atrophy of the ovaries. In severe cases, prolapse of the vulva and rectum may occur. Boars exhibit enlarged mammae and atrophied testes.

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Zearalenone is present in the meat from animals feeding on contaminated grain as well as in bread baked from contaminated wheat. While cases of poisoning of humans are rare there is concern about the effect of the long term exposure of humans to such an estrogenic activity.

Inactivation of mycotoxins, including zearalenone, using epoxidase or lactonase is

disclosed in WO9612414.

There is a need for further methods of detoxification of animal feed products, e.g., such as fermentation by-products, including distiller's wet and dried grain, contaminated by the mycotoxin zearalenone.

SUMMARY OF THE INVENTION

The inventors of the present invention have discovered that zearalenone in a feed product can be degraded by treating the feed product with a cutinase. Accordingly, in a first aspect the invention provides a process for degrading zearalenone in a feed product which process comprises treating said feed product with a cutinase.

In a second aspect the invention provides a use of a cutinase for degrading a mycotoxin.

In a third aspect, the present invention provides a use of a cutinase for degrading the mycotoxin zearalenone.

Unless the context clearly requires otherwise, throughout the description and the claims, the words "comprise", "comprising", and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of "including, but not limited to".

Detailed description of the invention

Zearalenone

In the context of this invention the term "zearalenone" comprises the mycotoxin zearalenone produced from certain *Fusarium* sp. The IUPAC name is (*4S,12E*)-15, 17-Dihydroxy-4-methyl-3-oxabicyclo[12.4.0]octadeca-12, 15, 17, 19-tetraene-2, 8-dione. The term "zearalenone" also comprises any derivative of zearalenone which comprises an internal carboxylic ester bond susceptible for modification by a cutinase.

Animal feed products

The term "animal" includes all animals, including human beings. Examples of animals are cattle, (including but not limited to cows and calves); mono-gastric animals, e.g. pigs or swine (including, but not limited to, piglets, growing pigs, and sows); poultry such as turkeys and chicken (including but not limited to broiler chicks, layers); and fish (including but not limited to salmon).

The term "feed" or "feed product" means any compound, preparation, mixture, or composition suitable for, or intended for intake by an animal.

The feed product may be a product which apart from an unwanted level of

zearalenone is suitable for consumption by an animal. The feed product can also be a product suspected of comprising an unwanted level of zearalenone, and/or a product having an unknown level of zearalenone, including products not comprising a detectable level of zearalenone.

Preferably the feed product is a grain-based product. Preferably the grain based product comprises cereal(s), e.g., one or more of corn, wheat, barley, rye, rice, sorghum and millet. Also preferred are grain based product comprising material derived from one or more of corn, wheat, barley, rye, rice, sorghum and millet. In one embodiment, the feed product

may e.g. be derived solely from cereal(s), and in another embodiment partly from legumes, e.g. from soybean, and partly from cereals. The grain based product may comprise whole or milled grain, e.g., wet or dry milled grain, including grain based product comprising fractions of wet or dry milled grain, e.g., gluten, protein, starch, and/or oil fractions. Also preferred are products comprising a by-product from brewing and/or fermentation processes, e.g., spent grain. Spent grain is the by-products from the production of alcoholic beverages and ethanol fuels. Brewers' spent grain (BSG) is the residue of beer making in breweries, which use malted barley as the major raw material. Distiller's spent grain (DSG) is the product left in distilleries after alcohol is removed by distillation from the fermented grains such as corn, wheat, barley, rice, and rye. Distiller's spent grain is also known as distiller's grain. Wet distiller's grain (WDG) is dried to produce dried distiller's grain (DDG) which is used primarily as animal feed.

Cutinases

In the context of this invention the term "cutinases" include enzymes comprised by the enzyme classification E.C.3.1.1.74. Preferred are the below mentioned enzymes as well as enzymes with homologous sequence, especially recombinant and/or substantially purified enzymes.

The cutinase may be derived from a fungus. Particularly, the cutinase may be derived from a strain of *Humicola*, particularly *H. insolens*, more particularly *H. insolens* strain DSM1800 (US 5,827,719) or from a strain of *Fusarium*, e.g. *F. roseum culmorum*, or particularly *F. solani pisi* (WO 90/09446; WO 94/14964, WO 94/03578). The fungal cutinase may also be derived from a strain of *Rhizoctonia*, e.g. *R. solani*, or a strain of *Alternaria*, e.g. *A. brassicicola* (WO 94/03578). The cutinase may also be a variant of a parent cutinase such as those described in WO 00/34450, or WO 01/92502, all of which are hereby incorporated by reference. The cutinase may be the variant of the *Humicola insolens* cutinase comprising the substitutions E6Q, G8D, A14P, N15D, E47K, S48E, R51P, A88H, A91H, A130V, E179Q and R189V, which is disclosed at p. 24, line 11 of 10038.204-WO.

SEQ ID NO:1 is the amino acid sequence of the *Humicola insolens* cutinase (corresponding to the mature part of SEQ ID NO:2 of US 5,827,719, and of SEQ ID NO:1 of WO 01/92502), and SEQ ID NO:2 is the amino acid sequence of the *Fusarium solani pisi* according to Fig. 1D of WO 94/14964.

The cutinase must be present in the medium to be detoxified in effective amounts. Preferably the cutinase is present in concentrations of 0.01-100 mg enzyme protein pr. kg dry matter, preferably 0.1-10 mg enzyme protein pr. kg dry matter, or more preferably 1-5 mg enzyme protein pr. kg dry matter.

The medium

In an embodiment the cutinase is degrading the zearalenone in a medium comprising the feed product. The medium is preferably aqueous and may be a liquid, a paste or a slurry. To form a suitable medium water may be added to the feed product. The cutinase be comprised in solid or liquid formulations suitable for application to said medium.

In a embodiment the cutinase is degrading the zearalenone to an extent whereby the content of zearalenone per kg dry matter feed product is reduced to less than 50%, preferably less than 60%, more preferably less than 70%, and most preferably to less than 80% of the initial amount.

The detoxifixation efficiency of the invention depends on e.g. availability of water, pH, temperature and buffer of the medium. For example, the treatment may take place at a pH-value at which the relative activity of the actual cutinase is at least 50, or 60, or 70, or 80 or 90%. Likewise, for example, the treatment may take place at a temperature at which the relative activity of the actual cutinase is at least 50, or 60, or 70, or 80 or 90%. The relative activity is calculated relative to the activity at the pH value where the highest activity is observed.

pH in the medium

Depending, inter alia, on the characteristics of the cutinase employed, the pH in the medium employed should normally be in the range of 5-11, preferably in the range 6-10, e.g. 6.5-8.5.

Temperature in the medium

Preferably a reaction temperature is applied which is close to the optimum temperature for the cutinase. In numerous embodiments of the invention, temperatures in the range of 10-65°C, more preferably 30-50°C, should be employed.

Treatment duration

The duration of treatment depends, inter alia, on the treatment type, the type of item to be treated, the properties of the medium, e.g. temperature and pH and the type and amounts of enzyme employed.

The enzymatic reaction is continued until the desired result is achieved, following which it may or may not be stopped by inactivating the enzyme, e.g., by a heat-treatment step.

For detoxification purposes treatment times in the range of 1 minute to 1 week may be employed. In many cases a treatment time in the range of 6 to 48 hours will be suitable.

Identity

The relatedness between two amino acid sequences or between two nucleotide

sequences is described by the parameter "identity".

For purposes of the present invention, the degree of identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends in Genetics* 16: 276-277), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

(Identical Residues x 100)/(Length of Alignment – Total Number of Gaps in Alignment)

For purposes of the present invention, the degree of identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *supra*), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled "longest identity" (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

(Identical Deoxyribonucleotides x 100)/(Length of Alignment – Total Number of Gaps in Alignment)

Homologous sequence

The term "homologous sequence" is defined as a predicted protein that gives an E value (or expectancy score) of less than 0.001 in a tfasty search (Pearson, W.R., 1999, in *Bioinformatics Methods and Protocols*, S. Misener and S. A. Krawetz, ed., pp. 185-219) with a specified sequence.

The term "homologous sequence" may also be defined as a sequence that has a degree of identity at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or even 100%, to a specified sequence.

EXAMPLES

Example 1

Enzyme: A recombinantly produced enzyme composition comprising the variant of the cutinase from *Humicola insolance* disclosed at p. 24, line 11 of 10038.204-WO.

Assay: Reactions were performed in 300 microL volumes in eppendorf tubes comprising

zearalenone 30 microM, Tris 100 mM and enzyme 0.1 mg EP/mL. In control reactions the enzyme volume was substituted an equivalent amount of H_2O. The reactions were incubate 24 hours at 37°C before being terminated by adding 600 microL of a 100 microM acetonitrile stop solution. Reactions were stored at -20° C until chromatographic analysis.

Chromatographic analysis: Samples were centrifugated and the supernatant analysed for zearalenone by HPLC-DAD as described by Smedsgaard (*J. Chromatogr. A*, 1997, *760,* 264-270). The DAD scanned from 200-600 nm. Separation was done on a Phenomenex (Torrance, CA) Luna C18(2) 10×2 mm ID, 3 micrometer, column 2, using a linear gradient moving form 5% to 100% acetonitrile in 20 min. Residual zearalenone was calculated relative to the control. The results are presented in tables 1.

Table 1. Residual zearalenone after 24 hours incubation with or without a cutinase at pH 7.

Enzyme	**Residual zearalenone (%)**
Control	100
Cutinase	19

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CLAIMS

1. A process for degrading zearalenone in a feed product which process comprises treating said feed product with a cutinase.

2. The process according to claim 1 wherein the dosage of the cutinase is 0.01-100 mg enzyme protein pr. kg dry matter, preferably 0.1-10 mg enzyme protein pr. kg dry matter, or more preferably 1-5 mg enzyme protein pr. kg dry matter.

3. The process according to claim 1 or claim 2 wherein the feed product is a grain-based feed product.

4. The process according to any one of claims 1 to 3 wherein the feed product comprises one or more selected from corn, wheat, barley, rye, rice, sorghum and millet.

5. The process according to any one of claims 1 to 4 wherein the feed product is an animal feed composition.

6. The process according to any one of claims 1 to 5 wherein the feed product is a by-product from a fermentation process.

7. The process according to any one of claims 1 to 6 wherein the feed product comprises brewer's spent grain, distiller's spent grain, distiller's wet grain, and/or distiller's dried grain.

8. The process according to any one of claims 1 to 7 wherein the feed product is a swine feed product.

9. The process according to any one of claims 1 to 8 wherein the cutinase is a cutinase having the sequence shown in SEQ ID NO:1 or a homologous sequence.

10. The process according to any one of claims 1 to 9 wherein the cutinase is a variant of the cutinase shown in SEQ ID NO:1 comprising one or more, including all of the substitutions G8D, N15D, S48E, A88H, N91H, A130V and R189V.

11. The process according to any one of claims 1 to 10 wherein the cutinase is a cutinase having the sequence shown in SEQ ID NO:2 or a homologous sequence.

12. A use of a cutinase for degrading the mycotoxin zearalenone.

13. The use according to claim 12 in a grain-based feed product.

14. The use according to claim 12 or claim 13, wherein the dosage of the cutinase is 0.01-100 mg enzyme protein pr. kg dry matter, preferably 0.1-10 mg enzyme protein pr. kg dry matter, or more preferably 1-5 mg enzyme protein pr. kg dry matter.

15. A process for degrading zearalenone in a feed product, substantially as herein described with reference to any one or more of the examples but excluding comparative examples.