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(54) **Eljárás mikotoxinok enzimatikus lebontására szolgáló adalékanyag előállítására, adalékanyag és alkalmazása**

Az európai szabadalom ellen, megadásának az Európai Szabadalmi Közlönyben való meghirdetésétől számított kilenc hónapon belül, felszólalást lehet benyújtani az Európai Szabadalmi Hivatalnál. (Európai Szabadalmi Egyezmény 99. cikk(1))

A fordítást a szabadalmas az 1995. évi XXXIII. törvény 84/H. §-a szerint nyújtotta be. A fordítás tartalmi helyességét a Szellemi Tulajdon Nemzeti Hivatala nem vizsgálta.

METHOD FOR PRODUCING AN ADDITIVE FOR THE ENZYMATIC DEGRADATION OF MYCOTOXINS AND ADDITIVE AND USE OF THE SAME

The present invention relates to an additive for the enzymatic degradation of fumonisins, in vegetable raw materials and mixtures containing vegetable raw materials, as well as the use of genes.

Mycotoxins very frequently occur on agricultural vegetable products and, depending on the type of mycotoxins, inflict severe economic damage, in particular, in the foods produced from agricultural products and even in animals and humans fed with such foods, said damage being extremely manifold. Numerous methods have already been developed, trying to detoxify or degrade, or render harmless, such mycotoxins in order to inhibit any damage caused by mycotoxins in the fields of animal and human nutrition, animal breeding, food and feed processing and the like.

Known mycotoxins comprise a plurality of structurally interrelated mycotoxins such as, for instance, fumonisins, among which fumonisin B1 is the most frequently occurring toxin of the group. There are, however, numerous derivatives and related molecules which are also known to exhibit noxious effects in humans and animals. Thus, it is known that fumonisins impair the sphingolipid metabolism by interacting with the enzyme ceramide synthase. Sphingolipids not only are components of cell membranes, but also play an important role as signal and messenger molecules in many elementary cellular processes like cell growth, cell migration and cell binding, in inflammatory processes and intracellular transport procedures. Due to this impairment of the sphingolipid metabolism, fumonisins have been made responsible for the toxic effects on various animal species and also humans. It could, thus, demonstrated that fumonisins have cancerogenic effects in rodents, and, based on epidemiologic data, they have been associated with esophageal cancer and neural tube defects in humans. They have been held responsible for the typical toxicosis caused by pulmonary edemas, for instance, in various animal species such as, e.g., swine. In this context, fumonisins constitute an almost ubiquitous contamination source on various cereal crops, in particular corn as well as nuts and vegetables, and this strongly negative effect relating to the health of humans and animals is not to be neglected.

The microbial degradation of fumonisins has already been described in EP-A 1 860 954, according to which microorganisms are used to detoxify fumonisins and fumonisin derivatives by adding to feeds detoxifying bacteria or yeasts selected from precisely defined strains for detoxifying fumonisins.

Catabolic metabolic paths for the biological degradation of fumonisins and the genes and enzymes responsible therefor have already been described too. Thus, EP 0 988 383, for instance, describes fumonisin-detoxifying compositions and methods, wherein the fumonisin-degrading enzymes used are

above all produced in transgenic plants in which the detoxification of fumonisins is effected using an amine oxidase that requires molecular oxygen for its enzymatic activity.

Moreover, WO 2004/085624 describes transaminases, deaminsases and aminomutases as well as compositions and methods for the enzymatic detoxification to detoxify, in particular, aminated toxins, e.g. fumonisins. In this context, polypeptides possessing deaminase activity are used for detoxification.

From WO 00/04158, the use of fumonisin-degrading amine oxidases in the production of foods or feeds and in the processing of vegetable raw materials has become known.

Hitherto known methods, however, have in common that, in order to detoxify mycotoxins, they require molecular oxygen for the described catabolic metabolic paths, yet the amine oxidases, which are particularly required, cannot work under oxygen-independent conditions. The use of such genes and enzymes for the detoxification of feeds, for instance in the digestive tracts of animals, is not possible because of the substantially oxygen-free environment in the digestive tracts of animals, the known genes and enzymes thus exhibiting no activity.

The invention aims to provide an additive for the enzymatic degradation of fumonisins, by which it is feasible in a safe and reliable manner to degrade or detoxify such mycotoxins, particularly in an oxygen-independent environment.

Vegetable raw materials in this context include cereals or cereal products, grasses, fruits or vegetables and intermediate products containing these substances for the production of foods and feeds such as, for instance, silage, fruit mash or the like.

Additives in this context include feed additives, food additives as well as additives for the production of bioethanol.

The nucleic acid sequences used for degradation of mycotoxins or the enzymes expressed in prokaryotic and eukaryotic host cells by said nucleic acid sequences and catalytically acting in an oxygen-independent environment, are listed below.

Nucleic acids

Sequences:

>Seq ID 1 (*fum* (fumonisin catabolism) gene cluster, 15,420 bp)

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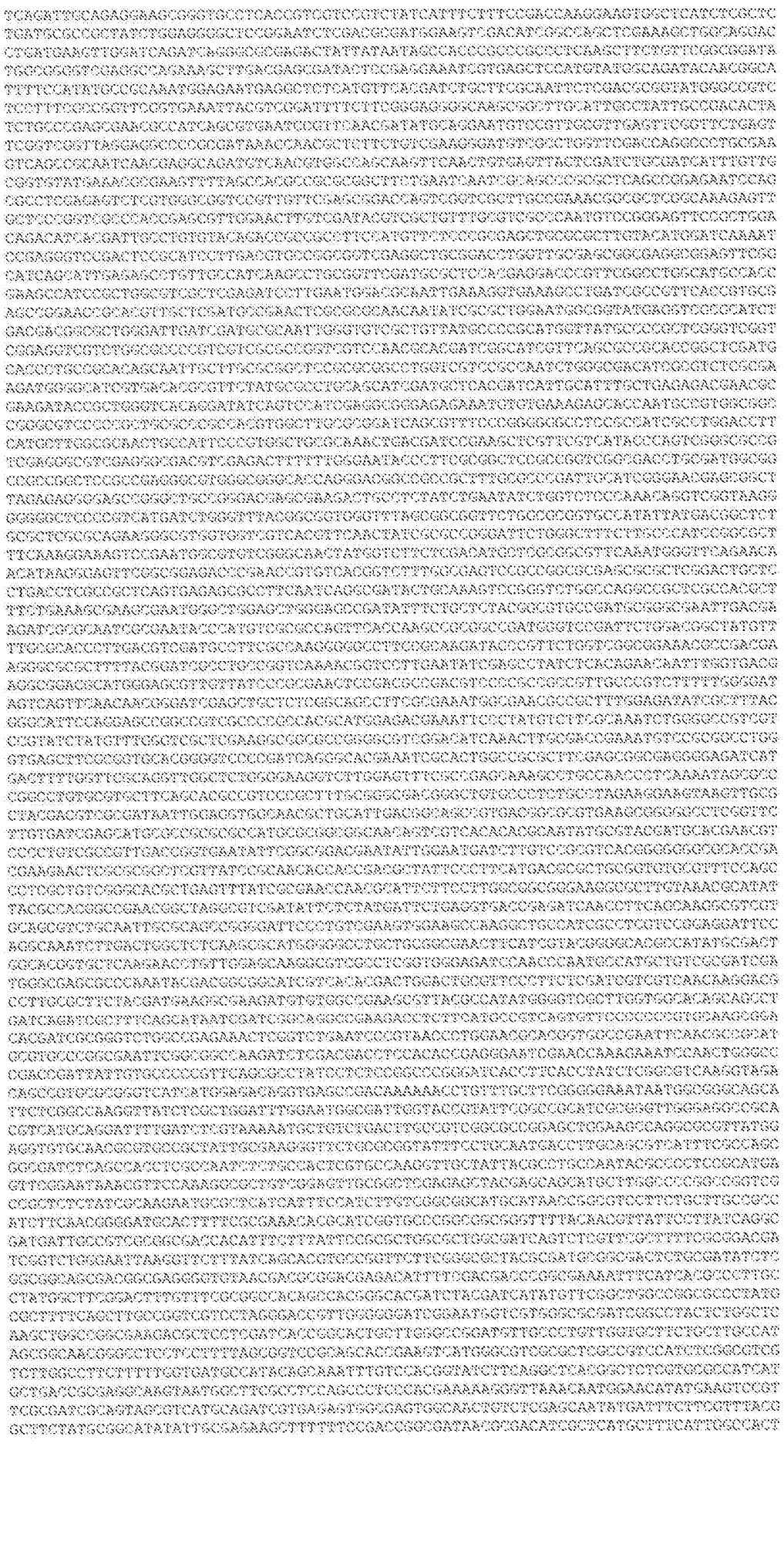

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>Seq ID 18 (*fumI*)

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Enzymes

Sequences:

>Seq ID 9 (FumD)
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LATEMSAAWVSFAVHGVPDQGTKSHWFRFERFGEIMTFGSQVGSGEGLGVSFSKACQPSK

>Seq ID 19 (FumI)
MANGTRQKDLRERAERVIPGGMYGHESTRLLPPEFFQFFRRALGARIWDADEQFYIDYMC
AYGPNLLGYRQSEIEAAADAQFLLGDTMTGPSEIMVNLAEAFVGMVFHADWAMFCKNGSD
ATSTAMVLARAHTGRKTILCAKGAYHGASPWNTFHTAGILASDRVHVAYYTYNDAQSLSD
AFKAHDGDIAAVFATPFFHEVFEDQALAQLEFARTARKCCDETGALLVVDDVRAGFRVAR
DCSWTHLGIEPDLSCWGKCFANGYPISALLGSNKARDAARDIFVTGSFWFSAVPMAAAIE
TLRIIRETPYLETLIASGAALRAGLEAQSQRHGLELKQTGPAQMPQIFFADDPDFRIGYA
WAAACLKGGVYVHPYHNMFLSAAHTVDDVTETLEATDRAFSAVLRDFASLQPHPILMQLA
GA

To solve these objects, an additive of this type is characterized in that it contains an enzyme of the sequence ID No. 9 as well as optionally, in addition, a cosubstrate for at the used enzyme, an enzyme of the sequence ID No. 19 and an inert carrier.

Such an additive which contains an enzyme of the sequence ID No. 9 as well as optionally, in addition, a cosubstrate for the used enzyme, an enzyme of the sequence ID No. 19 and an inert carrier, excels by selectively degrading, and hence detoxifying fumonisins. The use of an additive according to the invention, which essentially consists of isolated enzymes as well as, optionally, their cosubstrates and carriers, offers the advantage that the former will keep their catalytic activities in an environment and under conditions in which, for instance, complete microorganisms would not or hardly be active, while, at the same time, allowing for significantly higher specific activities and the catalyzation of defined reactions with the avoidance of undesired side reactions.

In addition, problems caused according the prior art on agricultural raw products by the use of reproducible germs will be safely avoided, and additives merely containing isolated enzymes will, moreover, provide an enhanced formulation aptitude for a selective and controlled activation, i.e., for instance, in a particular site of the digestive tract, as well as the avoidance of an undesired, elevated consumption of substrate.

Furthermore the sphingolipid metabolism impaired by the interaction of fumonisins with the enzyme ceramide synthase is maintained while, at the same, biologically degrading the fumonisins to non-toxic substances. Finally, technological detoxification applications will be achieved.

Such an additive, on the one hand, allows for to completely and reliably degrade, for instance, mycotoxins directly on raw materials, with the specific enzymes produced by this method catalyzing the degradation of fumonisins and intermediates of the degradation path, and, on the other hand, allows for to degrade mycotoxins, for instance directly during the production of bioethanol in the mash for the production of alcohol, or to degrade and render harmless mycotoxins even during the production of foods directly in the production process.

By designing the additive in a manner that the enzyme is used sheathed with a protective coating, as in correspondence with a preferred further development of the invention, it will be safeguarded that the enzyme will be secured against any premature loss of activity so as to safely and reliably develop their action in the intended site, for instance in the gastrointestinal tract.

By encapsulating the enzyme in a protective coating, it is feasible to transport the enzyme to its destination of use, for instance, in particular, into the digestive tract without being changed and, in particular, degraded or damaged, so that the enzyme will not start acting before the dissolution of the protective coating, for instance in the gastrointestinal tracts of humans or animals, thus ensuring an even more selective, rapid and complete degradation of the mycotoxins in the oxygen-independent environment of the gastrointestinal tract while, at the same time, preventing fumonisins from exerting their noxious effects on living creatures which have taken in the same together with food.

By preferably further developing the additive in a manner that the enzyme is a carboxylesterase of the sequence ID No. 9 the enzyme qualified for substrate catabolism will be substantially applied so as to ensure, in addition to a reduced amount of the enzyme to be applied, that no undesired side reaction will occur when using said enzyme.

According to a preferred further development of the invention, the additive is designed such that it contains a carboxylesterase of the sequence ID No. 9, an aminotransferase of the sequence ID No. 19, an α-keto acid as a cosubstrate and an inert carrier. By the additive containing a carboxylesterase, an aminotransferase, an α-keto acid as a cosubstrate besides an inert carrier, it is, in particular, feasible to initially hydrolyse fumonisins contained in foods by cleaving tricarballylic acid residues from the fumonisins using carboxylesterase, and to subsequently further react the thus hydrolysed fumonisin under the action of the aminotransferase and α-keto acid as a cosubstrate, preferably pyruvic acid in the present case, by substituting a keto group for an amino group of the hydrolysed fumonisin molecule so as to form

a 2-keto-hydrolyzed fumonisin, which is totally harmless, for instance, for mammals and can be excreted unchanged, and alanine as a side product, which too does not exert or have any negative effects, for instance, on organisms.

According to a preferred further development of the invention, the additive is further developed such that it contains a carboxylesterase of the sequence ID No. 9, at least one adsorbent like a clay mineral as well as, optionally, an inert carrier. When using but one carboxylesterase of the sequence ID No. 9 and at least one adsorbent, the detoxification of the fumonisins may also be performed in a manner that only the tricarballylic acid residues are cleaved and the thus formed, hydrolysed fumonisin is adsorbed on said adsorbent. By cleaving the tricarballylic acid residues by the aid of carboxylesterase, a substantially long-chain molecule is formed, which can be readily and reliably adsorbed so as to ensure the complete detoxification merely by the selected use of a single enzyme, in particular, by the oxygen-independent degradation of fumonisin and subsequent adsorption.

By additionally using at least one adsorbent selected, in particular, from clay minerals when using the carboxylesterase of the sequence ID No. 9, it is possible to render fumonisins totally harmless even without the addition of any further enzymes, by cleaving the two tricarballylic acid side chains by the carboxylesterase from the fumonisin molecule in a first step and forming what is called hydrolysed fumonisin. Hydrolysed fumonisin, which is a substantially chain-like molecule, can subsequently be adsorbed, for instance, on clay minerals so as to enable fumonisins to be rendered completely harmless even in a one-step enzymatic degradation process.

By degrading the fumonisins in an oxygen-independent manner, it is feasible to further develop the method according to the invention to the effect that the nucleic acid sequences of genes or enzymes will perform the degradation reactions safely and reliably without any addition of molecular oxygen so as to make the thus produced additive usable in any oxygen-independent or anaerobic media where mycotoxins will possibly have to be degraded, such as, for instance, in foods for humans and animals, in the production of bioethanol, but also for the production of genetically modified agricultural crops.

By selecting the enzymes from the carboxylesterase of the sequence ID No. 9, and optionally the amino-transferase of the sequence ID No. 19, fumonisins can be smoothly and completely degraded in an oxygen-independent environment. In this case, the transcription of the open reading frames in the FUM gene clusters isolated from the gene cluster of the nucleic acid of the sequence ID No. 1, which is derived from a prokaryotic strain having the accession number DSM 16254, is controlled by a bidirectional promoter located between FumA and FumI, as is apparent from Table 1 below. The clusters encode proteins which are involved in the regulation of the gene expression, like e.g. FumB and FumC, in the sampling of the substrate and its transport, like e.g. FumA, FumJ, FumG, and in the catabolism of the

substrate, like e.g. FumD, FumE, FumF, FumH, FumI, FumK. From these nucleic acid sequences which encode special genes and enzymes, those genes were selected according to a preferred further development of the method according to the invention, which are responsible for the catabolism of the substrate, thus enabling the respectively formed enzymes to completely catabolise the substrate, i.e. fumonisins.

In this case, open reading frames selected, for instance, from the gene cluster of the nucleic acid sequence having ID No. 1 are expressed in prokaryotic or eukaryotic host cells. The transcription of the open reading frames contained in the gene cluster having SEQ ID No. 1, in the bacterial strain with the accession number DSM 16254, takes place in a manner controlled by a bidirectional promoter located between fumA and fumI, as is apparent from the annexed Fig. 1. The genes encode proteins which are involved in the regulation of the gene expression, such as in the recognition of the substrate and its transport, like e.g. FumA, FumJ, FumG, and in the catabolism of the substrate, like e.g. FumD, and FumI.

In the Table 1 below, the designations of the genes of the fumonisin-catabolized gene cluster are listed, wherein O indicates the orientation, namely f forward and r reverse.

Table 1

Gene	Sequence ID	O	Start	Stop	Length	Designation
fumD	8	f	8294	9916	1623	carboxylesterase
fumI	18	r	5063	3795	1269	aminotransferase

In that, as in correspondence with a further development of the invention, the additive is used in an oxygen-independent environment during the production of bioethanol, along with a mash or a vegetable starting material, by selecting the additive such that the enzymes contained therein are completely derived from bacteria catalysing the catabolism of fumonisins via a highly specific degradation path, it is feasible to use the same with high specificity, activity and efficiency so as to enable the additive to be also used technologically in an oxygen-independent environment.

Finally, the present invention relates to the use of genes as represented in the sequences ID Nos. 8 and 18, or of a cosubstrate, a carboxylesterase sequences ID No. 9, optionally an aminotransferase sequences ID No. 19 as well as an α-keto acid as a cosubstrate for producing an additive for the degradation of fumonisins, in the processing or use of vegetable raw materials. An additive produced in this manner allows for the complete and reliable degradation of fumonisins, particularly in an oxygen-independent environment. Especially such use allows for the safe and reliable degradation to harmless components of the total of fumonisins in, for instance, vegetable raw materials or starting materials.

A further use is the use of a carboxylesterase, at least one adsorbent, such as a clay mineral, as well as an inert carrier. When using a carboxylesterase of the sequence ID No. 9 and at least one adsorbent, it is feasible to safely and reliably detoxify fumonisins by the mere use of a single enzyme in that the tricarballylic acid side residues are cleaved from the fumonisin by, or by the aid of, said enzyme and the thus formed long-chain hydrolysed fumonisin is subsequently adsorbed on the adsorbent so as to render the toxin harmless in a safe and reliable manner.

According to a further use, the additive according to the invention is used for the oxygen-independent or anaerobic treatment of a vegetable starting material or a mash in the production of bioethanol. In this case, it is feasible to safely and reliably render the mycotoxins contained in the vegetable starting material or raw material harmless during the production of bioethanol in an oxygen-independent environment so as to subsequently allow for the use of the residue from ethanol production, namely the pomace or dried vinasse, either directly or after drying and pelletizing without further processing and, in particular, detoxification as a feed that is free of fumonisins.

By using the additive in a vegetable starting material to be fermented or in a mash for the production of bioethanol, it is feasible to free coproducts occurring in the production of ethanol, namely pomace, i.e. the dried grain residues and undissolved components, or dried vinasse (dried distiller's grains with solubles – DDGS) from fumonisins or mycotoxins, particularly in an oxygen-independent environment.

In the following, the invention will be explained in more detail by way of exemplary embodiments and Figures. Therein:
Fig. 1 depicts the fumonisin-catabolic gene cluster;
Fig. 2 illustrates the Michaelis-Menten curve for fumonisin carboxylesterase FumD;
Fig. 3 shows a degradation curve of hydrolyzed fumonisin B_1;
Fig. 4 illustrates the conversion of fumonisin FB1 into hydrolysed fumonisin HFB1 after the addition of carboxylesterase ID No. 9; and
Fig. 5 illustrates the degradation of hydrolysed fumonisin HFB1 by the addition of aminotransferase ID No. 19.

Fig. 1 depicts a fumonisin-catabolic gene cluster as a partial sequence of 15420 base pairs of a microbial strain having the accession number DSM 16254. In the *fum*-gene cluster of the prokaryotic strain DSM 16254, the transcription of the open reading frame is controlled by a bidirectional promoter located between *fumA* and *fumI*. The cluster encodes proteins involved in the regulation of the gene expression, like e.g. FumB and FumC, in the recognition of the substrate and its transport, like e.g. FumA, FumJ, FumG, and in the catabolism of a substrate, like e.g. FumD, FumE, FumF, FumH, FumI and FumK.
Examples

Example 1: The enzyme kinetics of fumonisin carboxylesterase

The *fumD* gene (sequence ID No. 8), which encodes a fumonisin carboxylesterase, was cloned and expressed in *Pichia pastoris* using standard procedures. The his-tagged enzyme was recovered and purified from the supernatant culture solution by affinity chromatography. The enzyme concentration was determined and the enzyme-kinetic parameters were determined with seven different substrate concentrations ranging from 50 µg to 25 mg FB_1 per litre and an enzyme concentration of 0.33 ng/ml. The reactions were buffered in 20 mM Tris-Cl buffer (pH 8.0) with 0.1 mg/ml bovine serum albumin and incubated at 30°C. Samples were taken after 0, 30, 60, 120 and 240 minutes of incubation and analysed by HPLC-MS/MS. Fumonisin B_1 (FB_1) and hydrolysed fumonisin B_1 were quantified, based on a calibration with the purified reference substances and a completely ^{13}C-labelled internal FB_1-standard.

Fig. 2 illustrates the Michaelis-Menten curve for the hydrolysis of fumonisin B_1 (FB_1) by fumonisin carboxylesterase FumD, which was determined at an enzyme concentration of 0.33 ng/ml in Tris-Cl buffer (pH 8.0), with initial enzyme speeds having been plotted against the substrate concentrations. The Michaelis-Menten curve shows a drop at higher substrate concentrations, since the enzyme speed was calculated based on the product, i.e. the formation of hydrolysed FB_1. Since hydrolysed FB_1 is formed from FB_1 in a two-step reaction via partially hydrolysed FB_1 with but one tricarballylic acid side chain which was retained and a side chain which was cleaved, the formation of the end product was delayed at high substrate concentrations. The Michaelis-Menten constant K_M was calculated as 0.90 µmol/l, which was equivalent to 650 ppb, and the conversion rate was 900 per second.

From Fig. 2 results that fumonisins can be rapidly and completely hydrolysed with the carboxylesterase in the relevant concentration ranges.

Example 2: The catalytic activity of HFB1 (hydrolysed fumonisin B1) aminotransferase

Sequences ID Nos. 18 and 24 were cloned using standard procedures and expressed in E. coli under the control of a bacteriophage T7 promoter. The bacterial cells were collected, resuspended in 50 mM sodium phosphate buffer and lysed under ultrasonic action. Hydrolysed fumonisin was added, and the samples were incubated at 25°C. Samples were taken at time intervals and analysed by HPLC-MS/MS. No reduction of the hydrolysed FB_1 concentration was observed. When a cosubstrate such as, for instance, an α-keto acid like e.g. pyruvic acid, or oxalacetate was added to the reaction, the complete degradation of the hydrolysed fumonisin to 2-keto-HFB_1 could be observed as illustrated in Fig. 3. This substance is totally harmless for mammals.

Example 3: Enzyme activity in the intestinal environment

To examine the enzymatic activity of FUM-carboxylesterase in the digestive tract, freshly butchered swine guts were used and transported to the lab under oxygen-exclusion and examined in an anaerobic sterile bench. Approximately 10-cm-long pieces of duodenum and jejunum were secured and cut out. Fumonisin B1, diluted to a final concentration of about 10 ppm in a concentrated aqueous solution, was injected by needles and mixed with intestinal contents. After this, 5 µg fumonisin carboxylesterase in an aqueous solution, or the same volume of water in the negative controls, respectively, was injected and incorporated. The intestinal sections were incubated at 39°C. Samples were drawn by the aid of needles and analysed by HPLC-MS/MS. It was shown that, at the time of the first sampling after two hours, fumonisin B1 had already been completely hydrolysed in the duodenum and jejunum.

Example 4: Determination of the temperature range of the activity of fumonisin carboxylesterase

To determine the temperature range in which fumonisin carboxylesterase is active, 1.6 ng/ml FUM-carboxylesterase in 20 mM Tris-Cl buffer, pH 7.0, was incubated with 0.1 mg/ml BSA and 10 ppm fumonisin B1 at different temperatures. It was shown that the temperature optimum for the enzyme was 30°C. Enzymatic activity was still clearly determined at 40°C and even 50°C. FUM-carboxylesterase is, thus, suitable for application under the temperature conditions prevailing in the digestive tract, or in the course of process steps in the production of foods and feeds, which take place at elevated temperatures.

Example 5: Determination of the pH range of the activity of fumonisin carboxylesterase

To determine the pH range in which fumonisin carboxylesterase is active, Teorell-Stenhagen buffer was used. This buffer can be adjusted over a range of 10 pH units with the same buffer capacity by the combination of citrate, phosphate and borate. FUM-carboxylesterase was incubated in this buffer with 10 ppm fumonisin B1 at different pH values and 25°C, at a concentration of 3.3 ng/ml. The highest activity was shown at pH 8.0, yet activity could be determined in the whole range from pH 5 to pH 10. This activity within this broad pH range has enabled the technological application of the enzyme as a feed additive or in the course of food and feed processing.

Example 6: Feeding test with piglets

The test was performed in a test stable with 12 stalls for 10 animals each. The stable was equipped with a slatted floor, pan troughs and a computer-controlled feeding system. The automats were arranged along the stall walls. Every day, the stable climate was automatically recorded, and the temperature was set according to the standard recommendations for piglet breeding.

For this test, 120 mixed-sex weaned pigs (age: about 4 weeks, average setting weight: 8.21 kg) were used. Each piglet was earmarked and individually weighed. The 120 piglets were randomly distributed among 12 stalls. All piglets came from the Austrian Breeding Program ÖHYB (= (large white x landrace) x Pietrain).

Immediately upon weaning, the piglets were fed with a starter feed for two days, after this settling-in period the changeover to the test feed took place. Feeding was effected in two phases: Weaning phase days 1 – 14, breeding phase days 15 – 42. The test feed was mixed individually per stall via the spotmix feeding installation and allotted in dry form twice a day as a function of the number of piglets, weight development and feed consumption. Water was available ad libitum. The 12 stalls were divided into four different application groups at three repetitions each and received the following admixtures in the above-described feed:

Group	
Negative control	no toxins, no enzyme addition
Positive control	4 – 5.5 ppm fumonisin B1
Test group 1	4 – 5.5 ppm fumonisin B1 + enzyme mix 1 (carboxylesterase, aminotransferase, pyruvate) 0.5 kg/t feed
Test group 2	4 – 5.5 ppm fumonisin B1 + enzyme mix 1 (carboxylesterase, aminotransferase, pyruvate, inert carrier) 1 kg/t feed

Respiratory problems were observed in the positive control with almost half of the animals, even one dropout occurred. All other groups appeared healthy.

Performance data

Group	Number of animals	Starting weight (average, kg)	Final weight (average, kg)	Drop-outs
Negative control	30	8.34	26.82	
Positive control	30	8.17	24.77	1
Test group 1	30	8.08	26.69	
Test group 2	30	8.25	27.03	

Example 7: Enzymatic degradation of fumonisins in bioethanol mash

Samples of corn mash for the production of bioethanol were taken and incubated at 30 to 65°C under stirring, the degradation of fumonisin B1 having been investigated after the addition of 770 units of carboxylesterase ID No. 9 per cubic meter of mash under stirring (stirring time in minutes). Samples were inactivated by boiling-up after having been taken and subsequently centrifuged for analysis, and an aliquot of the supernatant was evaporated. The residue was taken up in 200 µl sample buffer containing C^{13}-labelled internal fumonisin standard, shaken for 1.5 min, centrifuged off, and then subjected to LC-MS-analysis. From this results that, as illustrated in Fig. 4, fumonisin FB1 is completely converted into hydrolysed fumonisin HFB1. After the addition of aminotransferase ID No. 19, the hydrolysed fumonisin HFB1 is completely degraded to harmless components as illustrated in Fig. 5.

Example 8: Degradation of fumonisins and their derivatives in corn tortilla mush and cornflake mush

The activity of the fumonisin-degrading enzymes was examined in corn mush samples (corn grits) for the production of corn tortillas and cornflakes. Fumonisin-contaminated corn (about 1 ppm) was ground to corn flour, mixed with water and boiled up. For the production of tortillas, the corn mush cooled to about 50 to 60°C was supplemented with a mixture of proteinases in alkaline solution. After 30 to 180 min, when the pH had fallen below 9, preferably below 8, a mixture of carboxylesterase and aminotransferase (500 – 1000 U/m^3 each) was added and incubated for further 30 to 60 min. Concerning the production of cornflakes, a corn mush of ground corn and barley malt was boiled up in a pressure vessel for about one hour; after cooling to below 60°C (preferably 50°C), an enzymatic mixture comprising carboxylesterase and aminotransferase (500 - 1000 U/m^3 each) was added and incubated for further 30 to 60 min. Samples were then drawn from this mixture and examined for FB1 and HFB1 residues as in Example 7. HFB1 levels were below 80 ppb in all of the samples, the HFB1 formed of FB1 apparently had been continuously further reacted. The measured values for FB1 are indicated in the Table below.

Table: Enzymatic degradation of FB1 and HFB1 in corn mush; fumonisin concentration in ppb (µg/kg)

Treatmenttime with enzyme mix (min)	Tortilla mush (40°C, 500 units)	Tortilla mush (50°C, 1000 units)	Cornflake mush (35°C, 500 units)	Cornflake mush (40°C, 1000 units)
0	852	866	912	1053
10	116	134	51	97
30	32	71	17	37

Szabadalmi igénypontok

1. Adalékanyag növényi eredetű nyersanyagokban és növényi eredetű nyersanyagokat tartalmazó keverékben lévő fumonizinek enzimatikus bontására, azzal jellemezve, hogy 9. azonosítószámon megadott szekvenciájú enzimet, továbbá adott esetben legalább az alkalmazott enzim koszubsztrátját és egy inert hordozót tartalmaz.

2. Az 1. igénypont szerinti adalékanyag, azzal jellemezve, hogy védő burokkal borított enzimet alkalmazunk.

3. Az 1. vagy 2. igénypont szerinti adalékanyag, azzal jellemezve, hogy a 9. azonosítószámon megadott szekvenciájú karboxilészterázt, a 19. azonosítószámon megadott szekvenciájú aminotranszferázt, α-ketosavat és egy inert hordozót tartalmaz.

4. Az 1., 2. vagy 3. igénypont szerinti adalékanyag, azzal jellemezve, hogy a 9. azonosítószámon megadott szekvenciájú karboxilészterázt, legalább egy abszorbenst, pl. agyagot és egy inert hordozót tartalmaz.

5. Az 1-4. igénypontok bármelyike szerinti adalékanyag alkalmazása, azzal jellemezve, hogy az adalékanyagot oxigén-független környezetben alkalmazzuk bioetanol gyártása során cefrével vagy növényi eredetű kiindulási anyaggal együtt.

6. Az 1-4. igénypontok bármelyike szerinti adalékanyag alkalmazása, azzal jellemezve, hogy az adalékanyagot fermentálandó növényi nyersanyagban vagy cefrében alkalmazzuk bioetanol gyártása során.

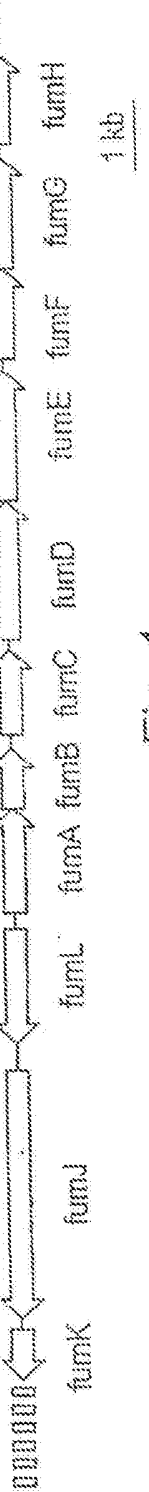
fumK
fumJ
fumL
fumA
fumB
fumC
fumD
fumE
fumF
fumG
fumH
1 kb

Fig. 1

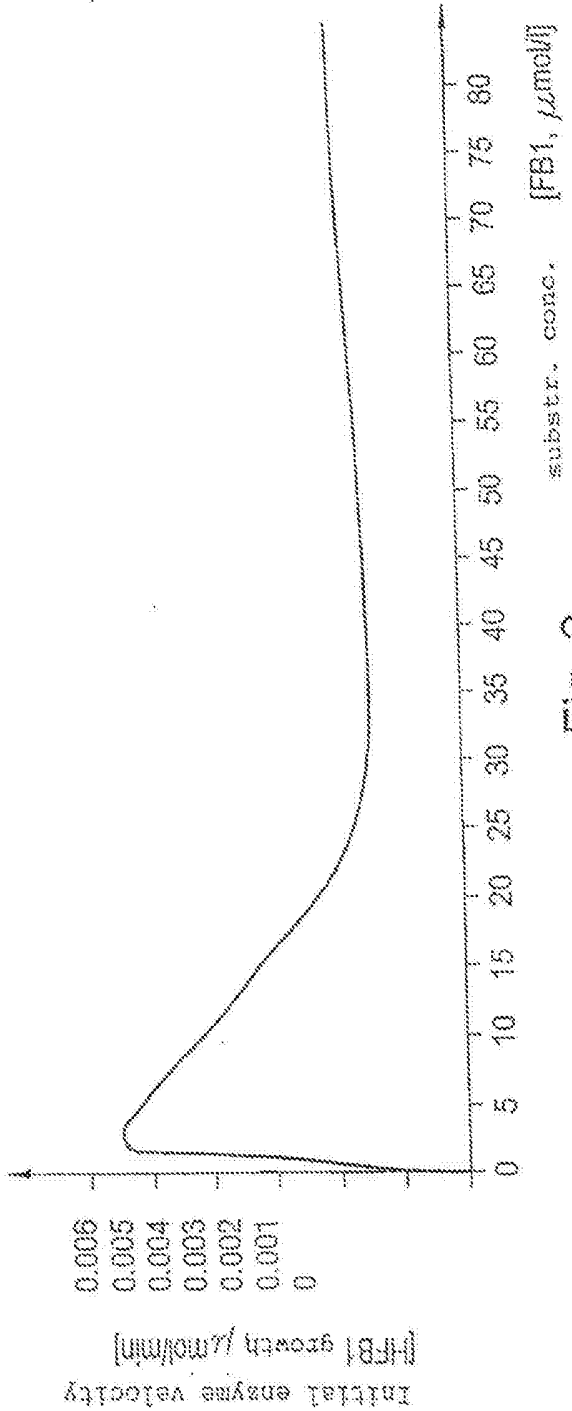
Initial enzyme velocity
[HFB1 growth µmol/min]
0.006
0.005
0.004
0.003
0.002
0.001
0
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
substr. conc. [FB1, µmol/l]

Fig. 2

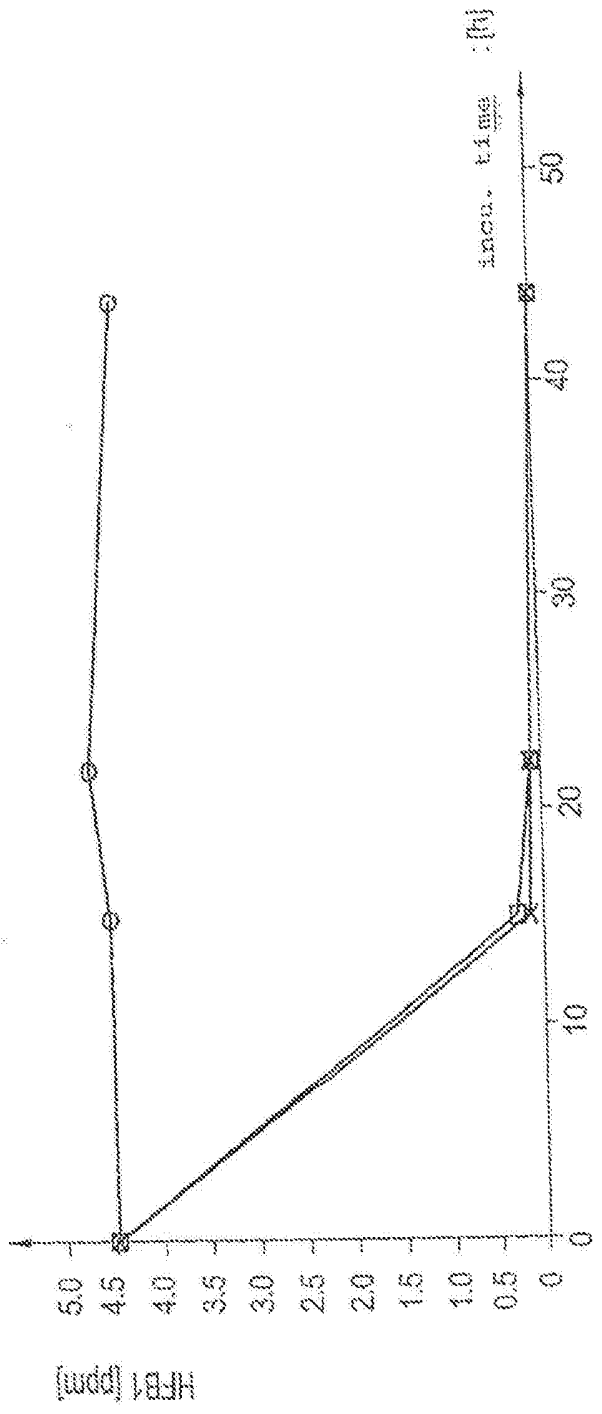
HFB1 [ppm]
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0
10
20
30
40
50
incu. time [h]
E.coli neg. comparison
Seq ID 18 expr. in E.coli
Seq ID 24 expr. in E.coli

Fig. 3

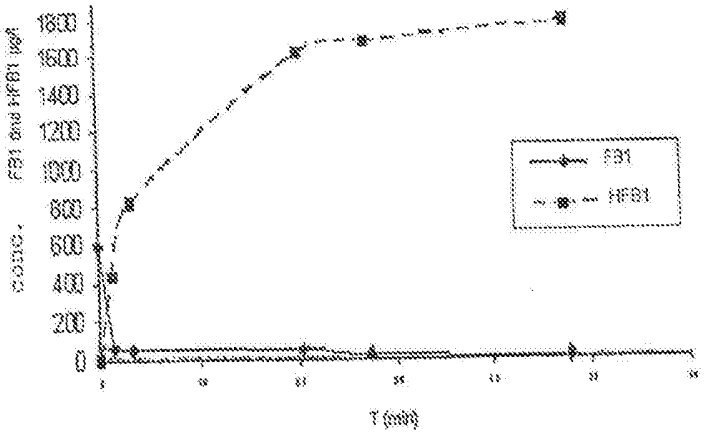
1800
1600
1400
1200
1000
800
600
400
200
0
FB1
HFB1
T (min)

Fig. 4

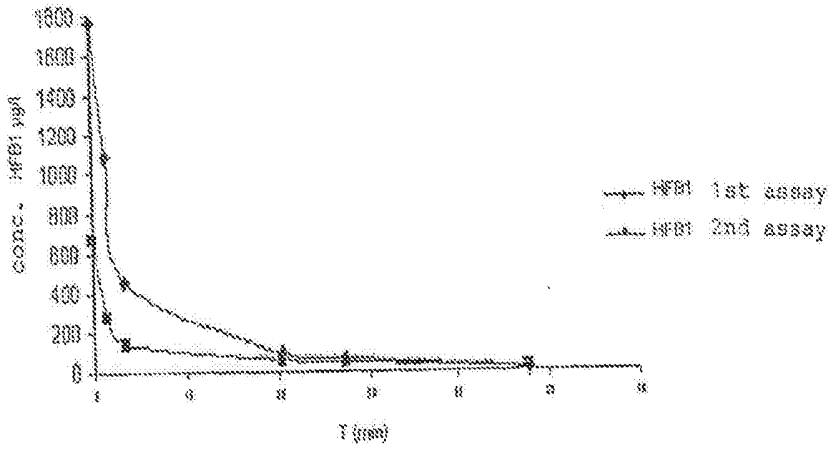
1800
1600
1400
1200
1000
800
600
400
200
0
HFB1 1st assay
HFB1 2nd assay
T (min)

Fig. 5