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MAZEN™ ANIMAL HEALTH

Ms. Bernadette Juarez
Deputy Administrator
US Department of Agriculture
Animal and Plant Health Inspection Services
Biotechnology Regulatory Services
4700 River Road, Unit 147
Riverdale, MD 20727

Re: Regulatory Status Review Submission for corn (*Zea mays,* L.) expressing β-mannanase from *Paenibacillus lentus*.

Dear Ms. Juarez,

Mazen Animal Health, Inc. is submitting this request for a Regulatory Status Review of the enclosed information regarding genetically engineered corn (*Zea mays*, L) that expresses -β-mannanase and a determination that this corn is not subject to further regulation under 7 CFR part 340. This corn has been grown for several years under permit by USDA-APHIS-BRS.

The attached document **does contain** confidential business information (CBI) which has been marked appropriately.

If you have questions, please contact me at your earliest convenience.

Best regards.
John M. Cordts

Agriculture and Biotechnology Consultant
Cordts Consulting LLC
Newark, DE

Mazen Animal Health, Inc. Regulatory Status Review Submission for Altered Seed Composition Corn (*Zea mays*, L.) Expressing β-mannanase from *Paenibacillus lentus*

CBI Justification Statement:

The Regulatory Status Review being submitted contains trade secrets and commercial or financial information that is protected from disclosure under section (b) (4) of the FOIA and is properly deemed as confidential business information (CBI). Given the highly competitive and rapidly evolving nature of biotechnology, this information is commercially valuable to Mazen and its collaborators and should not be disclosed. Mazen has maintained the information being submitted in secrecy and has agreed not to disclose this information to the public without the consent of our collaborators. Disclosure of this information by APHIS to a third party would cause substantial competitive harm to Mazen and breach our contractual agreements. The disclosure of the names of the products and descriptions, the gene sequences and descriptions, and other confidential processes would result in the loss of our competitive position and a loss in time and investment in the research and development of our products, as well as jeopardize our patent applications. It is our understanding that you will inform us in advance in the event you conclude that a third party is entitled to obtain access to these documents. If any questions should arise regarding these documents, please contact Mark Zylstra at the address or phone number listed in this application, or by email at mzylstra@mazemanimalhealth.com.

Mazen Animal Health, Inc. Regulatory Status Review Submission for Altered Seed Composition Corn (*Zea mays*, L.) Expressing β-mannanase from *Paenibacillus lentus*

Date: June 27, 2023

Ms. Bernadette Juarez
Deputy Administrator
Biotechnology Regulatory Services
Animal and Plant Health Inspection Services
US Department of Agriculture

Regulatory Status Review Submission for altered seed composition corn (*Zea mays,* L.) expressing β-mannanase from *Paenibacillus lentus*.

Submitted by

Mazen Animal Health, Inc., Iowa State University Research Park, 1805 Collaboration Place, Suite 1250, Ames, IA 50010
Mark Zylstra, mzylstra@mazenanimalhealth.com; 515-570-0125

Introduction:

Mazen Animal Health, Inc., hereafter Mazen, is submitting this document to USDA-APHIS Biotechnology Regulatory Services for a Regulatory Status Review as described in 7 CFR part 340.4. Mazen has been working with Applied Biotechnology Institute (ABI) on a project to commercialize ABI's corn product that produces β-mannanase in its seed. ABI has been growing this corn for many years under USDA-APHIS permits and Mazen intends to commercialize and scale up production of these corn lines for use in animal feed. The β-mannanase-producing corn is designed be used as a feed supplement to counter the negative effects of galactomannans and glucomannans on digestion from plant materials that are commonly used as feed.

Corn as a plant and crop

Zea mays, L., is a food, feed, fiber, and fuel crop plant. It is grown on approximately 85-95 million acres in the U.S. every year. Roughly forty percent of the crop goes to ethanol production as a gasoline additive. Another 30-40% goes to animal feed for cattle, hogs, and poultry. A small percentage is exported, and small amounts are used in human food products. The Organisation for Economic Co-operation and Development (OECD) has produced a "Consensus Document on the Biology of *Zea mays* subsp. *mays* (Maize)" (OECD, 2003) that describes in detail the biology and various uses worldwide for corn (maize).

Corn is an open pollinated crop with separate male and female flowers. It is most often, however, produced as a hybrid, and thus the male and female crosses to produce hybrids are controlled.

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Intended Trait and Phenotype

The intended trait and phenotype for Mazen's corn product is altered seed composition and it produces the enzyme β-mannanase preferentially in the seed. Plants are also herbicide tolerant as a result of insertion and expression of the phosphinothricin acetyl transferase (PAT) gene from *Streptomyces viridochromogenes.*

Genes inserted into corn

Mazen's collaborator, ABI, has generated several lines of corn producing β-mannanase. Minor variations in promoters and other regulatory sequences have been used in each case in order to identify the best lines intended for commercialization. The plasmid map and table of genetic elements reflect one of the produced lines and contains regulatory elements that target the expression of β-mannanase to the seed.

The endo-1,4,-β-D-mannanase gene (PGNpr25 on map) inserted into corn is the full length enzyme sequence found in the naturally occurring soil bacterium *Paenibacillus lentus* (Li, *et al.*, 2014).

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.**]** Both the enzyme and the strain are noted as being acceptable for use in animal feeds by the Association of American Feed Control Officials (AAFCO 2021).

Insertion of the gene into the corn genome was achieved using an avirulent *Agrobacterium tumefaciens* strain, EHA101 (Zambryski *et. al*, 1982**)**. The vector used to carry the T-DNA of interest was the super binary plasmid described in Ishida *et al*. (1996). Details of this construct are described in the following table:

Table 1: Genetic Elements and Their Functions in Mazen β-mannanase producing corn.
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The plasmid map used for the transformation is found here and the intended sequence insertion (between the right and left borders) is found in Appendix I.

Figure 1: Plasmid used for this work

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Mechanism of Action

Using a construct shown to target expression of the gene of interest to specific tissues, the β-mannanase enzyme will be expressed in a manner that drives high accumulation in the seed. The *Zea mays* globulin-1 promoter drives expression to the corn embryo while the barley alpha amylase signal sequence drives the protein expression to the embryo wall (Hood, *et. al,* 2003). This promoter has been shown to control expression tightly to these tissues and significant expression of β-mannanase to other parts of the plant is not expected.

In order to track and select plants that have been successfully transformed with the intended sequence, the phosphinothricin acetyltransferase (PAT) gene was also inserted and is driven by the cauliflower mosaic virus 35S promoter for constitutive expression in all tissues. This selectable marker results in resistance to glufosinate ammonium herbicides, allowing for effective removal of null plants (Strauch *et. al,* 1988).

Characteristics of endo-1,4,-β-D-mannanase

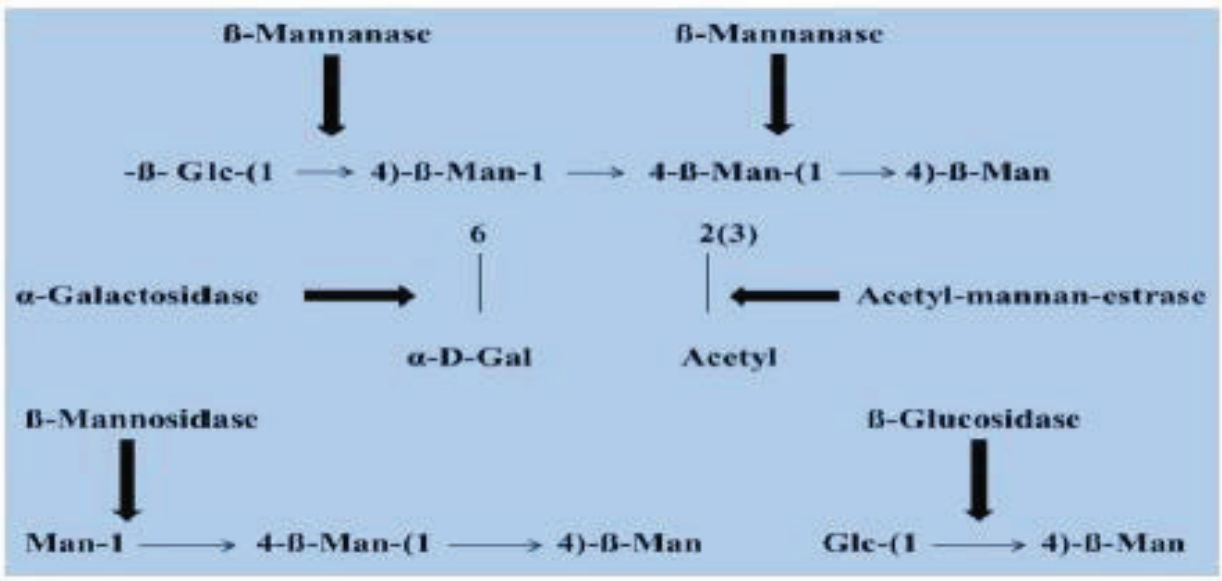
β-mannans are a group of hemicelluloses which are found in soybean meal and full-fat soybean and are important protein sources frequently added to poultry diets. However, β-mannans have been found to have anti-nutritional effects including high intestinal viscosity and low nutrient digestibility and to cause adverse effects on innate immune response and microbial proliferation in the gut (Shastak *et al.*, 2015). The addition of β-mannanase to animal feed has been shown to result in improved growth performance, ileal digestible energy (IDE) and decreases to intestinal viscosity in broilers fed diets with varying levels of mannans (Latham *et al*., 2018).

The details of the mode of action of β-mannanase in improving poultry performance are not fully understood, but many studies show the benefits of the addition of mannanase to the diet. This includes lowered viscosity of the intestinal digesta and therefore improved glucose and water absorption (Danicke *et al*., 2000). Reduction of the levels of beta-mannans in the gut are thought to improve the innate immune response as well as improved proliferation of beneficial microbes and resistance to pathogens. Improvements in body weight and cumulative feed conversion are observed (Saeed *et al.*, 2019).

Many microbial species naturally possess the ability to degrade mannans efficiently, the majority being gram positive bacteria such as *Bacillus* (David *et al*., 2018), and these enzymes have been used in numerous industries including oil drilling, detergents, textile, food, animal feed, and production of bioethanol (Dawood and Ma, 2020). β-mannanase is widely used in many feed additives and commonly used additives and mannanase enzymes from some species have Generally Regarded as Safe (GRAS) status (FDA GRAS notices 566 and 739 for use as food processing aides).

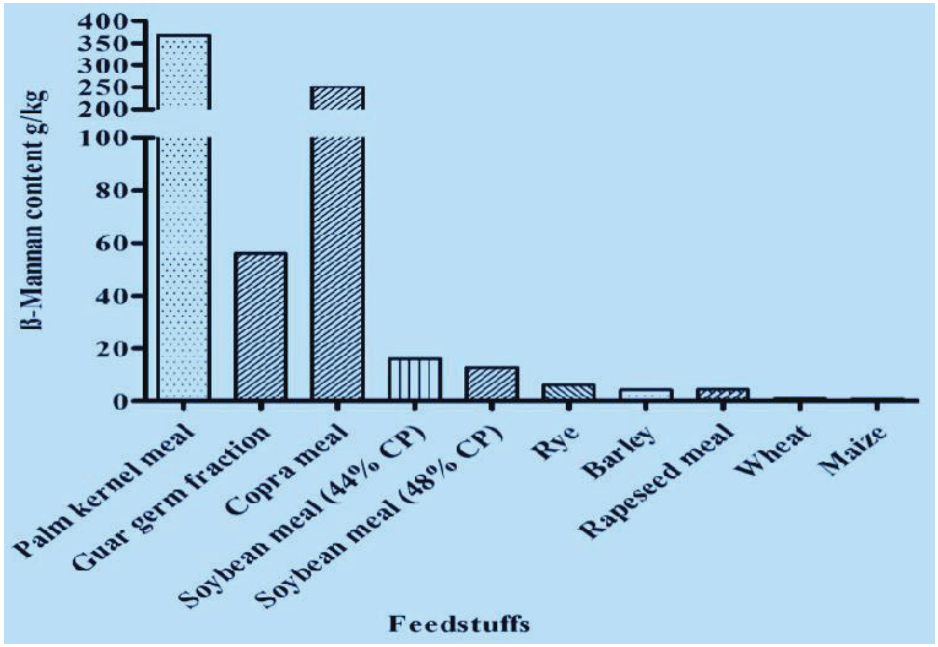
The primary mechanism of action is seen in the Figure 2 below. β-mannanase causes hydrolysis of β-1, 4-glycosidic linkages in β-mannans by random cleavage inside the 1, 4-β-D-mannan key chains of galactoglucomannan, galactomannan, and mannan (Ooi & Kikuchi, 1995; McCleary, 1986).

Figure 2: Mechanism of β-mannanase on β-mannans



The protein is exclusively targeted to the seed for accumulation in the kernel. Because the level of β-mannans in corn is extremely low (Figure 3), the enzyme is not active within the kernel, making it an attractive method of delivery (Saeed *et al.*, 2019). Maize is a staple component of animal feed; therefore, the product also fits nicely within current animal production procedures.

Figure 3: β-mannan levels in common components of animal feed



Conclusion

Finally, Mazen Animal Health, Inc. is requesting that USDA-APHIS-BRS determine that the β-mannanase-producing corn described in this document is not subject to its regulations at 7 CFR part 340.

Respectfully submitted,

Mark Zylstra
Mazen Animal Health, Inc.
Ames, Iowa

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Appendix I: Sequence of intended insert in Mazen Animal Health, Inc. mannanase-producing corn

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