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Item No.: 3172

FINAL DECISION DOCUMENT: TSCA SECTION 5(H)(4) EXEMPTION FOR ASPERGILLUS ORYZAE

I. BACKGROUND

In the September 1, 1994, Federal Register (59 FR 45526), the Environmental Protection Agency (EPA) proposed at 40 CFR Part 700 under section 5(h)(4) of the Toxic Substances Control Act (TSCA), Tier I and Tier II exemptions. These exemptions, which would be found at § 725.400, are exemptions from EPA review and expedited EPA review, respectively, for certain microorganisms under certain use conditions. EPA proposed to include Aspergillus oryzae at § 725.420 as a candidate recipient microorganism for the tiered exemptions. Aspergillus oryzae is an asexual, ascomycetous fungus used for hundreds of years in the production of soy sauce, miso and sake without recorded incidents. It has also been used in the fermentation industry for production of enzymes and other organic compounds.

This final decision document describes the basis for EPA's decision to include Aspergillus oryzae as a recipient microorganism at § 725.420.

II. CONDITIONS OF EXEMPTION

EPA recognizes that some microorganisms present a low risk when used under specific conditions at general commercial use. Therefore, EPA proposed to institute expedited regulatory processes for certain microorganisms under these specific conditions at the general commercial use stage. Microorganism uses that are exempt would meet criteria addressing: (1) performance based standards for minimizing the numbers of microorganisms emitted from the manufacturing facility; (2) the introduced genetic material; and (3) the recipient microorganism. Microorganisms that qualify for these exemptions, termed Tier I and Tier II, must meet a standard of no unreasonable risk in the exempted use.

To evaluate the potential for unreasonable risk to human health or the environment in developing these exemptions, EPA focuses primarily on the characteristics of the recipient microorganisms. If the recipient is shown to have little or no potential for adverse effects, introduced genetic material meeting the specified criteria would not likely significantly increase potential for adverse effects. As further assurance that risks would be low, EPA is also specifying procedures for

minimizing numbers of organisms emitted from the facility. When balanced against resource savings for society and expected product benefits, these exemptions will not present unreasonable risks.

A. Criteria for Minimizing Release from Manufacturing Facilities

The standards proposed for the Tier I exemption were the following: (1) the structure(s) be designed and operated to contain the microorganism, (2) access to the structure should be limited to essential personnel, (3) inactivation procedures shown to be effective in reducing the number of viable microorganisms in liquid and solid wastes should be followed prior to disposal of the wastes, (4) features to reduce microbial concentrations in aerosols and exhaust gases released from the structure should be in place, and (5) general worker hygiene and protection practices should be followed.

1. Definition of structure. EPA considers the term "structure" to refer to the building or vessel which effectively surrounds and encloses the microorganism. Vessels may have a variety of forms, e.g., cubic, ovoid, cylindrical, or spherical, and may be the fermentation vessel proper or part of the downstream product separation and purification line. All would perform the function of enclosing the microorganism. In general, the material used in the construction of such structure(s) would be impermeable, resistant to corrosion and easy to clean/sterilize. Seams, joints, fittings, associated process piping, fasteners and other similar elements would be sealed.

2. Standards to minimize microbial release. EPA is taking, for several reasons, a somewhat cautious approach in prescribing standards for minimizing the number of microorganisms emitted through the disposal of waste and the venting of gases. First, a wide range of behaviors can be displayed by microorganisms modified consistent with EPA's standards for the introduced genetic material. Second, EPA will not conduct any review whatsoever for Tier I exemptions. EPA believes the requirement to minimize emissions will provide a measure of risk reduction necessary for making a finding of no unreasonable risk. Taken together, EPA's standards ensure that the number of microorganisms emitted from the structure is minimized.

EPA's standards for minimizing emission specify that liquid and solid waste containing the microorganisms be treated to give a validated decrease in viable microbial populations so that at least 99.9999 percent of the organisms resulting from the fermentation will be killed. Since the microorganisms used in fermentation processes are usually debilitated, either

intentionally or through acclimation to industrial fermentation, the small fraction of microorganisms remaining viable after inactivation treatments will likely have a reduced ability to survive during disposal or in the environment. Moreover, industrial companies, in an attempt to keep their proprietary microorganisms from competitors and to reduce the microbial numbers to those permitted by local sanitation authorities, modify the microorganisms to increase the ability of their microorganisms to survive and perform their assigned tasks in the fermentor but decrease their ability to survive in the environment external to the fermentor.

EPA requirements also address microorganisms in the exhaust from the fermentor and along the production line. To address exhaust from fermentors, EPA is requiring that the number of microorganisms in fermentor gases be minimized by the use of standard industry equipment prior to the gases being exhausted from the fermentor. EPA selected this standard based on an estimate of the numbers of microorganisms likely to be in the exhaust from an uncontrolled fermentor and common industry practice. Moreover, microorganisms that are physiologically acclimated to the growth conditions within the fermentor are likely to be compromised in their ability to survive aerosolization. EPA anticipates, therefore, that few microorganisms will survive the stresses of aerosolization associated with being exhausted with the gases from the fermentor. The provision requiring reduction of microorganisms in fermentor exhaust gases contributes to minimizing the number of viable microorganisms emitted from the facility.

EPA is also requiring that other systems be in place to control dissemination of microorganisms by other routes. This would include programs to control pests such as insects or rats, since these might serve as vectors for carrying microorganisms out of the fermentation facilities.

3. Worker protection. The requirement to minimize microbial emissions, in conjunction with the requirement for general worker safety and hygiene procedures, also affords a measure of protection for workers. Potential effects on workers that exist with microorganisms in general (e.g., allergenicity) will be present with the microorganisms qualifying for this exemption. As with other substances that humans may react to (e.g., pollen, chemicals, dust), the type and degree of allergenic response is determined by the biology of the exposed individual. It is unlikely that a microorganism modified in keeping with EPA's specifications for the introduced genetic material would induce a heightened response. The general worker hygiene procedures specified by EPA should protect most individuals from the allergenic responses associated with microorganisms exhausted from fermentors and/or other substances emitted along the production line. The EPA requirement that

access to the structure be controlled also addresses this consideration by reducing to a minimum the number of individuals exposed.

4. Effect of containment criteria. As further assurance that risks would be low, EPA is specifying procedures for minimizing the number of organisms emitted from the facility for the Tier I exemption. EPA is not specifying standards for minimizing the number of microorganisms emitted from the facility for microorganisms qualifying for Tier II exemption. Rather, the Agency requests that submitters utilize as guidance the standards set forth for Tier I procedures. The procedures proposed by the submitter in a Tier II exemption request will be reviewed by the Agency. EPA will have the opportunity to evaluate whether the procedures the submitter intends to implement for reducing the number of organisms emitted from the facility are appropriate for that microorganism.

B. Introduced Genetic Material Criteria

In order to qualify for either the Tier I or Tier II exemption, any introduced genetic material must be limited in size, well characterized, free of certain nucleotide sequences, and poorly mobilizable.

1. Limited in size. Introduced genetic material must be limited in size to consist only of the following: (1) the structural gene(s) of interest; (2) the regulatory sequences permitting the expression of solely the gene(s) of interest; (3) the associated nucleotide sequences needed to move genetic material, including linkers, homopolymers, adaptors, transposons, insertion sequences, and restriction enzyme sites; (4) the nucleotide sequences needed for vector transfer; and (5) the nucleotide sequences needed for vector maintenance.

The limited in size criterion reduces risk by excluding the introduction into a recipient of extraneous and potentially uncharacterized genetic material. The requirement that the regulatory sequences permit the expression solely of the structural gene(s) of interest reduces risk by preventing expression of genes downstream of the inserted genetic material. The limitation on the vector sequences that are components of the introduced genetic material prevents the introduction of novel traits beyond those associated with the gene(s) of interest. The overall result of the limited in size criterion is improved ability to predict the behavior of the resulting microorganism.

2. Well characterized. For introduced genetic material, well characterized means that the following have been determined: (1) the function of all of the products expressed from the structural gene(s); (2) the function of sequences that participate in the regulation of expression of the structural

gene(s); and (3) the presence or absence of associated nucleotide sequences.

Well characterized includes knowledge of the function of the introduced sequences and the phenotypic expression associated with the introduced genetic material. Genetic material which has been examined at the restriction map or sequence level, but for which a function or phenotypic trait has not yet been ascribed, is not considered well characterized. Well characterized would include knowing whether multiple reading frames exist within the operon. This relates to whether more than one biological product might be encoded by a single sequence, and addresses the possibility that a modified microorganism could display unpredicted behavior should such multiple reading frames exist and their action not be anticipated.

3. Free of certain sequences. In addition to improving the ability to predict the behavior of the modified microorganism, the well characterized requirement ensures that segments encoding for either part or the whole of the toxins listed in the proposed regulatory text for the TSCA biotechnology rule would not inadvertently be introduced into the recipient microorganism.

These toxins are polypeptides of relatively high potency. Other types of toxins (e.g., modified amino acids, heterocyclic compounds, complex polysaccharides, glycoproteins, and peptides) are not listed for two reasons. First, their toxicity falls within the range of moderate to low. Second, these types of toxins generally arise from the activity of a number of genes in several metabolic pathways (multigenic).

In order for a microorganism to produce toxins of multigenic origin, a large number of different sequences would have to be introduced and appropriately expressed. It is unlikely that all of the genetic material necessary for producing multigenic toxins would be inadvertently introduced into a recipient microorganism when requirements that the genetic material be limited in size and well characterized are followed.

Similarly, other properties that might present risk concerns result from the interactive expression of a large number of genes. For example, pathogenic behavior is the result of a large number of genes being appropriately expressed. Because of the complex nature of behaviors such as pathogenicity, the probability is low that an insert consisting of well characterized, limited in size genetic material could transform the microorganisms listed for exemption into microorganisms which display pathogenic behavior.

4. Poorly mobilizable. Poorly mobilizable means the ability of the introduced genetic material to be transferred and

mobilized is inactivated, with a resulting frequency of transfer of less than 10^{-8} transfer events per recipient. The requirement that the introduced genetic material be poorly mobilizable reduces potential for transfer of introduced genetic sequences to other microorganisms in the environment. Such transfers would occur through the interaction of the introduced microorganism with indigenous microorganisms through conjugation, transduction, or transformation. Through such transfers, the introduced genetic material could be transferred to and propagated within different populations of microorganisms, including microorganisms which may never previously have been exposed to this genetic material. It is not possible to predict how the behavior of these potential recipient microorganisms will be affected after uptake and expression of the genetic material.

Since EPA is not limiting the type of organism that can serve as the source for the introduced genetic material, some limitation is placed on the ability of the introduced genetic material to be transferred. This limitation mitigates risk by significantly reducing the probability that the introduced genetic material would be transferred to and expressed by other microorganisms.

The 10^{-8} frequency is attainable given current techniques. Plasmids with transfer rates of 10^{-8} exist or are easily constructed. Some of the plasmids most commonly employed as vectors in genetic engineering (e.g., pBR325, pBR322) have mobilization/transfer frequencies of 10^{-8} or less.

The criteria set for "poorly mobilizable" for transduction and transformation should be readily met since the majority of transfer frequencies reported for transduction and natural transformation are less than 10^{-8}. Higher frequencies are likely only under special circumstances, such as when the introduced genetic material has been altered or selected to enhance frequency. Because the risk concern EPA addresses with the 10^{-8} criterion is spread of the introduced genetic material broadly through microbial populations, exchanges between very closely related microorganisms, even if occurring at high frequency, is not a concern so long as the spread through populations does not occur at high frequency.

Fungal gene transfer has also been considered in development of the poorly mobilizable criterion. Although mobile genetic elements such as transposons, plasmids and double stranded RNA exist in fungi and can be readily transferred, this transfer usually is only possible between members of the same species during anastomosis, a process specific to fungi. Since anastomosis only occurs between members of the same species, the introduced genetic material would not be transferred to distantly related fungi as may occur with bacteria.

5. Effect of introduced genetic material criteria. The requirements placed on the introduced genetic material, in concert with the level of safety associated with *Aspergillus oryzae*, ensure that the resulting microorganisms present low or negligible risk. The probability is low that the insertion of genetic material meeting EPA's criteria into strains of *A. oryzae* will change their behavior so that they would acquire the potential for causing adverse effects. Risks would be mitigated by the four criteria placed on the introduced genetic material, the relative safety of *A. oryzae*, and the inactivation criteria specified for the Tier I exemption. In the case of Tier II exemption, risks would be mitigated in light of the four criteria placed on introduced genetic material, the relative safety of *A. oryzae*, and EPA's review of the containment conditions selected.

C. Recipient Microorganism Criteria

Six criteria were used by EPA to determine eligibility of recipient microorganisms for the tiered exemption. All of the criteria were used together to determine whether, on balance, the microorganisms would not present an unreasonable risk to human health or the environment. Microorganisms which EPA finds meet these criteria are listed as eligible recipients. The first criteria would require that it be possible to clearly identify and classify the microorganism. Available genotypic and phenotypic information should allow the microorganism to be assigned without confusion to an existing taxon which is easily recognized. Second, information should be available to evaluate the relationship of the microorganism to any other closely related microorganisms which have a potential for adverse effects on human health or the environment. Third, there should be a history of safe commercial use for the microorganism. Fourth, the commercial uses should indicate that the microorganism products might be subject to TSCA jurisdiction. Fifth, studies are available which indicate the potential for the microorganism to cause adverse effects on human health and the environment. Sixth, studies are available which indicate the survival characteristics of the microorganism in the environment.

After each microorganism was reviewed using the six evaluation criteria, a decision was made as to whether to place the microorganism on the list. The Agency's specific determination for *Aspergillus oryzae* is discussed in the next unit.

III. EVALUATION OF *ASPERGILLUS ORYZAE*

A. History of Use

1. History of safe commercial use. A. oryzae has been used for centuries in the production of many different oriental foods such as soy sauce, sake and miso. As a "koji" mold, A. oryzae has been used safely in the food industry for several hundred years. It is also used to produce livestock probiotic feed supplements. The koji mold enzymes were among the first to be isolated and commercialized nearly 100 years ago. A. oryzae is considered a Class 1 Containment Agent under the NIH Guidelines for Research Involving Recombinant DNA Molecules. In Europe, Aspergillus species are considered category 2 under the European Federation of Biotechnology guidelines and category 1 under the OECD containment scale.

2. Products subject to TSCA jurisdiction. A. oryzae is currently used in the production of organic compounds such as glutamic acid, and several enzymes that are of potential use commercially, for example, amylase, protease, b-galactosidase, lipase, and cellulase. While these enzymes could be used as TSCA products, several of them have been more often used in food processing. In 1989, EPA reviewed a premanufacture notice (PMN) for a strain of A. oryzae modified for enhanced production of a lipase enzyme to be used primarily in detergent formulations for the removal of fat-containing stains. In 1994, EPA reviewed a PMN for a similar strain of A. oryzae modified for enhanced production of a cellulase gene for use in detergents as a color brightening agent.

B. Identification of Microorganism

1. Classification. The genus Aspergillus represents a grouping of a very large number of asexual fungi whose taxonomy is based on morphological features. The genus has been divided into groups based on attributes of the spores, conidiophores, and sclerotia. Because this separation of individual species into groups is based on morphological or physiological characteristics, it has resulted in somewhat tenuous and overlapping classification. While it has been hypothesized that A. oryzae is a domesticated version of A. flavus, a species known to produce potent aflatoxins, this theory has not been fully accepted by the scientific community. Distinguishing between these two species can be difficult.

2. Related species of concern. A. oryzae is a member of the A. flavus group of Aspergillus species. Most of the members of this group are known to produce potent mycotoxins, including aflatoxins. Chromosomal DNA homology and other techniques have shown strains of A. oryzae and A. flavus to be essentially indistinguishable. It has been assumed that A. oryzae is a domesticated version of A. flavus that has been selected for use in foods because of its loss of ability for aflatoxin production.

C. Risk Summary

1. Studies regarding potential for adverse effects. A. oryzae strains can produce a variety of mycotoxins after extended fermentation; however, only a few strains are known to produce the more potent toxins. A. oryzae does not appear to be a significant human pathogen, nor has it been reported as a plant or animal pathogen. Although A. oryzae strains appear stable under cultivation, in theory, there remains a low probability that reversion to the A. flavus phenotype could occur, if rearrangement rather than deletion is the mechanism by which the A. flavus phenotype is lost. However, through centuries of use in the food industry, A. oryzae has not been shown to produce aflatoxins.

2. Studies regarding survival in the environment. There are conflicting opinions about whether A. oryzae can be isolated from the environment. A. oryzae seems to be a species created by domestication of the A. flavus wild type, and therefore, may have lost certain features important to survival in the environment. Although soil is a possible natural habitat for A. oryzae, the intrinsic features of domesticated strains are expected to limit their ability to survive in a natural environment.

IV. PUBLIC COMMENTS RELEVANT TO THE RISK ASSESSMENT

A request for comments was made in the Proposed Risk Assessment and the Proposed Decision Document for A. oryzae pertaining to (1) the conclusion that genetic modification of A. oryzae cannot inadvertently produce an aflatoxigenic strain, and (2) whether there is a need to differentiate between strains of A. oryzae having a long history of safe use and recently isolated strains. However, no comments were received on this specific microorganism either in general or addressing these two requests for comments. There were a number of comments received on the tiered exemption, however, and some of these comments are relevant to the criteria discussed at Section II.A. of this document considered in listing this microorganism as an eligible recipient microorganism at § 725.420.

Some of the general comments received on the exemptions addressed the six criteria EPA used to select candidate recipient microorganisms to include at § 725.420. None of these comments questioned the eligibility of A. oryzae for inclusion on the list at § 725.420. EPA's responses to comments on how it used the six criteria to select candidate microorganisms at § 725.420 are detailed in the Response to Comments document to be found in the docket for this rulemaking.

The other comments pertained to the proposed containment conditions at § 725.422. Commenters questioned two criteria, the first of which is at § 725.422(b): "limit entry only to those persons whose presence is critical to the reliability or safety of the activity". Commenters pointed out that under the requirement as proposed, managers may be precluded from allowing administrative personnel, customers, and school and other educational tours into the facility. EPA had not intended to constrain facility managers to this extent and reconsidered the standard. EPA has revised § 725.422(b) to read "Control access to the structure". Additional explanation for this revision is given in the Response to Comments document (Section III.C.4.a.) and the Preamble of the Final Rule. Both of these documents can be found in the docket for this rulemaking.

The other criterion in the proposed rule which commenters questioned was at § 725.422(e): "provide and document effectiveness of features to reduce microbial concentrations by at least two logs in aerosols and exhaust gases released from the structure". Commenters argued that the requirement as written would require retrofitting of equipment in order to permit measurement within the fermentor headspace of microbial concentrations in aerosols. EPA had not intended that manufacturers be required to retrofit their fermentation equipment in order to qualify for this exemption. Therefore, EPA re-examined the basis for this criterion by reviewing information submitted on physical containment and control technologies in the PMNs it had received for intergeneric microorganisms between 1986 and 1995. Examination of these PMNs revealed that the number of microorganisms potentially released through fermentor exhaust gases is negligible compared to the number contained in the liquid and solid waste streams. Even under a worst case scenario of an uncontrolled release, as evaluated in the accompanying risk assessment, the number of viable microorganisms aerosolized with the fermentor exhaust gases would still be low, and therefore, the risk would remain low. Moreover, the use of a criterion requiring controls to minimize microbial numbers released through aerosolization at § 725.422, as compared to the worst case scenario of an uncontrolled release, would result in lesser exposure, and therefore, lower risk than under the uncontrolled release scenario. Uncontrolled releases are not standard industry practice because there are a number of economic considerations driving the control of exhaust gases such as maintaining proper molality of the fermentation broth by the use of a vapor recovery system, maintaining sterility, and preventing release of microorganisms for proprietary reasons. Therefore, upon re-evaluation, the Agency decided that language requiring minimization of microbial concentrations in aerosols could be substituted for the requirement of the 2-log reduction performance criterion without affecting the no unreasonable risk finding necessary for a 5(h)(4) exemption under TSCA. The potentially increased exposure to this organism from the

modification of the containment criteria from the proposed 2-log reduction to minimizing microbial numbers in exhaust gases does not change the risk of using this microorganism for fermentation. Therefore, EPA has revised § 725.422(e) to read: "Use features known to be effective in minimizing viable microbial populations in aerosols and exhaust gases released from the structure, and document use of such features". The Response to Comments document (Section III.C.4.b.) and the Preamble of the Final Rule provide a thorough explanation for the change in requirements for microbial releases through exhaust gases.

V. BENEFITS SUMMARY

Substantial benefits are associated with this exemption. Aspergillus oryzae is already widely employed in general commercial use. Many of these uses, such as the production of soy sauce, miso, and sake, and the production of enzymes such as a-amylase, lactase, and b-galactosidase are food uses and not subject to TSCA. However, some enzymes such as a-amylase and protease, both of which are produced by A. orzyae, have wide applications in the detergent industry. The Agency has already reviewed two PMNs involving the use of A. oryzae for production of two enzymes, a lipase and a cellulase, which were for use in commercial detergents.

The Agency believes this exemption will result in resource savings both to EPA and industry without compromising the level of risk management afforded by the full 90 day review. The exemption will result in reduced reporting costs and a decrease in delay associated with reporting requirements. The savings in Agency resources can be directed to reviewing activities and microorganisms which present greater uncertainty. This exemption should also facilitate development and manufacturing of new products and the accumulation of useful information.

VI. FINAL ANALYSIS

1. Risks from use of the recipient microorganism A. oryzae are low. A. oryzae has a long history of commercial use. While some strains of A. oryzae are known to produce mycotoxins, these mycotoxins are not highly toxic to humans and their production under usual commercial conditions does not appear to pose a significant risk to human health.

A. oryzae does not appear to produce aflatoxins. Additionally, in the industrial setting, the use of proper safety precautions, good laboratory practices, and proper protective clothing, allay concerns for exposure of workers to mycotoxins potentially produced by this microorganism. A. oryzae appears to lack many survival features necessary for establishment in the

environment. Potential hazards to the public and the environment are also mitigated by limitations to exposure brought about by the conditions of contained use which are designed to limit release of the microorganisms to the environment.

2. Use of strains of A. oryzae which are eligible for the TSCA section 5(h)(4) exemption present no unreasonable risk. Concern has been expressed that genetic modification of A. oryzae could inadvertently produce an aflatoxigenic strain, although this is of low probability. Naturally occurring strains of A. oryzae are not known to produce aflatoxins; however, some scientists have suggested that A. oryzae is a domesticated version of A. flavus and may possess dormant genes for aflatoxin production. This exemption is for strains of A. oryzae and does not include A. flavus. As part of their eligibility for this TSCA section 5(h)(4) exemption, companies are required to certify that they are using A. oryzae. It is therefore expected that companies will have information in their files which documents the correct identification of their strains. Additionally, it is expected that companies will choose well-characterized industrial strains for further development through genetic modification. These expectations in combination with the use of Good Laboratory Practices should ensure the use of the correct species.

The limitations placed by the section 5(h)(4) exemption on the introduced genetic material, in particular the well-characterized and limited in size restrictions, should reduce the likelihood that any sequences relating to aflatoxin production could be introduced. The containment requirements would limit exposure to any mycotoxins produced.

Because the recipient microorganism was found to have little potential for adverse effects, introduced genetic material meeting the specified criteria would not likely significantly increase potential for adverse effects. As further assurance that risks would be low, EPA is specifying procedures for minimizing numbers of organisms emitted from the facility for the Tier I exemption and will be reviewing the conditions selected for the Tier II exemption.

Modification of the language of the two proposed containment requirements § 725.422 does not affect EPA's original determination that microorganisms that are eligible for and used under the conditions of the Tier I exemption will not present an unreasonable risk of injury to human health or the environment. Increased exposure to the microorganisms within or outside the facility resulting from these revisions will be minimal. The risk of using this microorganism in fermentation under the final conditions of this exemption is still low.

When balanced against resource savings for society and expected product benefits, this exemption will not present unreasonable risks.

VII. ACTION

Aspergillus oryzae is included as a recipient microorganism at § 725.420 for the tiered exemption.

Attachment I - Final Risk Assessment of Aspergillus oryzae

Note: For Attachment I to this Final Decision Document, see “Final Risk Assessment of Aspergillus oryzae” appearing elsewhere in the list of “Support Documents.”