September 2021

Safety evaluation of technical enzyme products with regards to the REACH legislation[1]

Introduction

According to the REACH guidance on Substance Identification, the safety documentation of an enzyme product consists of two elements:

- Safety of the enzyme.
- Safety of the non-enzymic constituents.

Safety of the enzyme

The only known safety risk linked to the active enzyme itself is respiratory allergy and for proteases the minor risk of skin/eye irritation. Potential adverse effects due to the catalytic activity of new enzymes are not likely but always assessed. The risk of respiratory allergy is valid for all types of enzymes and has been well described in the literature.

The above considerations also apply to protein-engineered enzymes. There are no reasons to suspect, and certainly no evidence to support, any concern that modifications made through protein engineering will affect enzyme safety. They exhibit variation that is similar to that observed in nature, and in most cases they share a much higher homology to their progenitors than that seen among naturally occurring isozymes. Accordingly, there is little basis for concern that simply changing some amino acids in an otherwise harmless enzyme might convert that enzyme to a toxic protein. Since the protein engineering has no effect on the strain, a sudden change in production output from the strain is not to be expected.

Safety of the non-enzymic constituents

Industrial enzyme products are practically non-toxic to humans and other animals based upon 35+ years of testing, use in commerce, and an in-depth knowledge of their properties.

[1] The scope of this policy is industrial enzymes (UVCB substances)

A review of the extensive literature, concerned with the safety of enzymes from microbial sources, strongly supports the general assumption that industrial enzyme preparations from non-pathogenic organisms are safe. They are not toxic by ingestion and do not exhibit reproductive or developmental toxicity, nor are they mutagenic or clastogenic. The industry's own historical toxicity data confirm this.

On the other hand, there is a considerable scientific literature base on various protein and non-protein microbial metabolites that may induce toxicity via the oral route (Pariza and Foster 1983; Pariza and Johnson 2001). These include food poisoning toxins which are proteins (for example Staphylococcal enterotoxins and the neurotoxins of Clostridium botulinum) and small molecular weight mold toxins such as the aflatoxins. This has been discussed at length elsewhere and supports the conclusion that the focus of safety evaluation should be to ensure that an enzyme production strain does not produce toxins that are active via the oral route (Pariza and Foster, 1983; Pariza and Johnson, 2001). These concepts and considerations form the basis for establishing production strain safety.

The primary concern in the safety evaluation of technical enzyme products is therefore the safety of the production strain. The historical use of certain species and strains thereof provides proof beyond reasonable doubt of the safety of these strains (see e.g. de Boer, A. S. and Diderichsen, B., 1991, Barbesgaard P. et al., 1992, Priest F. et al, 1994, Nevalainen H. et al., 1994, Schuster E., et al., 2002, van Dijck, P. W. M. et al., 2003). The experience from numerous studies is, that the non-enzymic constituents originating from the fermentation as substrate residues or produced by the organisms have no toxicological or ecotoxicological potential (cf. e.g. Pariza and Johnson, 2001, HERA risk assessments on protease , and on amylase, lipase and cellulase, 2005, Zofia S. Olempska-Beer et al., 2006). Again, the industry's own toxicity data including documentation generated for regulatory approval confirm this.

As an illustration, in numerous sub-chronic toxicity studies in rats and dogs with strains of the *Aspergillus* species (a.o. *A. oryzae and A. niger*) and *Bacillus* species (a.o. *B. subtilis / amyloliquefaciens and B. licheniformis*), only a few insignificant adverse effects have been observed.

The observed effects in blood chemistry (liver and kidney function) was never accompanied by histological organ changes and thus deemed to be of minor toxicological relevance. Often the effects were attributed to a high protein and salt

(used as an additive after fermentation) content of the batches used. In the few cases where irritancy and/or inflammation were observed, protease activity was determined to be the cause (HERA risk assessment on amylase, lipase and cellulase, 2005).

Numerous *in vitro* mutagenicity studies, Ames tests and chromosome aberration tests, have not revealed any adverse effects.

These studies were performed with enzyme fermentation batches from wild-type strains, classical mutants and genetically modified strains.

The Safe Strain Lineage Concept

The toxicity test programs on GRAS determined enzyme products from industrial production strains of e.g. *Aspergillus* or *Bacillus* have not shown adverse effects *in vitro* or in animal studies. The lack of effects and the lack of expecting such effects based on the increased genetic knowledge of the organisms qualify the discontinuation of extensive toxicological test programs required for food or feed application by authorities, including the use of animal testing. If strains from a certain strain lineage have been tested and used for several years, and further improved by e.g. deleting genes coding for potentially harmful metabolites, then one must conclude at a certain point in time that a strain from this strain lineage can be declared safe for use without further testing by extensive programs including animal testing. This strain should be designated as "parental strain" of a "Safe Strain Lineage", and be used as a start point for further development and improvement for production strains.

The term "safe strain lineage" refers to a cluster of related strains that have all been derived by genetic modification from a single isolate ("parental strain") that was thoroughly characterized and shown to be non-toxigenic and non-pathogenic before the modifications to improve enzyme function were initiated so as to produce the cluster.

This "Safe Strain Lineage" concept is depicted in Figure 1.

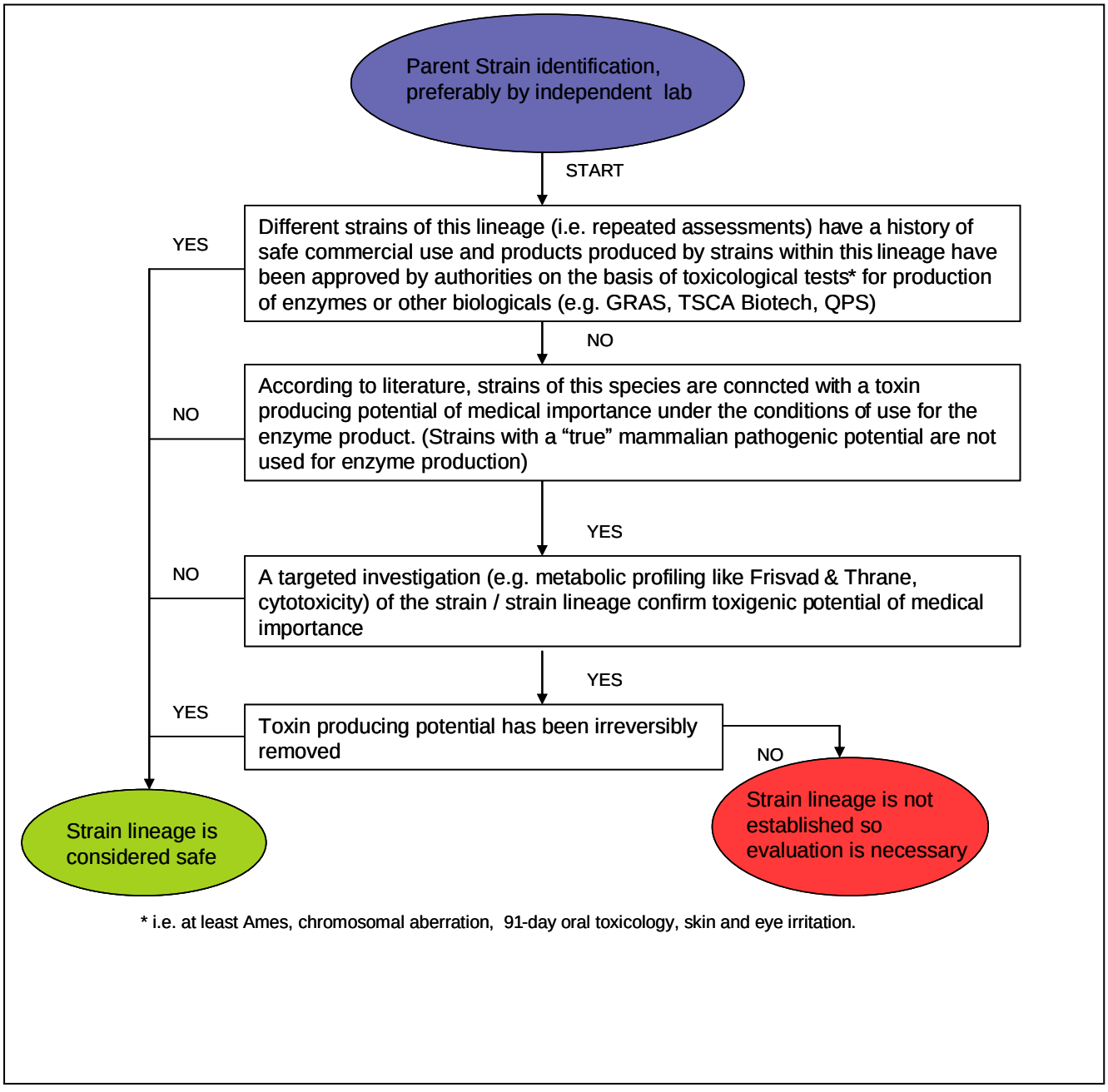

Figure 1: Safe Strain Lineage concept

In theory, the chance that genetic modification or an unexpected mutation may cause the expression of an unknown dormant gene coding for a toxic metabolite is never zero, but will be an unlikely event. Depending on the amount of available

data for the strain lineage *in vitro* tests on the enzyme preparation may be necessary to detect a potential adverse effect.

Based on this, it is our conception, that:

A newly constructed strain using a host strain derived from a "Safe Strain Lineage", or any changes made to a safe production strain or production process, should require at most a limited *in vitro* test programme on the enzyme preparation, to cover the minor theoretical possibility of activating silent genes coding for a toxin, if the following requirements are fulfilled:

- Thoroughly conducted toxicological studies have elucidated the properties of a given enzyme and the enzyme has a history of safe use.
- The "parental strain" belongs to a documented "safe strain lineage"
- The genetic history of the recipient microorganism ("strain line-tree") is documented back to the isolate from which it was derived ("parental strain").
- Strain development and improvements by introduced genetic modifications are well characterized in compliance with the definitions and requirements of regulatory bodies in the EU and US for "introduced genetic material". (These modifications include deletions, rearrangements, amplifications, point mutations, and/or plasmid loss within a single genome, either spontaneously or through use of chemical or physical mutagens).

These requirements are in accordance with the published recommendations (cf. e.g. Pariza and Johnson , 2001, Zofia S. Olempska-Beer et al., 2006).

To facilitate the implementation and acceptance of the safe strain lineage concept we have to distinguish between the safety requirements for a "parental strain" and the safety requirements for production strains or new strains to be introduced as production strains.

An example

As an example, a strain line from *A. oryzae* can be used. Several toxicological programmes on enzymes from the original "parental strain" as well as on derived strains in the strain line tree have been carried out.

There was no observed toxicity, therefore, any of these strains could theoretically be called "parental strain". For practical purposes a well-defined strain in the strain line tree was selected and used as "parental strain". This strain has several advantages: there is toxicity data directly on products produced by this strain, the absence of several moderately toxic metabolites and undesirable side activities is documented and there are no longer (antibiotic) resistance markers present.

The results of the several 13-week oral toxicity studies carried out can be summarized as the following: No adverse effects were observed at the highest (maximum) dosing concentrations. These concentrations differ slightly from enzyme to enzyme depending on the amount of Total Organic Solids (TOS = 100% - water% - ash% - diluents %) and the maximum possible dosing amount (10ml). The calculated safety factors, based on these concentrations and the specific application, lie between 1000 and 100.000 in worst case scenarios.

Both A*. oryzae* and its enzymes are accepted as constituents of food (FAO/WHO JEFCA, 1987). The safety of *A. oryzae* has been evaluated by a comprehensive literature survey in medical databases and by Barbesgaard *et al.* (1992). *A. oryzae* has a long history of safe use. In the Orient it has been used to produce koji, a complex enzyme preparation for the production of soy sauce, miso and sake for more than 2000 years. In Europe, it has been used since the beginning of the last century in the production of enzymes for baking and brewing and in the last decade as a recombinant organism for production of a variety of bioindustrial products. Published data show that these *A. oryzae* strains can be regarded as non-pathogenic. The wild type strain contains a non-functional cluster of genes homologous to the aflatoxin biosynthetic genes of *Aspergillus flavus.* Due to the general concern about aflatoxins the enzyme manufacturers using *A. oryzae* production strains have to ensure that these strains do not revert to an aflatoxin producing strain. One way to accomplish this is to delete the gene cluster[2] in the parental key strain in the development of the enzyme production strain. Another way would be to demonstrate that the strain does not produce aflatoxins under production conditions. Certain strains may produce one or more of the secondary metabolites cyclopiazonic acid, kojic acid and beta-nitropropionic acid. The toxicity of these metabolites is low to moderate (Burdock and Flamm, 2000) and there are no reports that their production has resulted in adverse effects on human health.

[2] N.B. to delete this gene cluster a license will be required.

Based on these data, the production strain can be considered a safe microorganism.

The genetically modifed *A. oryzae* production strain meets the criteria for a safe production microorganism as outlined by several expert groups (Berkowitz and Maryanski 1989, International Food Biotechnology Council 1990, EU Scientifc Committee for Food 1991, Organization for Economic Cooperation and Development 1992, 1993, FAO/WHO 1996, Jonas *et al.* 1996, Pariza and Johnson 2001). It is constructed by common transformation procedures, using well-known plasmid vectors with strictly defined and well-characterized DNA sequences that are known not to encode or express any harmful or toxic substances. The development of the production strain has been evaluated at every step to assess incorporation of the desired functional genetic information and to ensure that no unintended sequences were incorporated (Yaver *et al.* 1996).

Several other examples of the safe strain concept are given by Zofia S. Olempska-Beer et al., 2006.

Safety evaluation principles

When a strain is to be used which is not a member of a safe strain lineage, several of the principles described above can be used to evaluate the safety of the enzyme product. This can be the case e.g. when using a natural isolate.

In those cases also the identity of the microorganism should be determined by an independent laboratory. If a literature search shows that the species is not associated with toxin production of concern, and is a species historically used for enzyme production, then the strain can be considered safe.

If the species has not been used in industrial enzyme production before it's possible pathogenic / toxicogenic potential should be considered. For a first time industrial use, the concentrate produced by the strain should be tested at least through Ames, chromosomal aberration, and 91-day oral toxicology, plus usually skin and eye irritation for worker safety. Assuming the results from that testing is acceptable, a GRAS assessment of the enzyme preparation can be done through a paper exercise, comparing the TOS levels from the tox. lots used in testing with the new enzyme preparation to do consumption analysis and acceptability.

If the literature search showed that the species is associated with toxin production, a test for the toxin under inducing conditions should be done. If the toxin is not found, one can proceed as above.

If the toxin is found, and sufficient genetic information is available for the species, one can proceed with the deletion of one or more of the genes involved in the toxin synthesis if the genetical background is known.

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