ENZYME SAFETY
PANEL DISCUSSION

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BIO WORLD CONGRESS – *ENZYME SAFETY PANEL*

	A. History & Regulation	B. Enzyme aspects	C. Strains & Manufacture	D. Safety
Panel moderator Jim LaMarta, ETA	0. History of enzyme use	0. Enzyme identity & structure basics	0. Fermentation basics	0. Intro
Member 1 Diane Shanahan, ETA	1 Food uses and CFSAN scope – human food & supplements	2. Food allergenicity	3. Process controls / BioTSCA (strains & containment)	1. Basic safety assessment
Member 2 Dave Mason, ETA	2. Technical uses and TSCA scope	3. GHS	4. Process controls / FSMA	2. Type of tox studies
Member 3 Vince Sewalt, ETA	3. Animal feed uses & CVM scope	4. Occupational health & stewardship	2. Strain safety, SSL	3. Use of tox studies in GRAS / PMN
Member 4 Alice Chen, Keller & Heckman LLP	4.EU approval requirements	1. Protein engineering	1. GE Strains	4. Safety of : by-products co-products
	Q&A	Q&A	Q&A	Wrap-up, Q&A

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Agenda

- Welcome
- Introductions
- Discussion Topics
  - History & Regulation
  - Enzyme chemistry & worker safety
  - Manufacture & Production Strains
  - Toxicology & by-products
- Summary & Close

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History of Enzyme Use

- Early advantageous use
  - Leavened bread
    - Beer (amylases)
  - Yoghurt
    - Cheese (rennin)
  - Fermented vegetables
  - Vinegar

Industrialization

- 1833 – Payen & Persoz isolate the enzymes found in barley malt
- 1874 – Christen Hansen isolates rennet from calves stomachs
- 1876 – William Kuhne cell-free extracts of yeast
- 1894 – Jokichi Takamine isolates diastase from *Aspergillus oryzae*
- 1917 – Boidin & Effront use amylase from *Bacillus subtillus* to desize cotton
- 1906 – Otto Rohm extract from manure - leather
- 1913 - Otto Rohm – trypsin from pancreas - textiles
- 1959 – E. Jaag alkaline bacterial protease
- 1961 – Novo develops subtilisin for detergents
- 1960's – glucoamylase – corn syrup
- 1970's – lactase - treatment of milk
- 1975 – restriction enzymes – the birth of modern biotechnology
- 1984 – feed enzymes, ß-glucanase for poultry diets containing barley
- 2000 – cellulases for bioethanol production

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A1. History & Regulation
Food uses and CFSAN scope-human food & supplements

- A variety of commercial enzymes are available for use in food (e.g., baking, brewing, juice, and dairy, and other food processing applications)
- Enzymes are also available as dietary supplements (digestive aids).
- Any substance that is reasonably expected to become a component of food, or affect the characteristics of food, is a food additive
- Food additives are subject to premarket approval, unless the substance is generally recognized as safe (GRAS)
- Previously via GRAS affirmation process and now under GRAS Notification process, enzymes are well suited to the GRAS process given the general availability of scientific data supporting enzyme safety and the peer-reviewed methodology and decision trees for evaluating the safety of microbial enzymes
- 600 GRAS Notices have been filed through 2015 of which approximately 14% are related to food enzymes
- Enzymes as Dietary Supplements are regulated under the Dietary Supplements Health and Education Act (DSHEA). Ingredients that were already in use prior to 1994 are grandfathered in.
- Any new supplements and/or ingredients must go through premarket notification for a new dietary ingredient (NDI) (21CFR190.6)

A2. Technical uses and TSCA scope

Technical uses of Enzymes: Enhancing efficacy, safety while reducing the environmental impact

- **Prevalent Technical Applications of Enzymes:**
  - **House Hold Care: Laundry Detergents, Cleaning Products, Deodorizers**
  - **Manufacturing: Textiles, Leather Processing, Coloring, Paper, Lumber**
  - **Bioenergy: Fuel Ethanol (1G), Cellulosic Ethanol production (2G), Biodiesel**
  - **Industrial Hygiene and Process Optimization: De-foaming, Bleaching (de-inking), De-greasing**

- **Benefits of Industrial Enzymes:**
  - **Replace chemicals or processes presenting safety, environmental concerns or enhance efficiency**
    - **Reduce the use of sulfide in tanneries**
    - **Clothes can be washed at lower temperatures with reduced energy requirements**
    - **Enhanced industrial hygiene**
- **TSCA :**
  - **Section 5 compliance for Biotechnology**
    - **Tier 1 production organisms**
    - **PMN**
    - **MCAN**

For more information: http://www.enzymeassociation.org/wp-content/uploads/2013/09/benefits_paper.pdf

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A3. History & Regulation

Food uses and CVM scope – Production Animals and Pet Food

- Enzymes are used widely in animal feed, to make nutrients in the feed more bio-available, or they are used in commodity processing resulting in animal feed (e.g., DDG) and pet food (e.g., plant or animal waste fractionation/protein processing)
- Enzymes widely used animal feed include phytases, proteases, and various carbohydrases
- Similar to human food, any feed ingredient or processing aid that is reasonably expected to become a component of animal food, or affect its characteristics, is a food additive. Regardless of whether the enzyme is added to animal feed or used as processing aid, GRAS is the exemption to premarket food additive approval.
- GRAS Notification to FDA/CVM is a recently established process; data (publication) requirements, although initially stipulated by FDA/CVM, may still be subject to negotiation, but will be defined in the final GRAS Rule expected August 2016.
- At the State level, feed enzymes are also regulated, and coordination takes place within the American Association of Feed Controls Officials (AAFCO).
- A process to get GRAS Notified feed ingredients incorporated into the AAFCO list is under development.

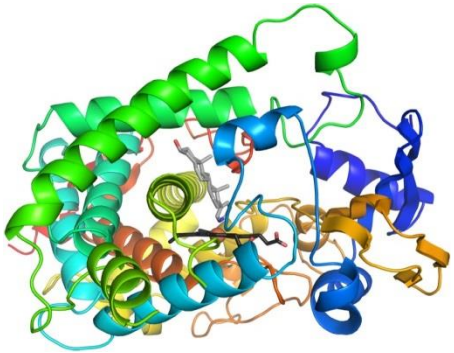
A4. EU approval requirements

- **Food enzymes:** Most countries do not require formal approval (except DK and FR) , but EU-wide food enzyme approval is being implemented under EU Regulation 1332/2008. Currently, enzymes can be marketed in accordance with national law in EU Member States
- **Technical enzymes:** Placed on, manufactured in or imported in quantities above 1 metric ton per year per legal entity- must be registered under the EU REACH Regulation (Regulation 1907/2006)
- **Feed enzymes**: Dossier review by European Food Safety Authority (EFSA)
- **Some notable differences in EFSA data requirements:**
- **Genetically Modified Organism(food and feed):**
  - Genetic stability confirmation at gene level
  - Detection of recombinant DNA in product
- **Food:**
  - Generally requires toxicity data specific to the enzyme/strain combination (possible exception EFSA's Qualified Presumption of Safety list)
- **Feed:**
  - Generally requires toxicity data specific to the enzyme/strain combination
  - Processing aid: no specific EU legislation/authorization procedure
  - Functional in the feed product: authorization needed for a feed additive under EU Regulation 1831/2003
  - Approval is product-specific (including registration of enzyme blends) and specific to animal species/production type, with species-specific data needs
  - Animal efficacy studies to support production label claims

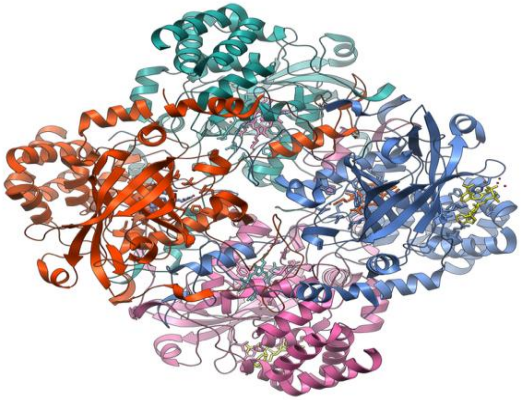
Enzyme Identity & Structure

- Identity is based upon function
- Reaction it catalyzes
- From the general to the specific
  - hydrolase, reductase, oxidase
    - Beta galactosidase, dihydrofolate reductase, monoamine oxidase
- IUBMB Name and EC number
- For Food and Pharma the source is important

B. Enzyme Structure



Cytochrome P450: cyp17A1
E. Scott U. Kansas



Catalase
Protein database

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B1. Protein Engineering

- Changes to the amino acid sequence of the enzyme relative to the native sequence
- Evaluation of amino acid changes
  - Enzyme characterization
  - Allergenicity potential
  - Toxin or virulence factor potential
- Toxicity testing possibility
- Impacts to regulatory filings examples when the enzyme is further engineered after submission

B2. Enzyme Aspects - Food Allergenicity

- In general, ingestion of microbial enzymes is not likely to be of concern with regard to food allergy (Bindslev-Jensen et al, 2006).
- Nevertheless, evaluation of the enzyme component should also include the consideration of its potential to cause an allergenic response upon ingestion.
- The model for the assessment of allergenicity is the 2009 Codex guidance published by FAO/WHO (Codex, 2009) and uses a weight of evidence approach.
- This approach uses the sequence of the enzyme protein as the first step in the assessment to evaluate its relationship to known allergens.

B3. GHS

GHS: “The hazards have not changed, only how we communicate them”

Hazard Communication Standard are summarized in the link below.

Hazard Category Cut-off Concentration Triggering Classification Of An Enzyme* Mixture

- Respiratory Sensitizer Category 1 ≥ 0.1%
  - Like all proteins enzymes are considered respiratory sensitizers
- Skin Irritant Category (protease) 2 ≥ 10%
- Eye Irritant Category 2B ≥ 10%

*Active enzyme protein, calculated on the basis of the declared enzyme activity

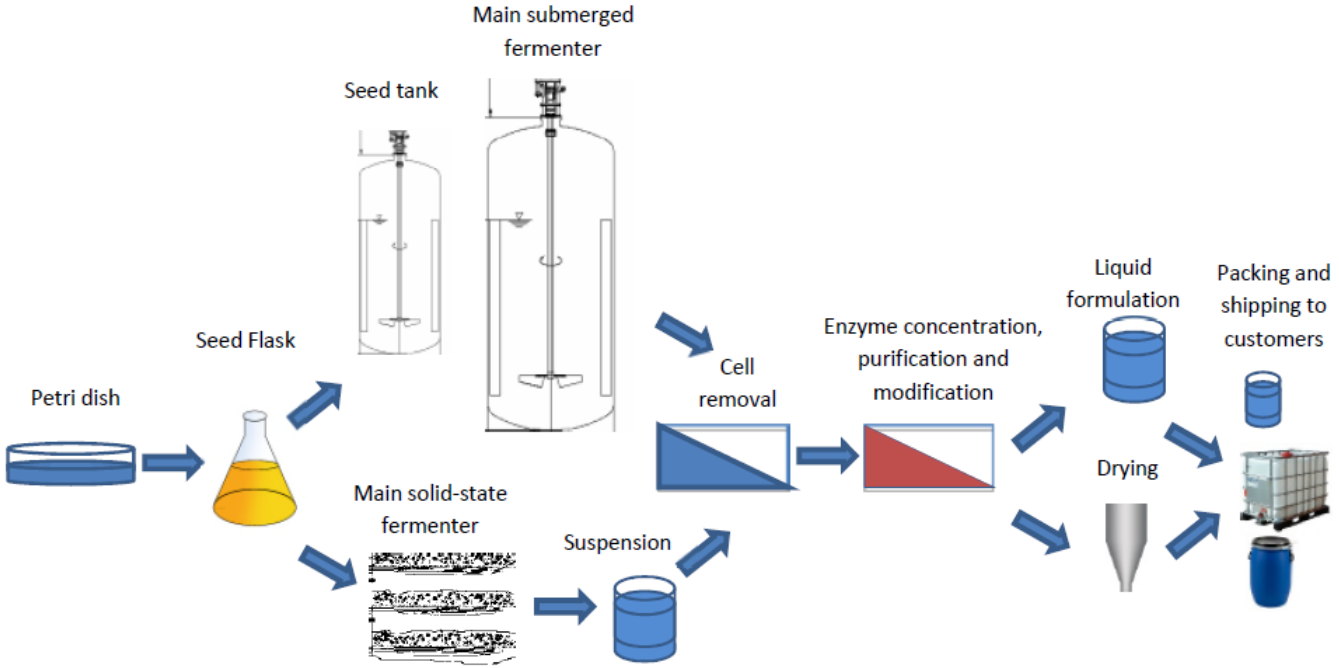
The presence of other hazardous components in the mixture may require additional classification

✓ http://www.enzymeassociation.org/wp-content/uploads/2013/12/ETA-GHS-Enzymes.pdf

B4. Enzyme Safe Handling & Stewardship

- Main stewardship focus for industrial enzymes in the workplace relates to controlling inhalation exposure, as enzymes, like all proteins, are potential respiratory sensitizers.
- Exposure control is achieved by:
  - Product design (granules, liquid)
  - Engineering controls (equipment design, ventilation systems) to prevent/contain aerosols, avoid routine/uncontrolled spillage
  - Personal Protection equipment
  - Work practices that minimize formation/release of aerosols (housekeeping, cleaning, maintenance, program review/audit)
  - Training and awareness
- Choice of product form & application for consumer products is important to minimize avoid consumer exposure to aerosols.

C. Manufacture Basics



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C1. Genetic Engineering

- Genetic changes not including alterations to the amino acid sequence of the enzyme
  - Regulatory sequence
  - Signal peptides
  - Non-enzyme proteins
  - Safety by design (BSL-1)
- Evaluation of genetic modifications
  - Limited in size
  - Well-characterized (donor microorganism assessment)
  - Poorly mobilizable
  - Free of toxin and/or allergen encoding sequences
  - Antibiotic resistance markers removed if possible
  - Metabolic “spill over” effects

C2. Safe production organisms & strain lineages

- Enzyme manufacture leverages drop-in production platforms, consisting of optimized and well-characterized microbial production organisms that have been thoroughly tested for safe production of high titers of enzymes.
- A so-called Safe Strain Lineage (SSL) can be established based on repeated testing of members of the lineage and their products in toxicological studies.
- Additional members of the SSL can be developed with well-characterized and safe molecular tools.
- The safety of such new members of the SSL can subsequently be evaluated using a decision tree approach (e.g., Pariza & Johnson 2001).

C3. Manufacture Process Controls - strains & containment (TSCA)

- Manufacture of microbial enzymes uses microorganisms
- Microorganisms fall within the EPA definition of a chemical substance
- TSCA Biotech rule covers certain modified microorganisms
  - Microorganisms with intergeneric coding sequences and used for commercial purposes
- Containment of the microorganisms is important; potential to induce adverse effects directly or indirectly upon release
  - Physical – facility; structural
  - Process – air, liquid and solid streams inactivated
  - Introduced genetic material – well characterized; poorly mobilizable

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C4. Manufacture process controls – food/feed safety (FSMA)

FSMA empowers FDA to proactively enforce GMPs

- Plants are registered
- Enzyme Industry takes a proactive, risk based approach, that is cross functional
- Ensures evaluation extends outside the plant to process inputs
    - ❖ **Air**
    - ❖ **Water**
    - ❖ **Steam**
    - ❖ **Raw Materials**

✓ **There is no “certification” of FSMA compliance**

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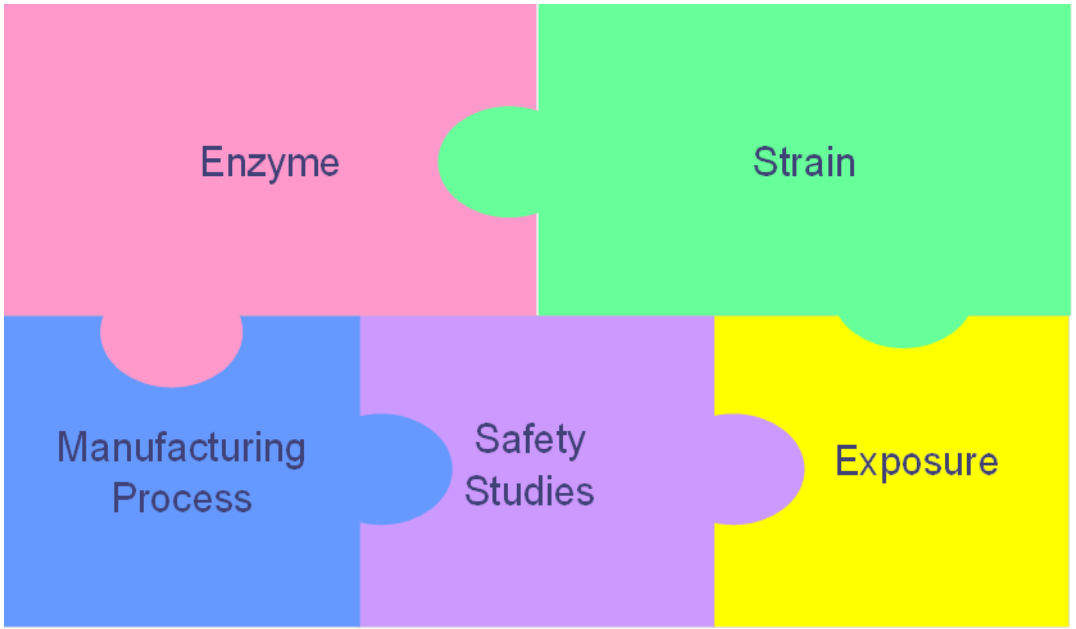
D. Safety



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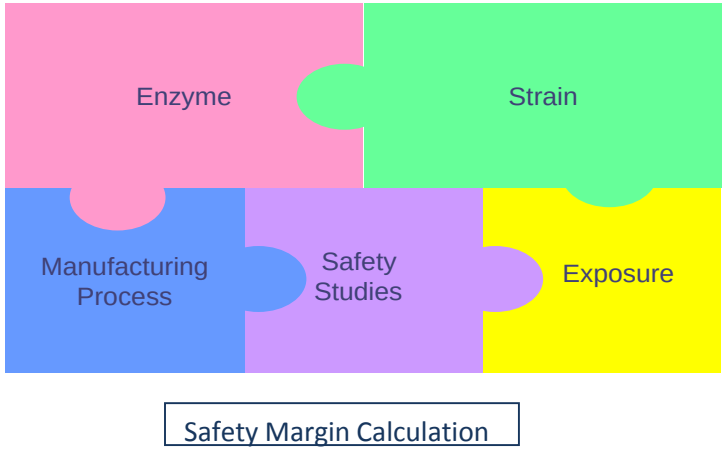
D1. Safety - Basic Safety Assessment



There are five main elements

- 1) the enzyme
- 2) the production microorganism
  3) the manufacturing process
- 4) safety studies
- 5) estimation of dietary exposure and safety margin calculation.

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D2. Components of Safety Assessments

Rational approaches to assaying safety in manufacturing, handling, transport and use

Risk assessment relies on a preponderance of the data to support safety in manufacturing, handling, transport and use.

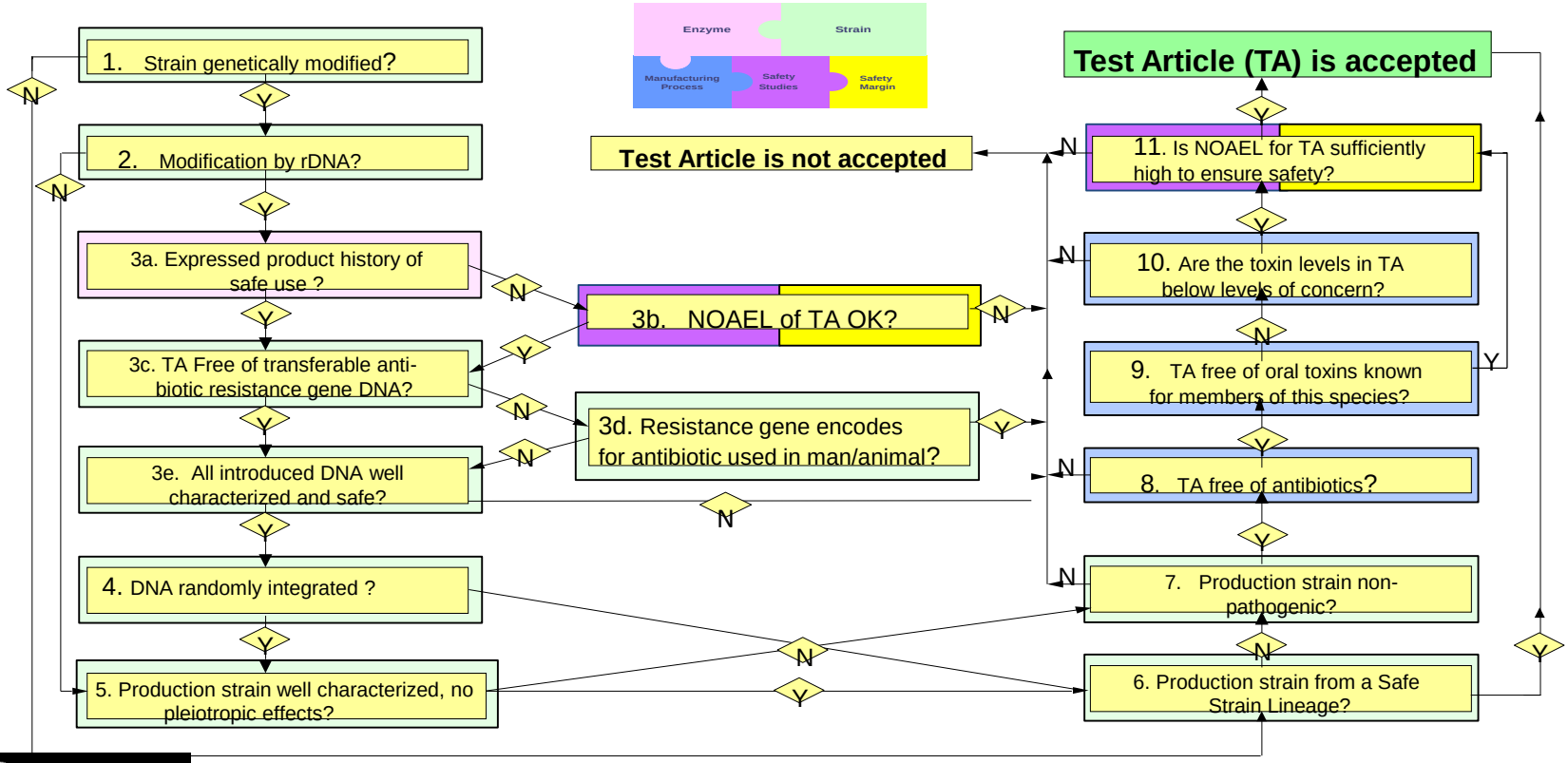
- A typical Safety matrix might include
  - Mutagenicity and cytotoxicity
  - Oral toxicity studies (7-90 days)
    - Overt toxicity/ Mortality, generally not observed
    - Adverse responses
    - Identification of a NOAEL or NOEL

*Additional studies maybe considered for specific intended uses or to address certain enzyme specific, production or handling concerns (ex. Skin irritation, Nutritional analysis)

D3. Selection of Tox Studies to Support GRAS

- Whether new toxicological studies are needed to support the safety of a food or feed enzyme preparation depends on the availability of safety data representative of related enzymes and strains from the same SSL.
- For this a decision tree is used that considers the following:
  - history of safe use of the newly expressed enzyme protein
    - If no history of safe use, this triggers new tox studies
  - prerequisite safe transformation methods and well-characterized modification(s) to the host
  - use of a strain that belongs a Safe Strain Lineage (SSL) as defined earlier
    - If belonging to an established SSL, the NOAEL from a prior study can be used to make sure the safety margin is sufficient
    - If not belonging to an SSL, then additional questions need to be addressed regarding the strain's ability to produce antibiotics & toxins, *and* new tox studies are to be conducted.

Pariza & Johnson (2001) Decision Tree



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D4. Safety of Co-products

- **Examples:**
  - spent biomass
  - extracted co-products (e.g., lipids, proteins, etc.)
- Inactivation status of production organism
  - Laboratory data
  - Commercial scale data
- Novel production microorganisms may require target animal feeding studies
- Color additive potential for some microorganisms

Questions ?

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