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Yes We GRAS !

Enzymes as Catalysts for Successful Application of the GRAS Process

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Agenda

- Welcome
- Introductions
- Topics
  - GRAS definition
  - Enzymes, a History of Safety in Use and Manufacture
  - Manufacture process & Enzyme Characterization
  - Production Strain Construction & Safe Strain Lineage
  - Toxicology & Margin of Safety
  - GRAS framework: Track record of enzymes
- Summary & Questions

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The GRAS definition

- GRAS concept is rooted in the LAW!
- Federal Food, Drug and Cosmetic Act says:

The term ‘‘food additive’’ means any substance the intended use of which results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of any food **if such substance is not generally recognized**, among experts qualified by scientific training and experience to evaluate its safety, as having been adequately shown through scientific procedures (or, in the case of a substance used in food prior to January 1, 1958, through either scientific procedures or experience based on common use in food) to be safe under the conditions of its intended use;

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The GRAS definition –what does it mean?

- GRAS substances are excluded from the definition of food additive
  - As such they **do not require "premarket approval"**
- The safety determination is always limited to the **intended use**
  - The same quantity and quality of data that is required for a food additive is also required for a GRAS substance
  - The scientific data and information must be widely known and there must be consensus among qualified experts-**the common knowledge element**

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History of Enzyme Use

- Enzymes are found in all living things
- Ubiquitous in fresh and processed foods throughout the human diet
- Used in ancient times before function of enzymes was even understood
  - Beer (carbohydrases for conversion of starch to sugar)
  - Leavening of bread (enzymes in yeasts that produce CO2)
  - Meat tenderizing (juice of the papaya to soften meat)
  - Cheese (milk clotting enzymes from animal stomachs)
  - Vinegar (converting alcohol to acetic acid)
  - Wine (oldest known reference to use of enzymes, 2100BC)

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Enzyme Use Historical Timeline

- **1833 – Brewing:** Payen & Persoz isolate barley malt enzymes
- **1874 – Cheese:** Christen Hansen isolates rennet from calve stomach
- 1876 – William Kuhne cell-free extracts of yeast
- 1894 – Jokichi Takamine isolates diastase from Aspergillus oryzae
- **1959 – Detergent Enzymes:** E. Jaag alkaline bacterial protease
- **1960's – Grain Processing:** glucoamylase for 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 – Biofuels:** cellulases for bioethanol production

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General Enzyme Safety

'Intrinsically safe' proteins (Olempska-Beer et al., 2006)

- Can be categorized as "practically non-toxic"… based on 40+ years of testing, use in commerce, and an in-depth knowledge of their properties
- Not reproductive or developmental toxins
- As with any protein, enzymes, when inhaled, have the potential to elicit an allergic response in atopic individuals
- Proteases can irritate skin and mucous membranes

Lack of Genotoxicity

- Enzymes are not mutagenic, and not clastogenic
- Supported by plethora of data, e.g. Pariza & Johnson (2001) reported over 100 Ames assays and over 60 Chromosomal Aberration studies, all testing negative.

Enzymes are used at very low levels

- The levels of any contaminants, if they were present, would be 'de minimis'.

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Generally Recognized Guidelines for Safety Evaluation

- **Enzymes**
  - Pariza and Johnson (2001), Pariza & Cook (2010)
  - FAO/WHO JECFA (Joint Expert Committee on Food Additives)
  - EU SCF (Scientific Committee for Food) and EFSA
- **Biotechnology**
  - IFBC (International Food Biotechnology Council)
  - OECD
  - FAO/WHO
- **Assessment of Potential Allergenicity**
  - FAO/WHO (2001), amended by Codex Alimentarius (2009)
  - Ladics et al., 2011
- Rigorous, peer-reviewed methodology; used by ETA members
- Provide FDA and other agencies with framework for assessment

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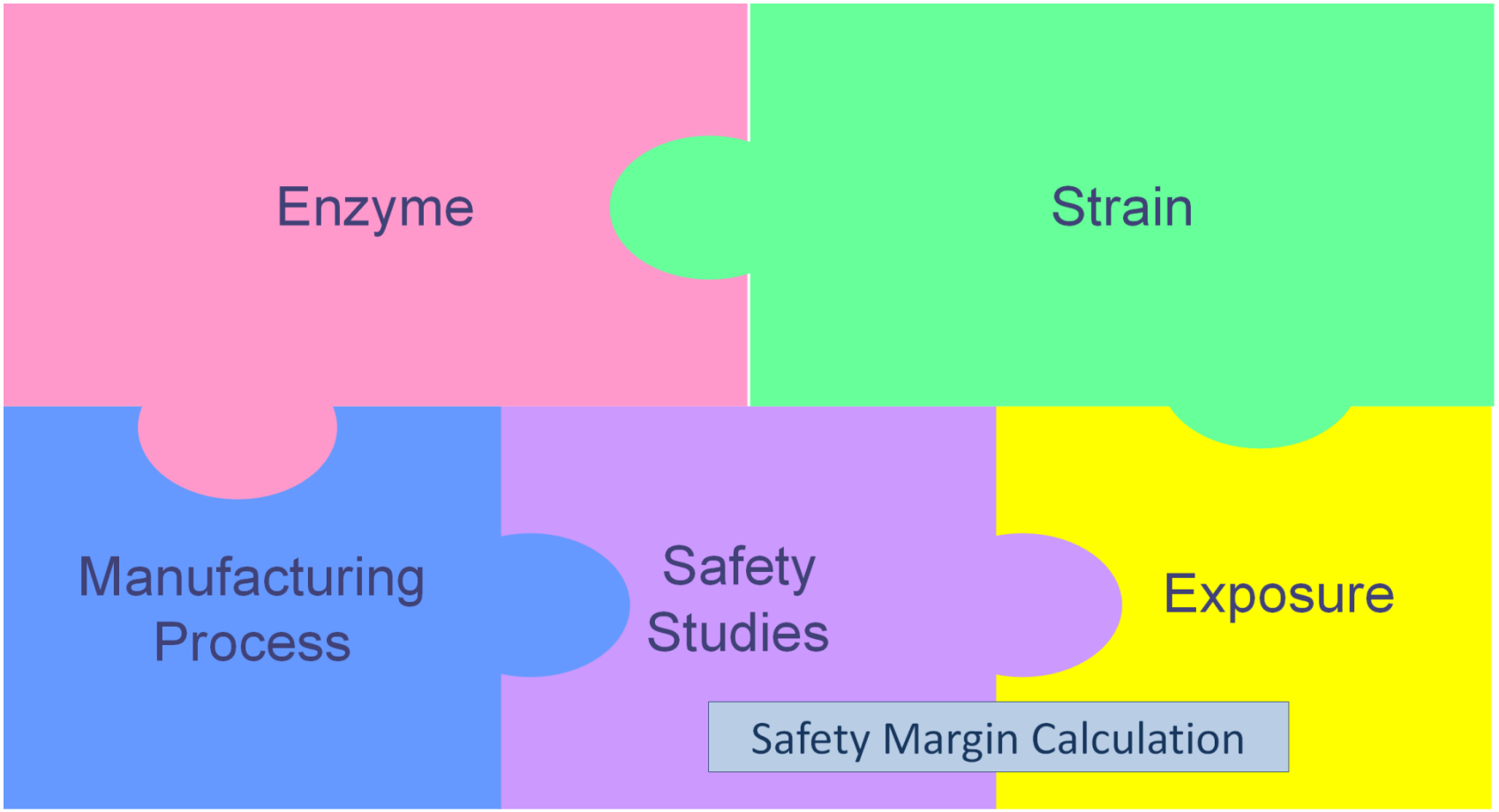
Safety Evaluation of Food Enzymes…
5 elements



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Enzyme

- Characterization
  - IUB and CAS classification
  - Source
  - Functionality
    - Specific reaction
  - Properties
    - MW, structure, protein sequence
  - History of use
  - Assessment for potential allergenicity
    - Considers source, type and history of safe use of protein
    - Sequence evaluation approach based on published methods

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Enzyme Identity

- Identity or enzyme class is based upon specific catalytic function
  - Logical to use this as the basis for classification
- International Union of Biochemistry & Molecular Biology – IUBMB is recognized authority on enzyme nomenclature
  - IUBMB record: lists Enzyme Commission # and corresponding CAS #
  - Enzymes classified into 6 major classes with more specific subclasses

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Enzyme Identity and Structure

- All enzyme proteins based on the same 20 amino acids
- Typically comprise several hundred AA's folded in a unique 3-D structure
- This structure determines an enzyme properties
  - catalytic activity, specificity and stability

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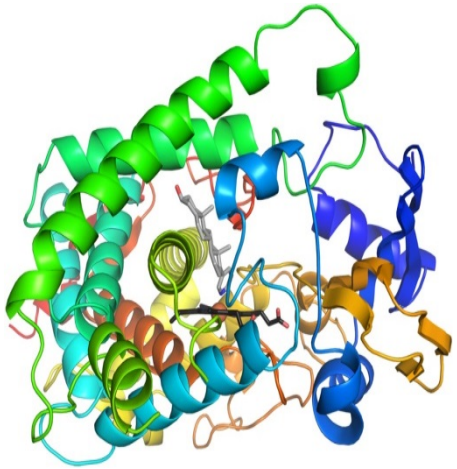
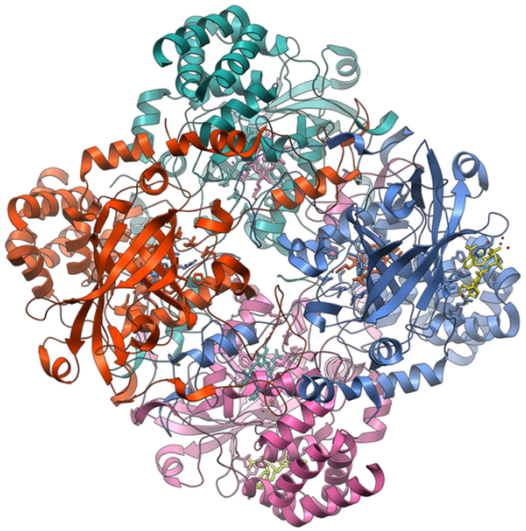
Enzyme Structure



Cytochrome P450: cyp17A1
E. Scott U. Kansas



Catalase
Protein database

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Enzyme Structure

- **Active site region is highly conserved**
- **Non-active site region has greater variation**
  - via natural adaptation to changing environments
- **Some adaptations may affect the enzyme's characteristics**
  - Temperature or pH optima
  - Rate of enzyme action
  - Yield during fermentation

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Protein Engineering

- First introduced over 25 years ago
- Intentional changes to the amino acid sequence of the enzyme relative to the native sequence
  - Typically does not involve the catalytic site and thus, the enzyme identity is not affected
  - Allows for enzymes with tailored characteristics
    - Improved thermal stability
    - Improved enzymatic efficiency
    - pH optimum for the application conditions

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Safety of Protein Engineered (PE) Enzymes

- **PE enzymes compared to their wild-type counterpart have the same risk profile**
  - Both manufactured using identical processes
  - Both structurally similar
  - PE enzymes have changes well within natural variation among wild-type enzymes
- **Evaluation of amino acid changes conducted**
  - Allergenicity potential
  - Toxin or virulence factor potential
- **Survey of toxicity data for PE enzymes** – 28 Ames tests, 26 Chromosomal Aberration, all testing negative (Pariza and Cook, 2010)

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Allergenicity Potential

- 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 uses a weight of evidence approach. Codex guidance published by FAO/WHO (Codex, 2009)
- Uses the sequence of the enzyme protein as the first step to evaluate its relationship to known allergens.
- Enzyme source, type, and history of safe use also a consideration

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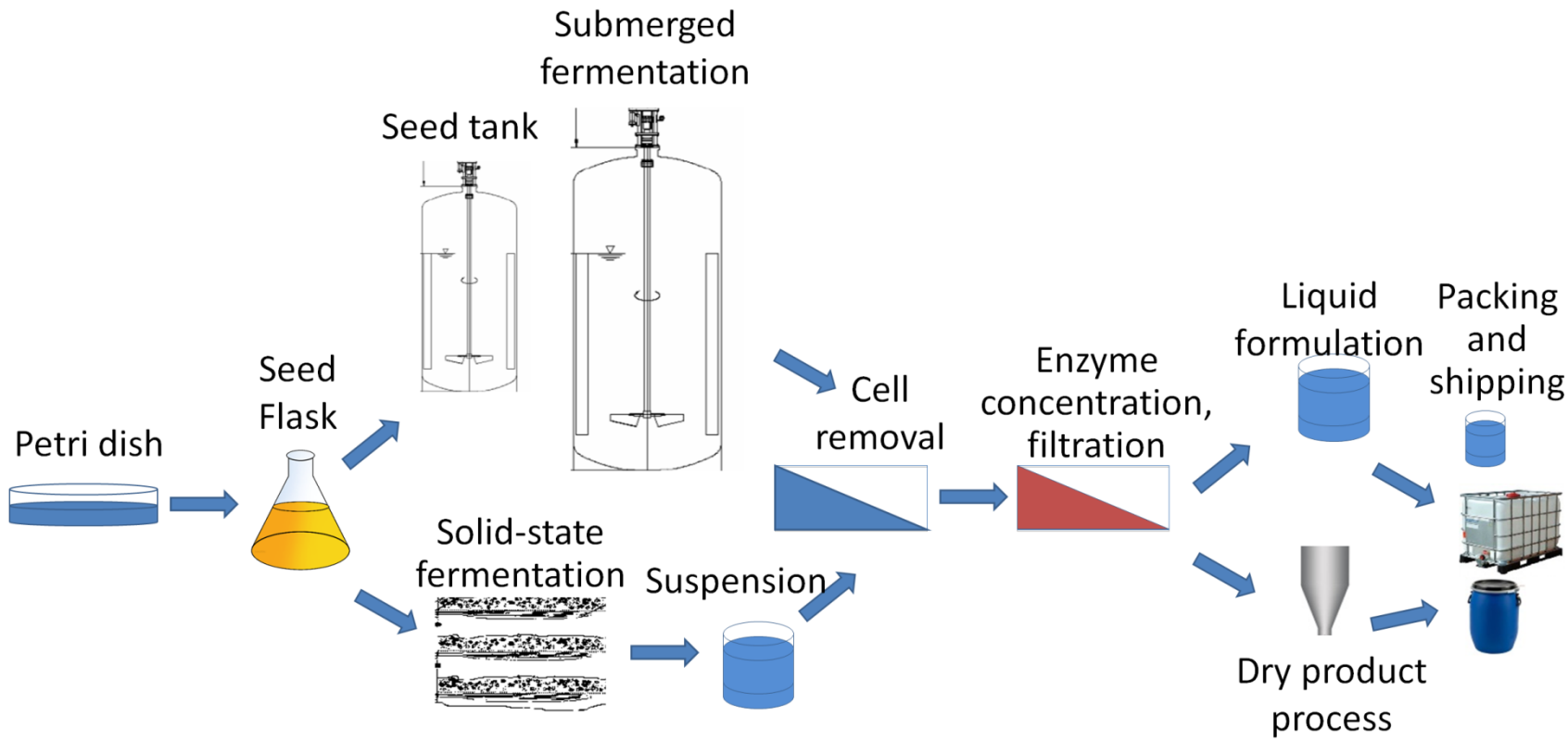
Manufacturing Process

- Products produced using Current Good Manufacturing Practices (cGMP)
- Raw materials and processing aids are food grade or safety assessed
- Quality Management system
- Controlled Manufacturing Process
  - Fermentation
    - Follow aseptic procedures and specific protocols to monitor growth of organism
    - Quality checks for contamination at critical control points
  - Follow standard industry practices for purification and formulation
  - Final products meet FCC and JECFA purity recommendations for enzymes used in human food

General diagram for the manufacturing process of enzymes



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Typical Composition of Commercial Enzyme Preparation

- Enzyme protein
- Formulation Ingredients
  - Materials to standardize and maintain activity
  - Improve handling
  - Safe and suitable for the intended use
- Non-enzyme fraction of the concentrate
  - Peptides, amino acids, carbohydrates, lipids, salts
- No viable production organisms present
- Testing of final product according to JECFA (2006) and current FCC specifications.

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Production platforms & safe 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 for enzyme manufacture 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).

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Production Strain development

- **Host Strain Selection**
  - Most productive natural expression system
  - Non-pathogenic & non-toxigenic strain – BL1
  - Multiple testing - Establish SAFE STRAIN LINEAGE
- **Modifications to the host strain**
  - knock out unwanted side activities
  - reduce survivability in nature
  - improve productivity
- **Addition of sequences to improve yield**
  - regulatory expression, secretion signals
- **Use of selectable markers**
  - Metabolic / nutritional / antibiotic resistance markers (ARM)
  - Used in strain selection, not typically in manufacture (especially ARMs)
- **Optimize the protein**
  - improve specific activity, purity
  - tailored to application conditions (temperature, pH)
  - results in lower dose – less resource wasted

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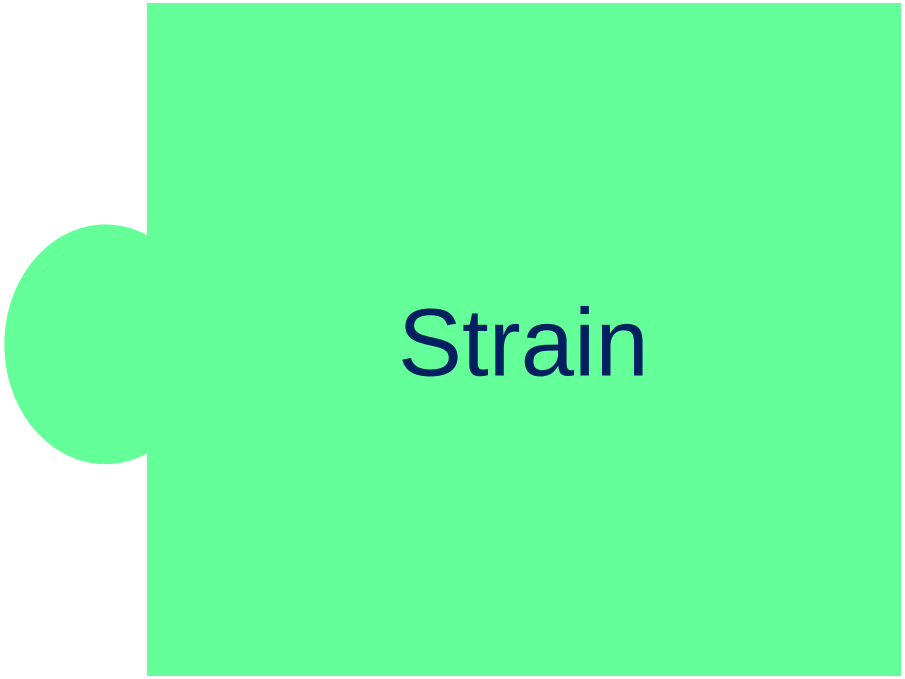
- **Strain Characterization**
  - Safety of production strain is key component to safety evaluation
    - Non-toxigenic (Non-pathogenic-usually no concern because no viable cells)
    - If the organism is safe then the ingredient produced should be safe.
  - Genetic modifications introduced into the recipient strain
    - Non-toxigenic
    - Do not encode or express any harmful substances (toxins, allergens)
  - Introduced DNA
    - Limited in size & poorly mobilizable
    - Well-characterized - sequence and donor(s)
    - Use common techniques, plasmids and selectable markers (no clinically relevant ARMs)



- Demonstration of Safe Strain Lineage
  - History of safe use in food and for production of enzymes
  - Safety demonstrated by repeated tox studies and analysis using Pariza and Johnson decision tree guidelines

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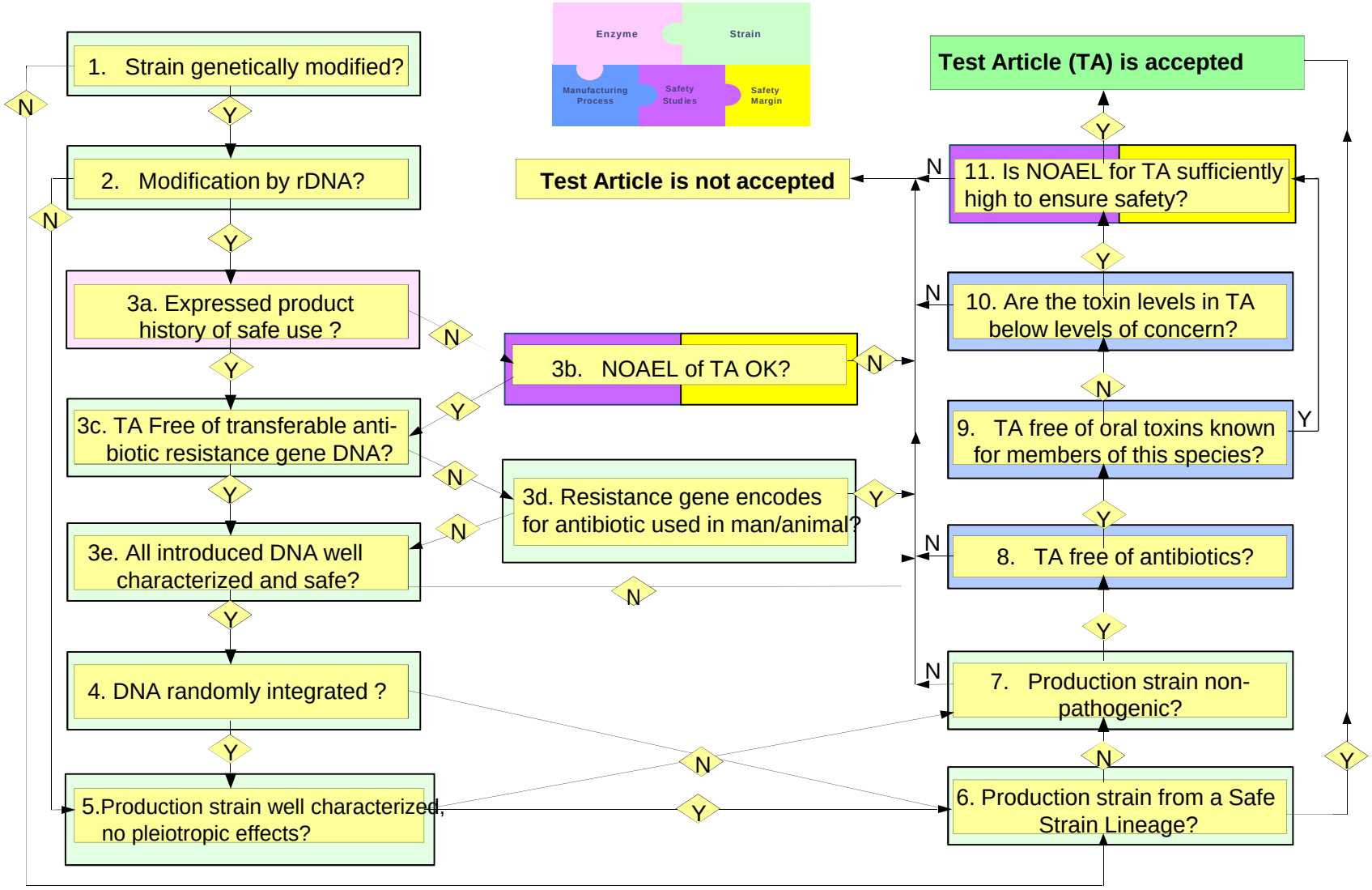
Pariza & Johnson (2001) Decision Tree



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- **Safety Studies in support of GRAS**
  - Pivotal information to be publicly available
  - Characterization of a test batch
    - Representative of the commercial products
  - Selection of toxicity data needed based on decision tree and FDA toxicology guidance, Red Book 2000

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Types of Safety Studies

Rational approaches to demonstrate safety in manufacturing, handling and intended use

Overall Safety Assessment relies on a preponderance of the data to support safety

(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.)

Ø A typical Safety matrix might include

- Genotoxicity (Ames test, chromosomal aberration)
- Allergenicity and toxin screenings
- Oral toxicity studies (rats, 7-90 days)
    - v Identify toxic dose
    - v Potential for adverse effects
    - v Determination of a NOAEL or NOEL (TOS/kg/day)

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

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- **Exposure**
  - Dosage
    - Use in application
    - Total Organic Solids (TOS) from the fermentation
  - Consumption
    - Daily consumption of food per person per kg/bw
  - Exposure
    - Estimated Daily Intake = Dosage x Consumption
- **Safety Margin**
  - Dose level with No Adverse Effect divided by the estimated human exposure
    - Conservative calculation
      - Assume enzyme used in all of the particular food
      - Assume all enzyme remains in food

Exposure & Safety Margin

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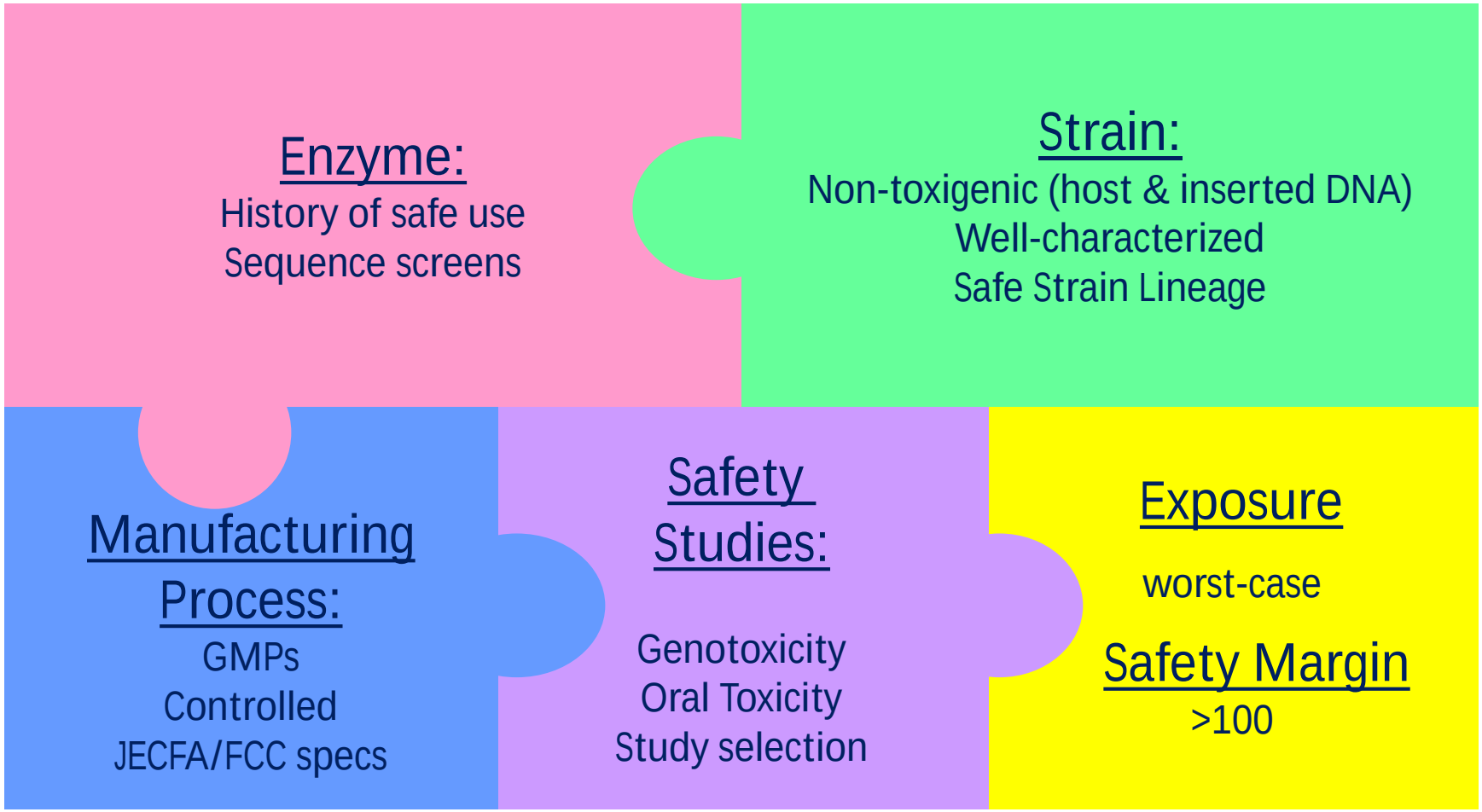
Safety Evaluation of Food Enzymes



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Fit of enzymes to GRAS process

- **Long History of safe use**
  - Use of Enzymes in food
  - Use of Production organisms in enzyme manufacture
- **Establishment of well-characterized production platforms**
  - Maximize cost-effectiveness of enzymes
  - Establish Safe Strain Lineages
- **Track record in GRAS Notification**
  - Transparency to stakeholders (FDA, Supply Chain, Consumers)
  - Facilitates down-stream & international approvals

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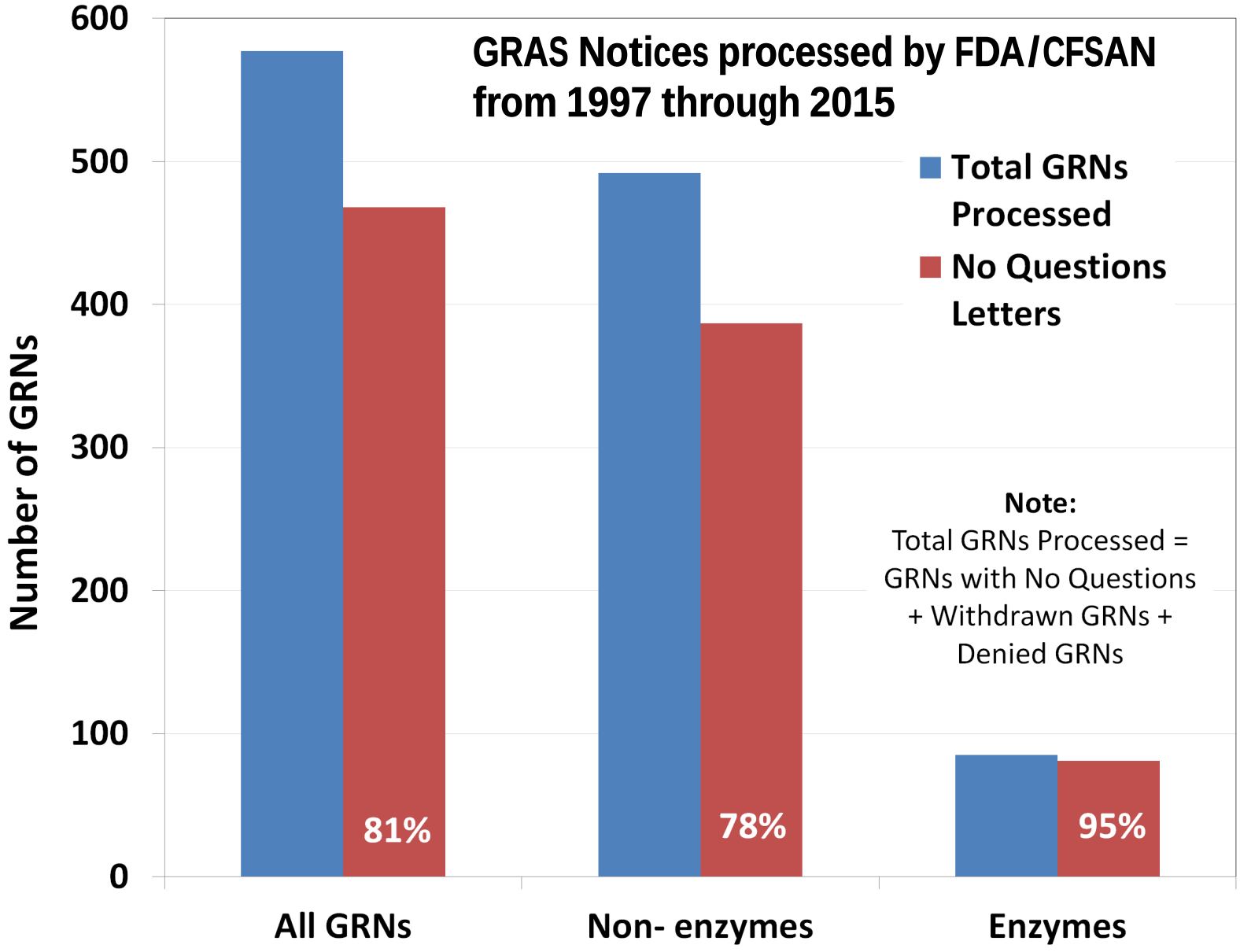

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Questions ?

Ask the Enzyme Technical Association GRAS Panel:

	Member Details	Member Topics
Moderator: Tony Pavel	Past General Counsel of ETA Sr Food Lawyer, Cargill, Inc. tony_pavel@cargill.com	GRAS definition Fit of enzymes to GRAS process GRAS Notice track record
Lori Gregg	Novozymes lobg@novozymes.com	Enzymes: A history of safety supported with data
Diane Shanahan	BASF diane.shanahan@basf.com	Enzyme identity, characterization & manufacture
Vince Sewalt	DuPont vincent.sewalt@dupont.com	Production strain development Safe Strain Lineage & Decision Tree
Jim LaMarta	DSM james.lamarta@dsm.com	Toxicology studies, exposure assessment, and overall enzyme safety evaluation

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