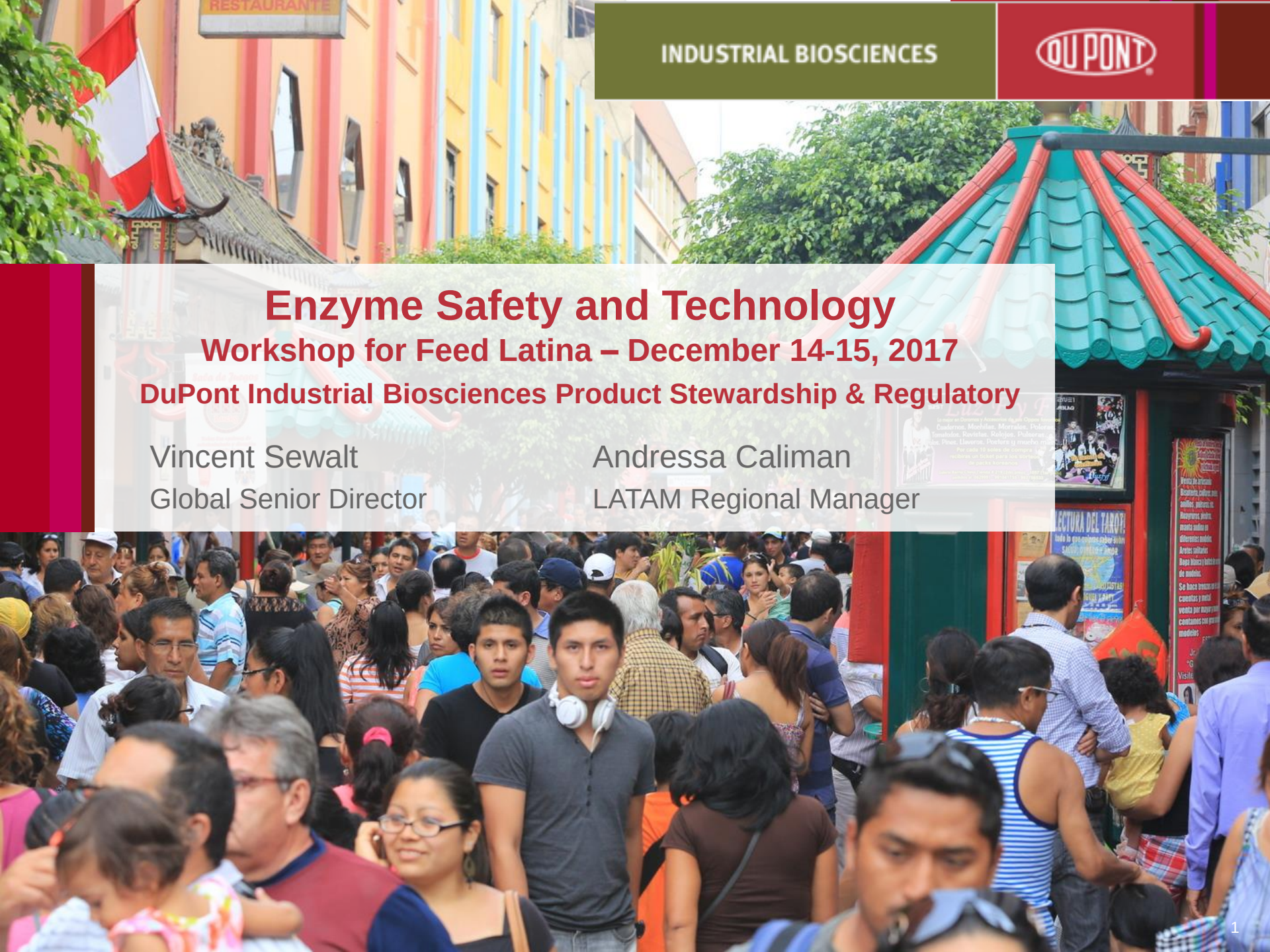

INDUSTRIAL BIOSCIENCES

DUPONT

Enzyme Safety and Technology

Workshop for Feed Latina – December 14-15, 2017

DuPont Industrial Biosciences Product Stewardship & Regulatory

Vincent Sewalt
Global Senior Director

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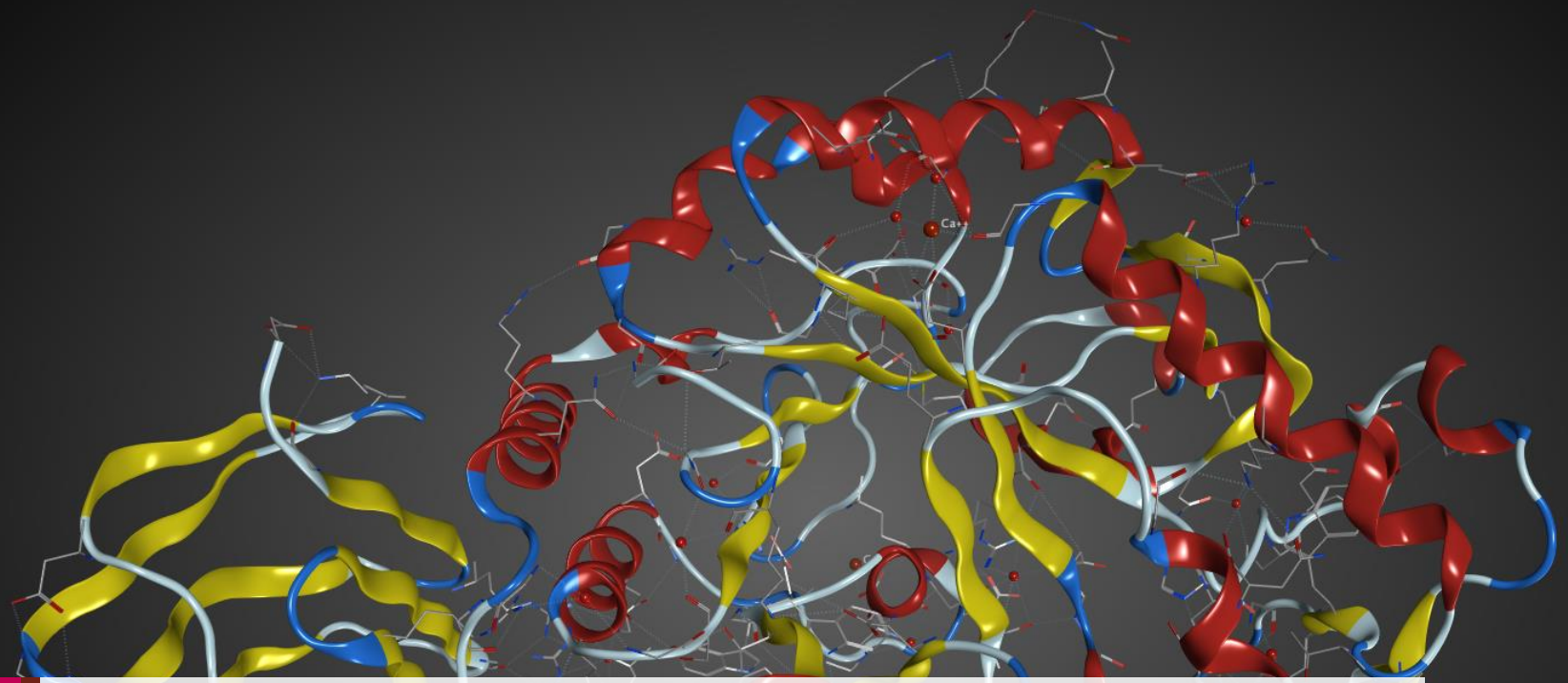
LATAM Regional Manager

Agenda

Timing	Agenda	Topics
Thursday	Enzyme Safety	1. What are enzymes (basics) 2. Enzyme uses 3. Enzyme safety evaluation
Friday	Enzyme Technology	1. What are enzymes (more detail) 2. How are enzymes developed and manufactured - Production organisms - Fermentation - Downstream processing 3. Storage and handling

Enzyme Technology

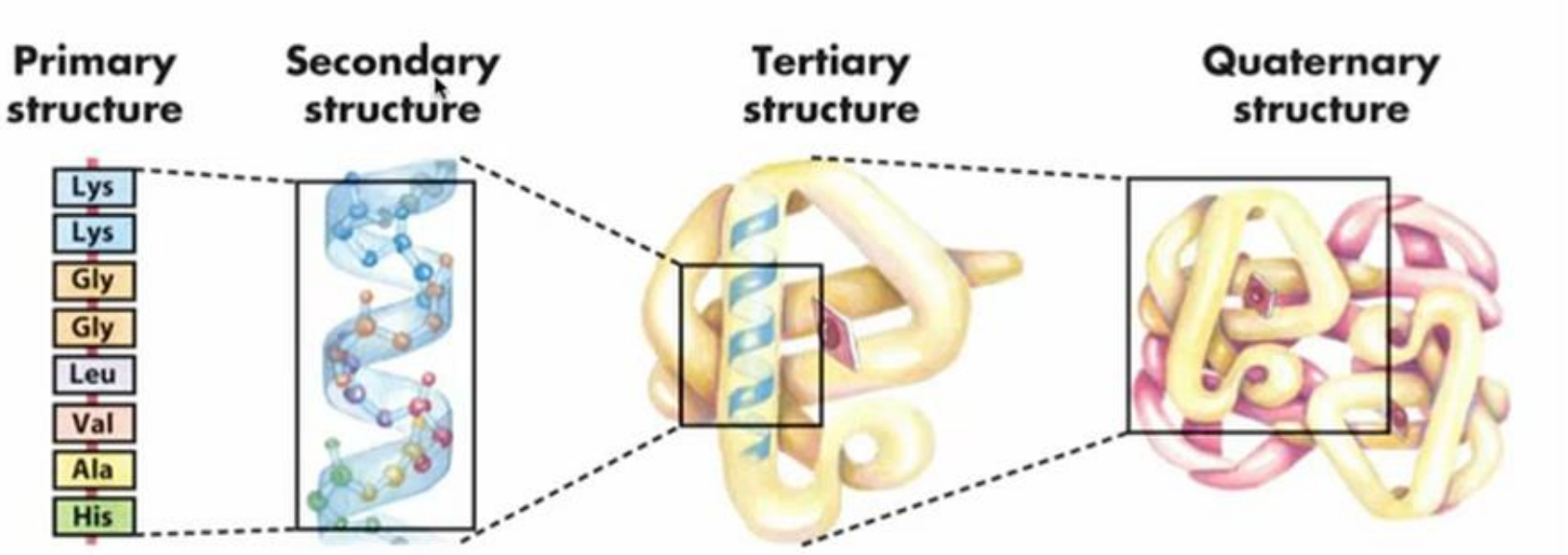
- What are enzymes (in detail)
- How are enzymes developed & manufactured?
  - » Production organisms – production platforms
    - » Safe Strain Lineages
  - » Fermentation
  - » Downstream processing
- Storage and handling



What are Enzymes ?

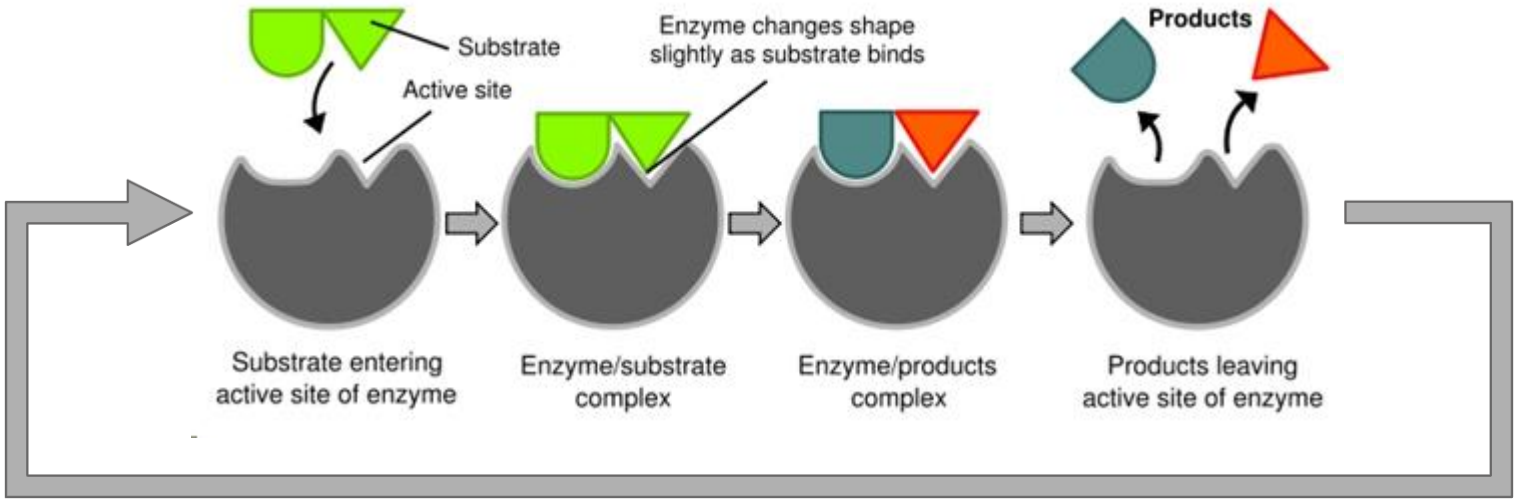
Enzymes Are Proteins

- Chains of amino-acids (20)
- Folded into a 3-dimensional structure
- Produced by plant, animals and microorganisms (naturally occurring)



Enzymes Are Natural Catalysts

- They speed up chemical reactions, lowering the energetic threshold
- They are specific (substrate + reaction)
- Required in very small amounts as they are not consumed during the reaction



Classification according to reaction catalyzed by the enzyme:

- International Union of Biochemistry and Molecular Biology
- http://www.chem.qmul.ac.uk/iubmb/enzyme/

Benefit of Using Enzymes

- Highly specific & efficient
- Versatile - enzymes catalyze wide variety of reactions
- Enzymes can be selected or optimized to operate under extreme conditions (high temperature, low pH)
- Bio-based from renewable resources
- More efficient use of resources:
  - Cost savings
  - Less environmental impact (reduced pollution, reduced waste)

Variation of Enzymes in Nature

Enzymes in nature with a given designated activity:

- can be from a wide diversity of organisms
- are naturally adapted to the environment of the host organism and therefore may have wide variation in temperature stability, salt tolerance, pH etc.

- Can have divergent amino acid sequences, but have conserved active (catalytic) site sequences
  - General safety profile is the same within a class of enzymes

Variation of Enzymes in Nature

% amino acid sequence identity	*B. amyloliquefaciens*	*B. licheniformis*	*G. stearothermophilus*	*A. niger*	*A. oryzae*	*Z. mays*	*O. sativa*	*H. vulgare*	*P. vulgaris*	*H. sapiens*
Bacillus amyloliquefaciens	100									
Bacillus licheniformis	80	100								
Geobacillus stearothermophilus	65	65	100							
Aspergillus niger	21	21	22	100						
Aspergillus oryzae	23	24	24	66	100					
Zea mays (corn)	24	26	25	28	27	100				
Oryza sativa (rice)	25	27	25	27	26	89	100			
Hordeum vulgare (barley)	25	23	24	25	28	70	69	100		
Phaseolus vulgaris (bean)	26	27	25	24	27	67	65	64	100	
Homo sapiens (human)	25	33	29	22	28	23	22	23	24	100

α-amylases in nature have divergent

amino acid sequences but have the same catalytic activity and IUBMB number

α-amylase catalytic amino acids are highly conserved

	Region 1	Region 2	Region 3	Region 4
B.licheniformis	DVVINH	GFRLDAVKH	EYWQ	FVDNHD
B.amyloliquefaciens	DVVLNH	GFRIDAAKH	EYWQ	FVENHD
G.stearothermophilus	DVVINH	GFRLDAVKH	EYWS	FVENHD
A.oryzae	DVVANH	GLRIDTVKH	EVLD	FVENHD
H.vulgare	DAVINH	GFRIDASKH	EVID	FVDNHD
H.sapiens	DAVFNH	DGRLDWGPH	EVWD	FVDNHD

Properties of α-amylase from different species

Source Organism	*P.woesei*	*G.stearothermophilus*	*H.vulgare*	*P.haloplanctis*
optimum growth temperature	97-100 °C	55 °C	24 °C	20-25 °C
enzyme stability	stable at 98°C	$T_{1/2}$ (90°C): 50 min	$T_{1/2}$(60°C) 26 min	T_m = 44°C

How Are Enzymes Made?
-Production Organism

What Are Some Requirements for Enzyme Production?

- Enzymes produced for industrial scale need to:
  - Be stable
  - Be sufficiently pure
  - Have desirable activity adapted to the application
  - Be produced in high yields in shortest possible fermentation time

- Crucial to select the right microorganism to do the fermentation
  - Does it produce the right activity?
  - Is it adapted to the desired application conditions (pH, Temp)?
  - Is it productive at scale?
  - Is it SAFE ?

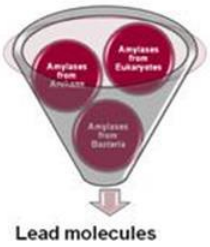
- Once selected, repeated use allows for:
  - Strain optimization for productivity
  - Safe Strain Lineage development

Why Microbial Enzymes?

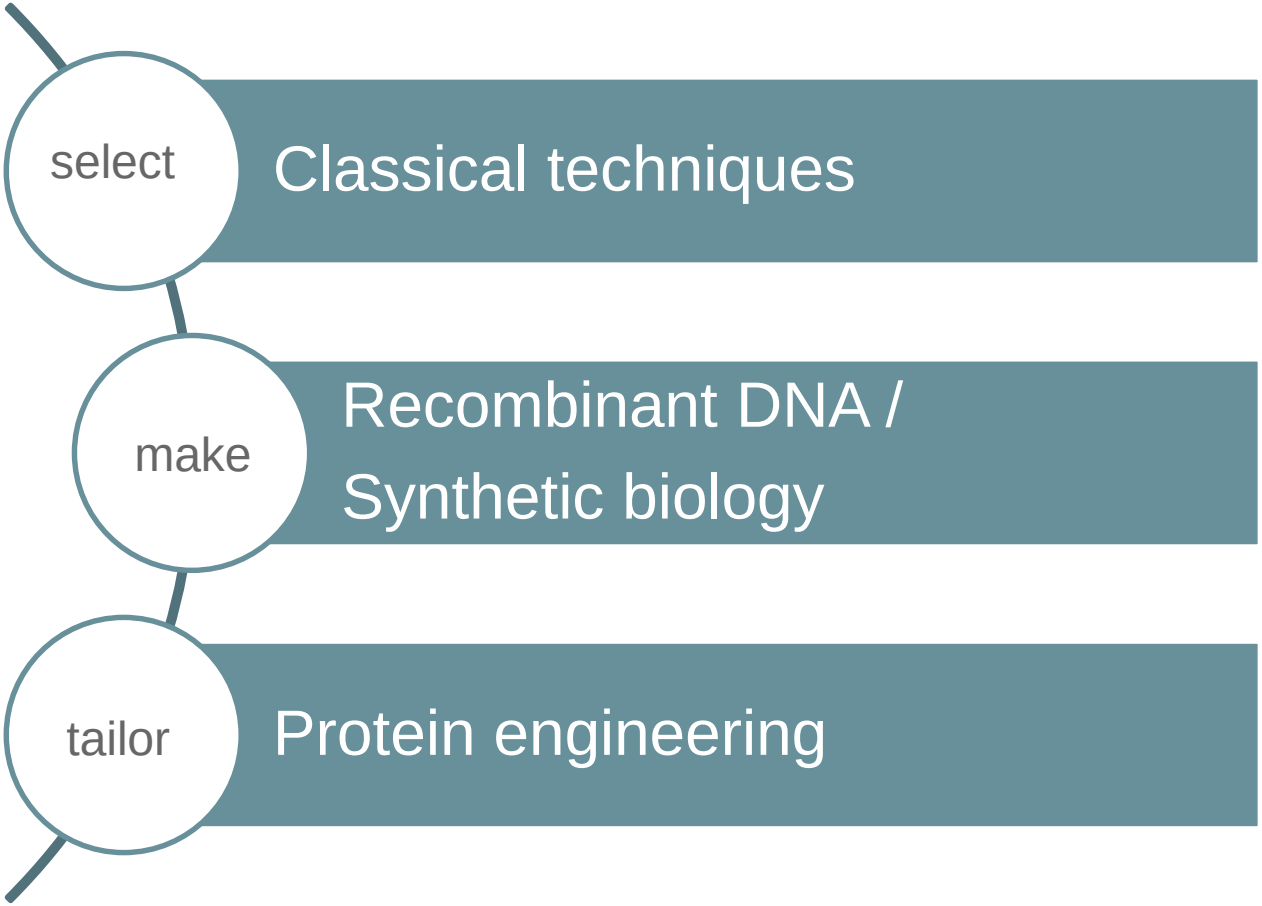
- Sourced from bacteria, fungi and yeast
- Preferred over the enzymes sourced from plants and animals because
  - ✓ Can tailor the enzyme to the application
  - ✓ More controlled process
  - ✓ More consistent in quality
  - ✓ More cost-effective to produce
  - ✓ Safer
- Well-established published safety evaluation procedures for enzymes produced with modern biotechnology, which take into account the enzyme, the production organism, the manufacture process, and safety studies:
  - Pariza & Cook (2010); Sewalt et al. (2016)

The four steps of enzyme innovation

1. **Discovery of** new enzymes
2. **Applications** research to determine their value potential
3. **Protein Engineering** of the best enzymes to improve them / adapt them to industrial conditions
4. **Producing** these enzymes at commercial cost structure through engineered bacterial / fungal strains, fermentation / recovery / formulation processes



Approaches to the Development of Enzyme Products



Development of Enzyme Products Using Classical Techniques

- Wild-type strains
  - » natural source of enzyme
  - » nonpathogenic and non-toxigenic
  - » commercial use requires
    - ability to grow on industrial scale
      - **large fermentation tanks**
      - **low cost, bulk media**
    - mutagenesis to improve enzyme expression
      - **treatment with chemicals**
      - **treatment with irradiation**

Limitations of Classical Techniques

Classical Techniques

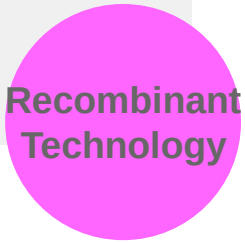
- Production Strain
  - limited number of strains able to grow on industrial scale
  - limited improvement in enzyme expression by random mutagenesis
  - Limited control over introduced changes – large screening effort for little ‘gain’
- Enzyme protein
  - available natural variations result in limits to characteristics, such as pH optimum, temperature stability
  - desired combination of characteristics found in nature is limited.

Benefits of Recombinant Technology

Recombinant Technology

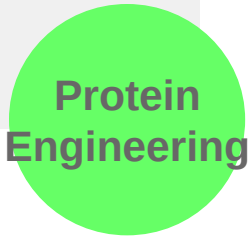
- Use established host strain – ‘domesticated’
  - » safe strain - history of safe use or scientific studies
  - » demonstrated ability to grow on industrial scale
- Increased enzyme yields - better use of all resources
  - » raw materials, energy, water, land,
  - » Reduced waste, CO_2 and other greenhouse gas emissions
- Enables commercialization of wide diversity from nature
  - » enzymes from unculturable microbes
  - » microbes with low enzyme yields (not economically viable)
- Ability to define and control change
  - » increased purity e.g. removal of side activities
  - » optimized protein sequence
- Continued use of safe microbial strains as production platforms allow for the establishment of **Safe Strain Lineages**

Development of Enzyme Products Using Recombinant Technology



- Heterologous expression of enzymes
  - » find the gene sequence coding for enzymes with desirable characteristics (“Donor species”)
  - » introduction into a selected microbial strain serving as ‘expression host’, which becomes the ‘production organism’
- Addition of sequences to improve yield
  - » regulatory expression
  - » secretion signals
- Targeted knock out of host strain genes with side activities
  - » sequences of concern if any
  - » maximize production of enzyme of interest

Benefits of Protein Engineering



- Protein tailored to a specific application
  - » enzyme products have greater specificity
  - » performance optimized e.g. pH, temperature, binding
- More efficient way to mimic natural diversity
- Changes controlled and defined
- Changes do not alter basic characteristics, classification, or safety

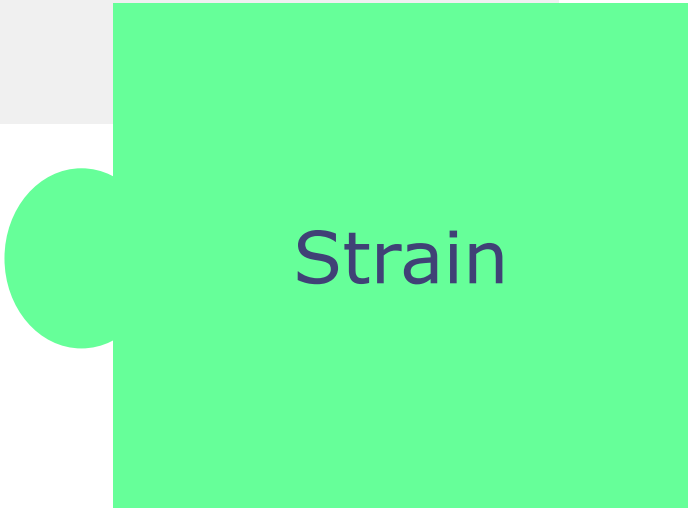
Development of Enzyme Products Using Protein Engineering

Protein Engineering

- Single amino acid changes
  - Published and patented technologies
  - One or more amino acids along the native protein
  - 20 possibilities at any amino acid site
  - Controlled, targeted and accurate
  - Occurs in nature – protein evolution is a natural process, protein engineering is a targeted &. accelerated version

1. Strain Characterization

- Safety of production strain is key component to safety evaluation
- Non-toxigenic
- Concept → If the production organism is safe then the ingredient produced is safe.



2. Genetic engineering of host

- Non-toxigenic
- Do not encode or express any harmful substances

3. Introduced DNA

- Well-characterized
- Use common techniques
- Description of source for expressed gene
- Well-known plasmids and selectable markers

(no transferable antibiotic resistance markers of clinical relevance)

4. <u>Demonstration of Safe Strain Lineage</u>

- History of safe use in food and for production of enzymes
- Safety demonstrated by repeated tox studies and analysis using decision tree guidelines

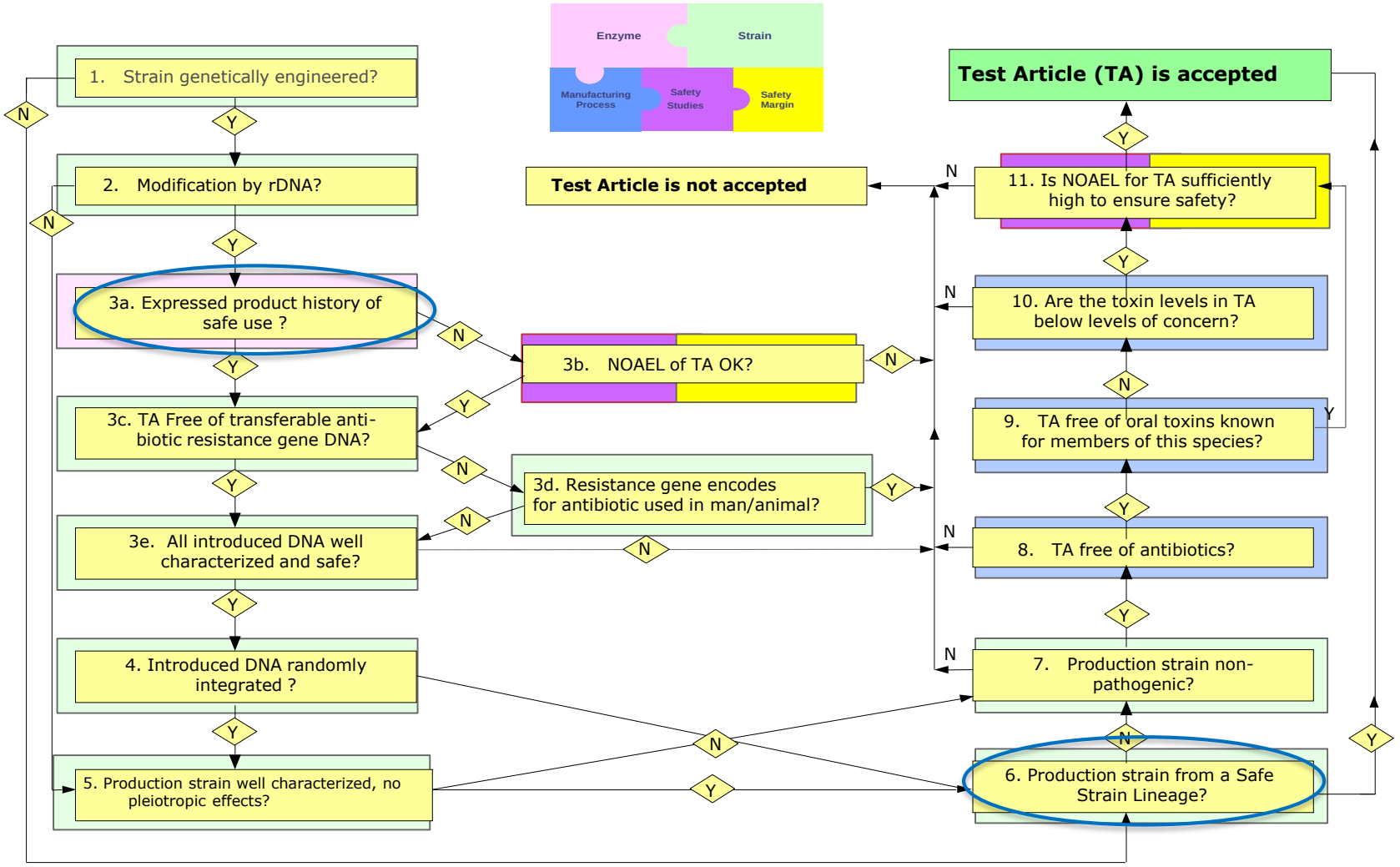
Definitions: back to basics

GM vs GE production organisms;
PE enzymes

Enzymes are GMO? → NOT

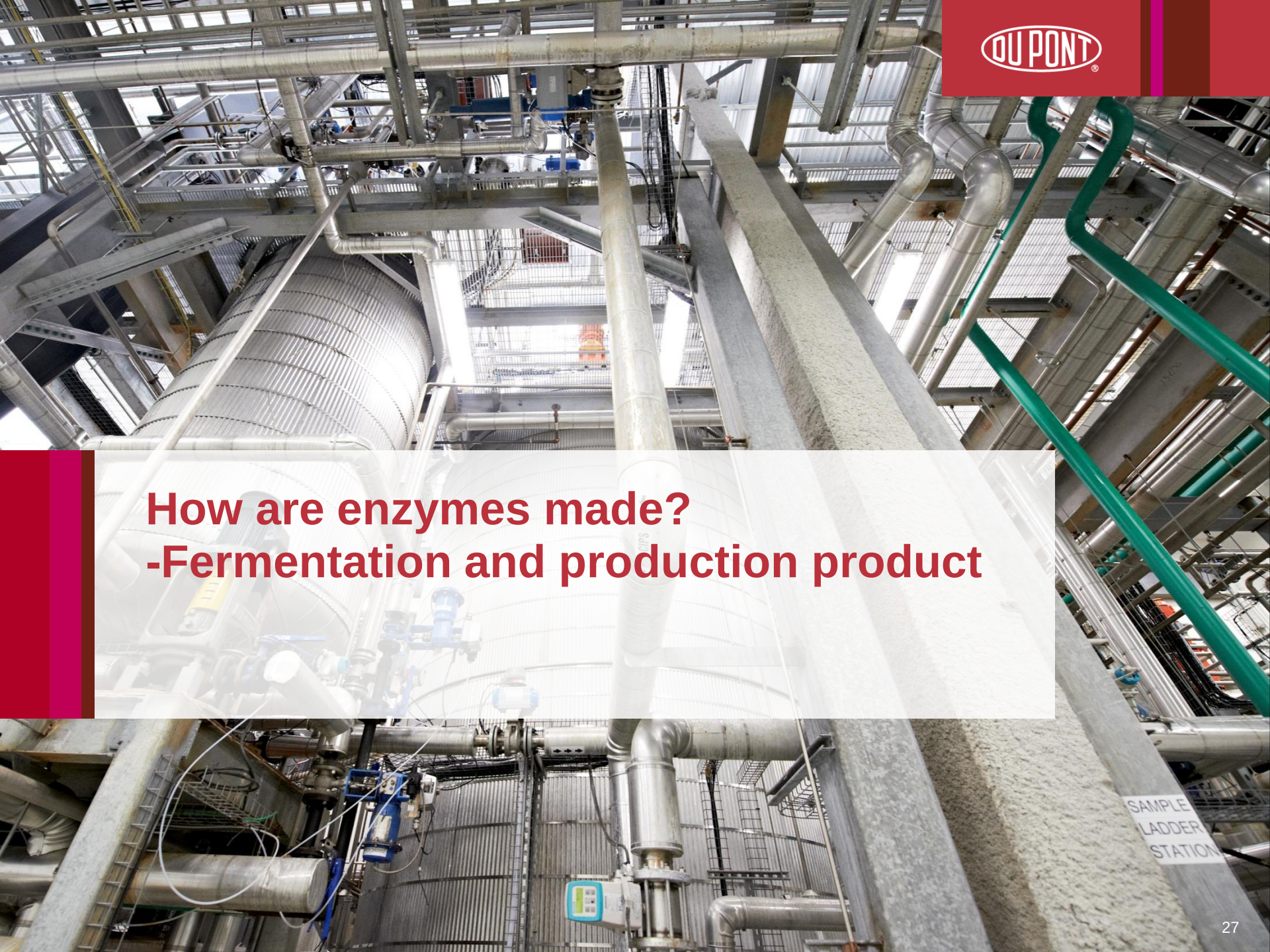
- The term ‘genetic modification’ is confusing – in North America this includes mutagenesis & selection, while in the EU it does not.
- The Pariza & Cook decision tree uses ‘rDNA manipulation’ as a term that is narrower than genetic modification.

Pariza & Cook (2010)
Enzyme Safety Evaluation Decision Tree



Enzymes are GMO? → ¡ NO !

- The term ‘genetic modification’ (GM) is confusing
  - » in North America ‘GM’ includes mutagenesis & selection
  - » in the EU it does not.
- The Pariza & Cook decision tree uses ‘rDNA manipulation’ as a term that is narrower than GM.
- US and Canadian regulators refer to rDNA manipulation as ‘Genetic Engineering’ (GE).
- Note that the enzyme itself is not - and cannot be - a Genetically Engineered Microorganism (GEM).
  - enzymes are substances and not organisms.
  - incorrect to refer to ‘GMO enzymes’ or ‘GM enzymes’ or GE enzymes
  - For enzymes we can say they are produced with a GMM or GEM
- Enzymes CAN be ‘protein-engineered’ (PE)



How are enzymes made?
-Fermentation and production product

Fermentation Processes Are Built from the Ground Up

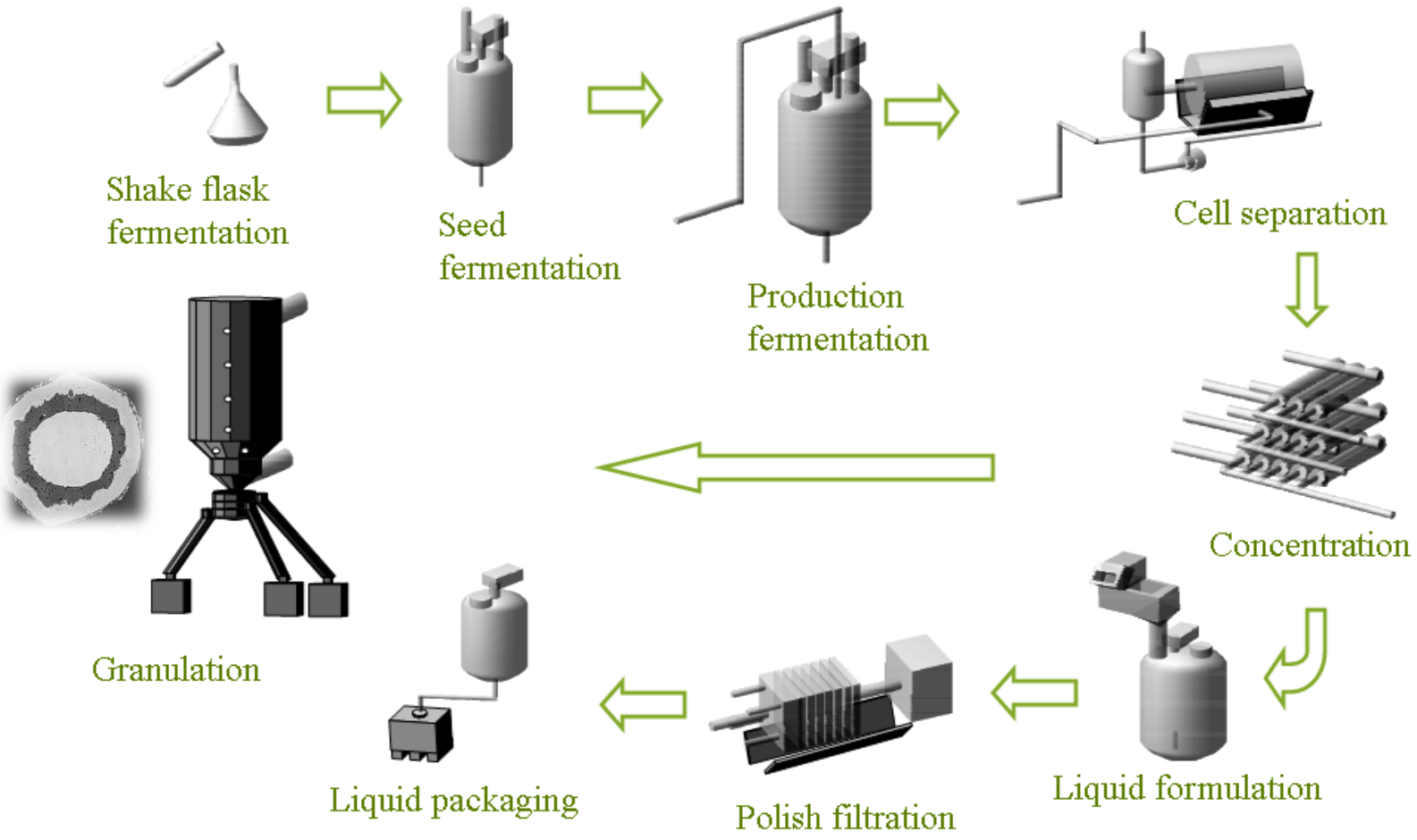
5 mL

250 mL

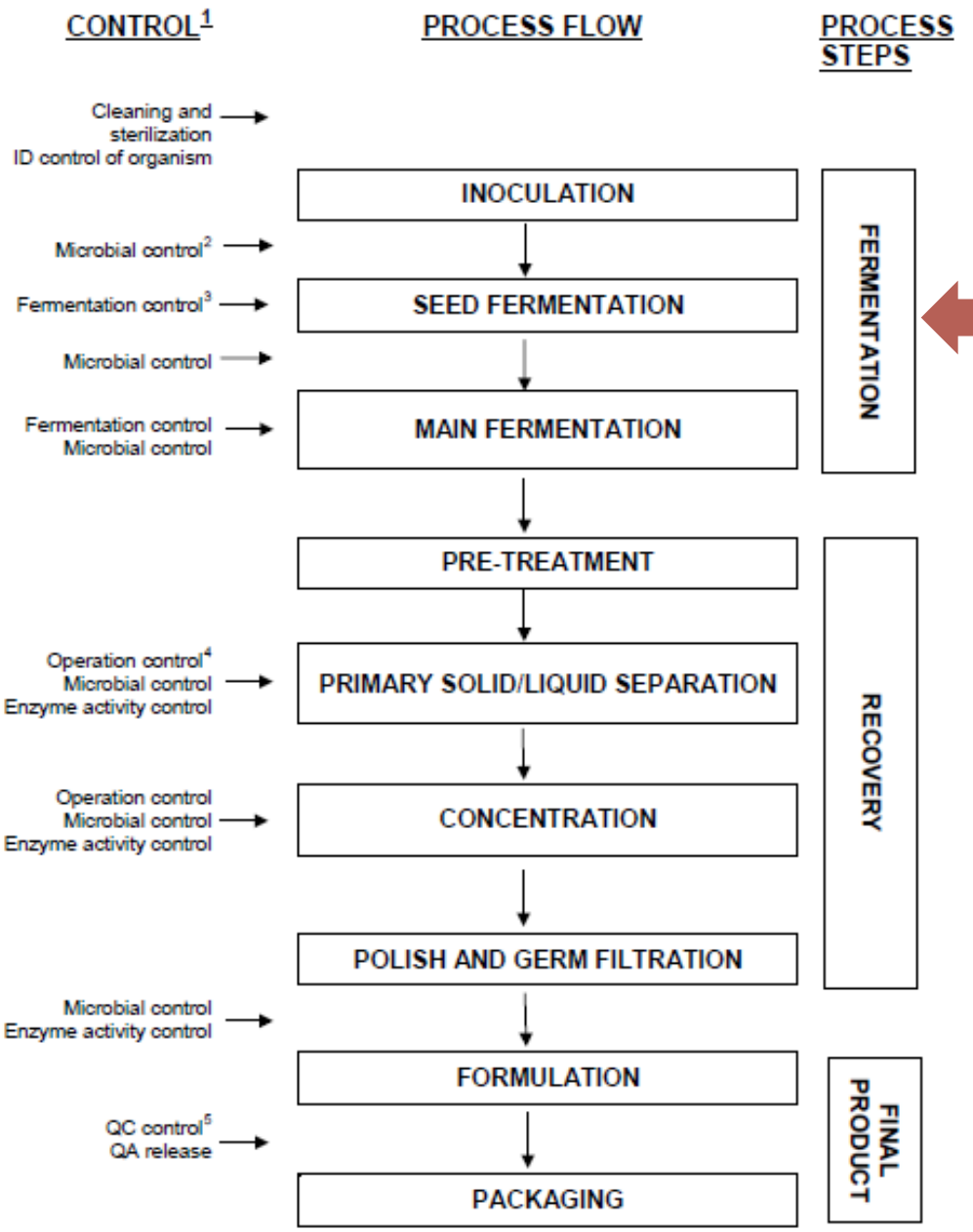
15 L

> 100,000 L

Manufacture of Enzymes



Production Process of Food enzymes from fermentation

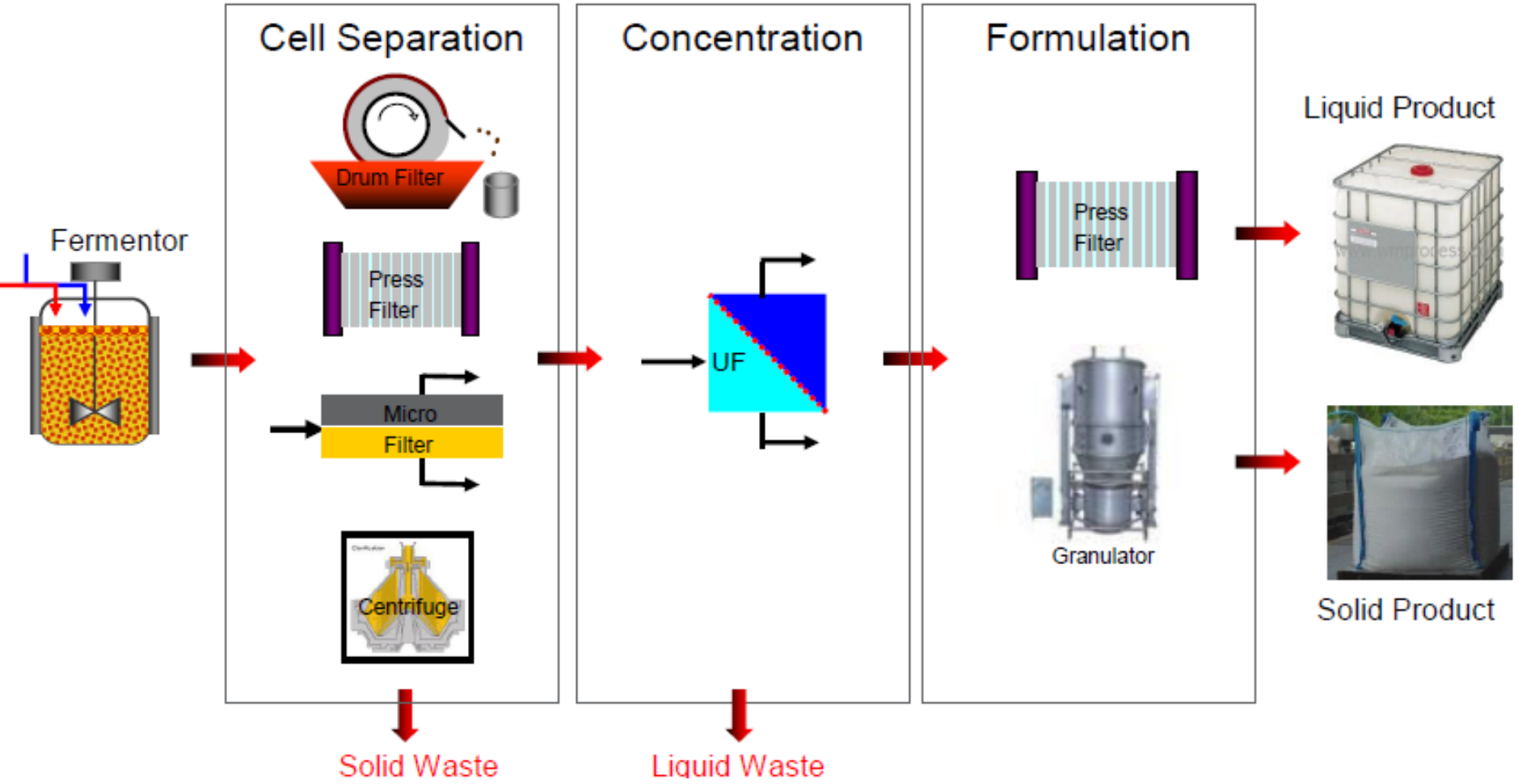


Carbons sources: Glucose syrup, Sophorose, Soy …

Nitrogen sources: Soy, Corn steep, ammonium salts …

Minerals: $MgCl_2$, NaCl…

Typical Industrial Enzyme Production Process



Finished Product - Requirements

- Enzyme preparation does not contain antibiotic activity
  (global requirement & product specification)

- GE production strain is not present in the finished product
  (global requirement, also for IP reasons). Test available for confirmation:
  - ❑ Production strain report

- Residual cell debris is not present
  requirement for exemption from formal regulation on Genetic Engineering in several jurisdictions (e.g., EU, Canada, Brazil).

- Note that the enzyme itself is not - and cannot be - a Genetically Engineered Microorganism.
  - enzymes are substances and not organisms.
  - it is not correct to refer to ‘GMO enzymes’ or ‘GM enzymes’

Enzyme Preparation

Enzyme preparation

= product that is actually sold / registered

Enzyme concentrate

= “feed enzyme”
Usually the subject of approval (positive list)

Enzyme protein

Pure substance

Laboratory

White crystals

Fermentation extract

Enzyme factory

Brown liquid

Formulation

Food manufacturers

Liquid or granulate

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PRODUCT DESCRIPTION - PD 236924-2.1EN

Enviva® Pro 201 GT

Direct Fed Microbial

Application

Enviva® Pro 201 GT is a probiotic feed additive for poultry diets.

Usage levels

Use at a rate of 0.5-2 lbs/short ton (US) or 0.25-1 kg/tonne (0.025-0.1%) of finished feed, included either directly or via a premix.

Processing stability

For optimum bioefficacy, do not exceed conditioning and pelleting temperatures of 95°C (203°F).

granular product.

Storage

Store in dry conditions. The active constituents will remain stable for at least 24 months when stored in original packaging at <25°C (77°F) and 6 months when included in a vitamin/mineral premix and stored at <25°C (77°F).

Packaging

Enviva® Pro 201 GT is available in 25kg foil lined paper bags.

Safety and handling

Provide for good ventilation and avoid dust formation. It is recommended to use protective glasses, respiratory mask and gloves during handling. In case of accidental contact with skin or eyes, the only action needed is copious flushing with water. See the Material Safety Data Sheet for further information.

Additional information

Storage

Store in dry conditions. The active constituents will remain stable for at least 24 months when stored in original packaging at <25°C (77°F) and 6 months when included in a vitamin/mineral premix and stored at <25°C (77°F).

Safety and handling

Provide for good ventilation and avoid dust formation. It is recommended to use protective glasses, respiratory mask and gloves during handling. In case of accidental contact with skin or eyes, the only action needed is copious flushing with water. See the Material Safety Data Sheet for further information.

DFM technology development

Identify the problem

Identify optimal strains

Screening studies

- Identify specific bacteria

In vitro studies

- Identify basic & functional properties of strains

In vitro studies

- Test strains through in-vitro model systems

In vivo studies

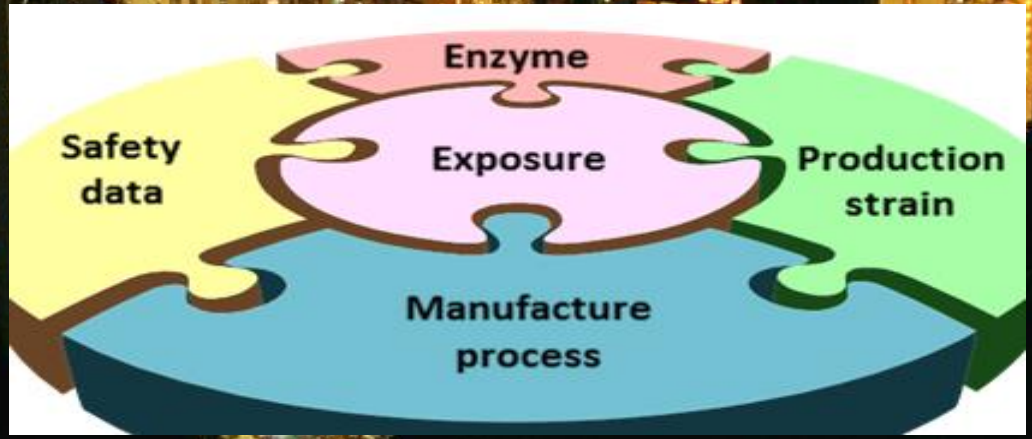
- Test strains in birds
- Pen trial
- House trial
- Regional trial

Demonstrate benefits

✓Helps establish a balanced gut microflora

✓More profitable animal performance

20,000 potential strains → ***Candidate strains***


Enzyme
Safety data
Exposure
Production strain
Manufacture process

Thank You!