

1. What is the significance of this research topic?

Ancient Egyptians (7000 BC) used enzymes in the production of bread, yogurt, and cheese. Our own body produces enzymes to facilitate digestion. Enzymes are specialized proteins acting as catalysts to speed up biochemical reactions or to perform specific metabolic reactions. They show up in a wide range of applications including production of beverages, infant foods, fish meal, cheese and dairy product, sweeteners, chocolate syrups, bakery products, fruit juice, soft drinks, vegetable oil, candy; in short, they are omnipresent in the food industry. Initially, the production of enzymes has been from selected strains, derived from a small number of microorganisms primarily *B. subtilis*, *B. licheniformis*, *A. niger* and *A. oryzae*. Over time, new type of microorganisms has been added, such as *E. coli K-12*, *F. venenatum*, and *P. fluorescens*. As the food processing industry became more complex, the demand for efficient production of enzymes with well-defined characteristics increased. In response, improved recombinant DNA techniques and other developments in biotechnology, such as protein engineering or *directed evolution*, have revolutionized the commercialization of enzymes. Today most enzymes are recombinant enzymes. In 2021, the industrial enzyme market has been valued at over USD 6Millions.

The microorganisms, used for the recombinant strains, are recognized as nonpathogenic, but research is still on going to study whether they are nontoxigenic. It has been established that some bacteria, may produce low levels of toxic secondary metabolites. In addition, several host microorganisms generate different extracellular enzymes which can degrade produced enzymes, with undesirable reactions in food. The toxicologic potential risks presented by the host strains are:

- **For bacterial host strains:**

- *Bacillus subtilis* and similar bacteria: the wild-type (WT) of *Bacillus* species can sporulate or produce extracellular proteases which can degrade the enzyme protein.
- *Escherichia coli K-12* and *P. fluorescens* can accumulate heterologous inclusion bodies which could be eliminated during the purification process.

- **For fungal host strains:**

- Certain strains of *A. oryzae* can produce low-levels of mycotoxins with low-to-moderate toxicity. Strain A1560 has shown to produce low levels of various acids (3- β -nitropropionic acid, kojic acid, and cyclopiazonic acid) under inducing conditions.
- Some *A. niger* strains produce several mycotoxins (like ochratoxin A) and secondary metabolites under specific fermentation conditions (nigragillin, nigerazine B, malformins, naphtho- γ -pyrones, and oxalic acid).
- *Fusarium venenatum* descendant of the WT strain A3/5, can produce mycotoxins (trichothecenes, culmorins, fusarins, and enniatin B).
- *Trichoderma reesei* is used in baking and alcohol production. A strain of *T. reesei* produced two metabolites, one identified as trichodermin, a trichothecene mycotoxin.

Advances in molecular biology, such as the development of DNA insertion techniques using expression vectors or cassettes, have created from production strains more efficient for the food industry, and safer enzymes which do not affect secondary metabolite pathways.

2. Who is working in this area?

- The major American and European food industry companies have R&D using genome sequencing and biochemical platforms to create enzymes: CHR HANSEN, Cargill, Biocatalysts, Clariant, Codexis, DSM and their European counterparts.
- In Academia, departments of Biochemistry and Molecular Biology are involved in various research projects directly or indirectly related to recombinant enzymes. One of them is the Arnold Group at California Institute of Technology (<http://fhalab.caltech.edu/>). Frances Arnold has received The Nobel Prize in Chemistry in 2018 for her work on directed evolution of enzymes.

3. What methods are used to study the concepts described in the paper?

- To evaluate the toxicity of host strains, their complete genome sequences, were determined and compared to existing pathogens to find similarities. For example, genome sequences of two *B. licheniformis* strains were compared to the sequences of two human pathogens: *B. cereus* and *B. anthracis* and found them homologous but distinct. In addition, the same strains were exposed to these pathogens; no antibodies from *B. licheniformis*, were observed.
- Animal models or cell cultures (mice, sheep's blood agar, mouse neuronal cell culture), were, also exposed to bacterial, or fungal host strains and evaluated for pathogenic signs.
- Molecular analysis of specific gene clusters were conducted to verify the production of toxins like in *A. oryzae*, in which genetic mutations were found preventing aflatoxin production.
- Different microorganisms were cultivated in media conducive to the synthesis of acids like kojic acid or cyclopiazonic acid or mycotoxin production.
- Some strains were also tested under conditions of industrial productions.
- Recombinant DNA technology has been used to create enzymes with desired properties. The gene encoding recombinant enzymes, is introduced into the host strains using expression vectors. The expression vector is a DNA plasmid that carries the expression cassette, which includes a promoter, the gene encoding the enzyme and a terminator. The promoter and terminator that control the transcription of the encoding gene, are derived from genes native of the host microorganism or related species. Bacterial cells with the desired enzyme are selected using antibiotic resistance marker or by complementation of an auxotrophic mutation with the functional gene to be expressed ¹. In addition, screening genetic techniques helped to discover or produce new enzymes from the environment host themselves:
 - Gene expression libraries have been created to identify in host microorganisms, enzymes with specific properties.
 - Directed evolution generates combinatorial libraries of enzymes by sequential random mutagenesis. Then it uses high throughput exploratory tools to identify enzymes with the desired characteristics.

¹ In some cases, the expression cassette and the marker gene are carried by two separate vectors which are conjointly used in the host strain. The enzyme-encoding gene can also be induced at a desired cell growth by a promoter. In addition, the expression cassette may be directed into specific loci to replace a host gene. Research has also created more complex promoters like inducible promoters that are activated by addition of an inducer to the fermentation medium.

4. Does the review article lead to new questions or hypotheses in this technical area (by the authors, by other researchers)?

The authors of the article emphasize that trend in the development of safer and with higher yield enzymes will intensify. Other researchers have expressed concerns about spreading the modified genes beyond the targeted encoding area, increasing the risk of tumors or in the case of plants the contamination of our biodiversity if engineered plants cross-breed with WT plants.

5. What are some practical applications of the research discussed in the article?

- One application of recombinant DNA (rDNA) technology has been to develop nonpathogenic and nontoxic microbial strains. For example, *F. venenatum* which is used for expression of xylanase, can produce trichothecenes. The MLY3 strain, was created by deleting from *F. venenatum*, the *tri5* gene encoding trichodiene synthase, blocking the production of trichothecenes.
- Genetic engineering also, has created bespoke enzymes with specific properties to food-processing conditions such as temperature or pH. As an example, DNA sequence modifications were made to the α -amylase from *B. licheniformis*, by replacing its amino sequence with the corresponding sequence from a *B. amyloliquefaciens* α -amylase and by introducing five additional amino acid substitutions to make the mutated enzyme active at low pH.
- In addition, rDNA techniques have improved industrialization of recombinant enzymes themselves. For example, the selectable marker *amdS* has been replaced with *URA3* gene which complements the *pyFR* mutation in the E. coli strain which allows to select mutated cells without the use of antibiotic resistance genes and uridine for growth.

6. How does this topic relate to other areas of cell biology, bioengineering, or medicine?

- **Medicine:** in human body, high levels of α -amylase can be indicative of severe medical conditions including acute inflammation of the pancreas, or perforated peptic ulcer. Lipases are used as source of new drugs, as digestive aids, or in the treatment of malignant tumors.
- **Transgenic animals (Tg):** animals with specific engineered genetic characteristics are used in studies about cells, or cellular functions.
- **Antibiotics, vaccines:** rDNA helps to improve their efficiency and production.
- **Use in Diagnosis:** Enzymes are used as drug targets, or marker enzymes. Acute pancreatitis and pancreatic injury can be determined by the level of lipases in blood. A test has been developed using lipases to measure canine pancreatic lipase.
- **Treatment of Damaged Tissue.** Proteolytic enzymes of plant and bacterial have been studied for the removal of dead skin of burns.
- **Treatment of diseases:** Lysozyme has also been found to have activity against HIV.
- **Treatment of Cancer.** Arginine-degrading enzyme (PEGylated arginine deaminase) can inhibit human melanoma and hepatocellular carcinomas, also, removal of chondroitin sulfate proteoglycans by chondroitinase AC and, to a lesser extent, by chondroitinase B, stops tumor growth, metastasis, and neovascularization.[1]

- [1] N. Gurung, S. Ray, S. Bose, and V. Rai, "A Broader View: Microbial Enzymes and Their Relevance in Industries, Medicine, and Beyond," *Biomed Res Int*, vol. 2013, p. 329121, 2013, doi: 10.1155/2013/329121.