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.orizae*. 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-charactered characteristics increased. In response, improved recombinant DNA techniques and developments in biotechnology, such as protein engineering and 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:

- ***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 are eliminated during the purification process.
- ***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- nitroproprionic acid, koji acid, and cyclopiazonic acid) under inducing conditions.
- *Some A.niger* strains produce several mycotoxins (ochratoxin) and secondary metabolites under specific fermentation conditions (nigragillin, nigerazine B, malformins, naphto-γ-pyrones, and oxalic acid).
- *Fusarium venenatum* descendant of the WT *strain A3/5* from can produce mycotoxins (trichothecenes, culmorins, and fusarins, and enniatin B).
- *Trichoderma reesei* is used in baking and alcohol production. A strain of T.reesei produced two metabolites, one identified as trichothecene mycotoxin.

Advances in molecular biology, such as expression vectors or cassettes, have allowed to create more efficient and safer enzymes from production strains including development of DNA insertion techniques 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?
The gene encoding recombinant enzymes are 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. For gene encoding in Bacillus strain, the plasmid can carry two other genes, one encoding the primary replication initiation protein (ORF alpha) and the other encoding the mobilization protein (ORF beta) to enable gene expression from one strain to the other. 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. Multiple copies of the encoding gene also, can be introduced within a cell. The expression cassette may be directed into specific loci to replace a host gene, or be added in the proximity of the host gene to create the desired enzyme property. Research has also created more complex promoters like inducible promoters that are activated by addition of an inducer to the fermentation medium.
Vector DNA are transferred using conjugation, electroporation or vector incubation with protoplasts. 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. In addition, screening genetic techniques have been used to discover or produce new enzymes from the environment samples:

- 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 and scores each enzyme protein with a “fitness” score based on the level of its performance in a specific task. Then it uses high throughput exploratory tools such as Machine Learning (ML) technologies to search efficiently and effectively the sequence space to identify enzymes with the desired characteristics.

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

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 nontoxigenic microbial strains. For example, *F.venenatum* which is used for expression of xylanase, can produce the mycotoxin, trichothecenes. The MLY3 strain, was created by deleting from *F.venenatum*, the *tri5* gene encoding trichodiene synthase, the enzyme that catalyzes the first step in the trichothecene pathway. Subsequent tests show that this strain or variants lost its ability to produce trichothecenes.
Genetic engineering also has created bespoke enzymes with specific properties to food-processing condition 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?