

MICROBIAL BIOTECHNOLOGY

Prof. Utpal Bora
Department of Biosciences and Bioengineering
Indian Institute of Technology Guwahati

Lecture-30

Lec 30: Health Benefits, Risks and Advances in Food Fermentation

Hello friends, welcome to my course on microbial biotechnology. We are in module number 9 where we are discussing food production involving microorganisms and their products. In today's lecture we are going to discuss about the health benefits, risks and advances in food fermentation. The lecture is broadly divided into two sections. In the first section, we will discuss about health impacts and advances in fermentation.

And in section two, we will be discussing about the precision fermentation. So let us straight away go to section number one, which is further divided into health benefits and risks of fermented foods and modern biotechnological advances in fermentation. So let's have a small discussion on probiotics and gut health. Probiotics maintain gut balance by promoting beneficial bacteria and inhibiting harmful pathogens. So they are very, very important for our health.

Content

Section 1: Health Impacts and Advances in Fermentation

Health Benefits and Risks of Fermented Foods

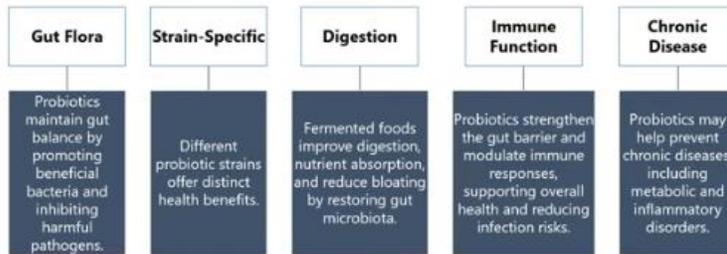
- Probiotics and Gut Health
- Nutritional Value
- Potential Risks

Modern Biotechnological Advances in Fermentation

- Industrial Fermentation Techniques
- Genetic Engineering
- Strain Improvement of Lactic Acid Bacteria (LAB).
- Exploration of Synthetic Biology in Food Fermentation
- Emerging Trends and Innovations of Engineered Food Fermentation

Different probiotic strains offer distinct health benefits, so the beneficial properties are actually strain specific. It helps in digestion, fermented foods improve digestion, nutrient absorption and reduce bloating by restoring gut microbiota. It has also an immune function. Probiotics strengthen the gut barrier and modulate immune responses, supporting overall health and reducing infection risks. It can also help us in addressing chronic diseases.

PROBIOTICS AND GUT HEALTH



(“Advances in Fermented Foods and Beverages” 2015)

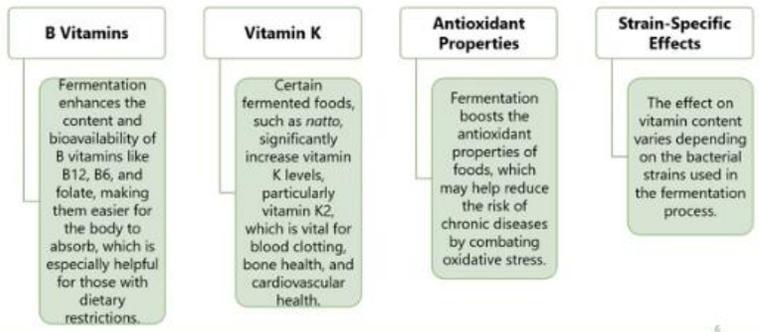
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Probiotics may help prevent chronic diseases, including metabolic and inflammatory disorders. Now, if we want to describe what probiotics are, they are live microorganisms, mainly bacteria, that offer health benefits when consumed in sufficient amounts. They are found in fermented foods and supplements, and we already know about the various characteristics and health benefits that probiotics offer us. From a nutritional point of view, fermentation helps increase vitamin content and also enhances antioxidant properties. Additionally, certain vitamins are enhanced by the process of fermentation, and bioavailability also increases, particularly for B12, B6, and folate, making them easier for the body to absorb.

This is especially helpful for those with dietary restrictions. Certain fermented foods, such as natto, significantly increase vitamin K levels, particularly vitamin K2, which is vital for blood clotting, bone health, and cardiovascular health. Fermentation also boosts the antioxidant properties of foods, which may help reduce the risk of chronic diseases by combating oxidative stress. The effect on vitamin content varies depending on the bacterial strains used in the fermentation process, so all these properties are actually strain-specific. So, let us look into the increased viability of nutrients and the beneficial compounds.

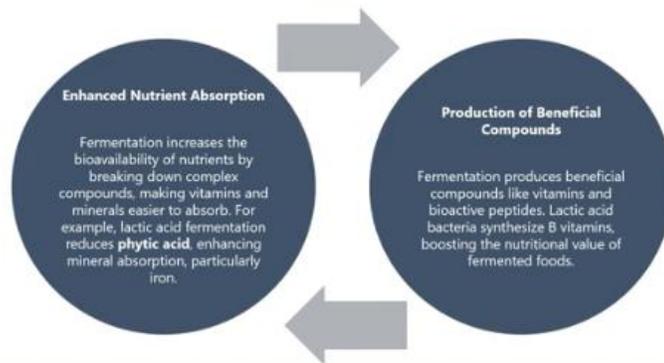
NUTRITIONAL VALUE

Impact of Fermentation on Vitamin Content and Antioxidant Properties:



Enhanced nutritional absorption is increased by fermentation. This is because it increases the bioavailability of nutrients by breaking down complex compounds, making vitamins and minerals easier to absorb. For example, lactic acid fermentation reduces phytic acid, enhancing mineral absorption, particularly iron. There is also the production of beneficial compounds by fermentation, such as vitamins and bioactive peptides. Lactic acid bacteria, for example, synthesize B vitamins, boosting the nutritional value of fermented foods.

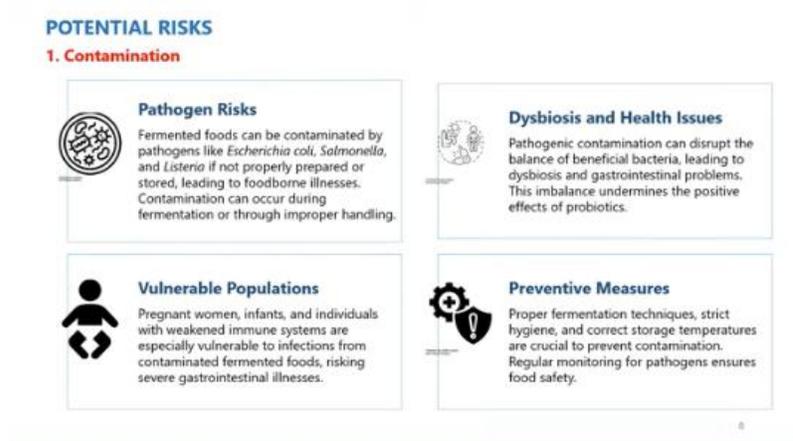
Increased bioavailability of nutrients and beneficial compounds



So, however, there are certain risks associated with fermented foods, in spite of the many benefits they offer to us. For example, there may be contamination, which can lead to pathogen risks. Fermented foods can be contaminated by pathogens like *Escherichia coli*, *Salmonella*, and *Listeria* if not properly prepared or stored. Leading to foodborne illnesses, contamination can occur during fermentation or through improper handling. And then it may lead to dysbiosis and health issues.

Ethosinic contamination can disrupt the balance of beneficial bacteria, leading to dysbiosis and gastrointestinal problems. This imbalance undermines the positive effects of the

probiotics. Then there are certain populations which can be actually vulnerable to these risks, particularly pregnant women, infants, and individuals with weakened immune systems, who are especially vulnerable to infections from contaminated fermented foods. Risking severe gastrointestinal illnesses. And proper fermentation techniques, strict hygiene, and correct storage temperatures are crucial to prevent contamination.



Regular monitoring for pathogens ensures food safety. Then there are also the risks from allergens and intolerances. For example, some people may be allergic to dairy products. Fermented dairy products like yogurt and cheese contain casein and whey, which can trigger allergic reactions in sensitive individuals. Then certain grain-based fermented foods, such as sourdough, may contain gluten, which may pose a risk for those with celiac disease or gluten intolerance.

Then there are fermented soy products like miso and tempeh, which can cause allergic reactions such as skin, respiratory, and digestive issues in vulnerable populations. Then, certain fermented foods may have high levels of histamine, which may cause headaches, rashes, or digestive problems in those with histamine intolerance. Then there is the risk of cross-contamination. Fermentation cross-contamination can introduce allergens like gluten into otherwise safe products. Then there is the risk of excessive consumption of fermented foods, which may lead to digestive issues like bloating, gas,

2. Allergens and Intolerances

 Dairy Allergens Fermented dairy products like yogurt and cheese contain casein and whey, which can trigger allergic reactions in sensitive individuals.	 Gluten Fermented grain-based foods, such as sourdough, may contain gluten, posing risks for those with celiac disease or gluten intolerance.
 Soy Fermented soy products like miso and tempeh can cause allergic reactions, such as skin, respiratory, or digestive issues.	 Histamine High histamine levels in fermented foods may cause headaches, rashes, or digestive problems in those with histamine intolerance.
 Cross-Contamination Fermentation cross-contamination can introduce allergens, like gluten, into otherwise safe products.	

and diarrhea due to high probiotic and fiber levels. And certain fermented foods have minor alcohol content. For example, kombucha contains small amounts of alcohol, posing a risk for those sensitive to alcohol. And there is also the issue of religious beliefs or other health reasons due to which these kinds of foods may cause certain risks or problems. Then there is nutrient imbalance.

Excess intake, especially of dairy-based ferments, can lead to nutrient imbalances, including increased saturated fat and calories. Then there may be a risk of probiotic overdose. Excessive probiotics may overwhelm the gut, causing issues in immunocompromised individuals or disrupting gut balance. Then there is a risk of food spoilage and microbial contamination. Improper preparation or storage of fermented foods can allow harmful bacteria like Salmonella or E. coli to grow, causing foodborne illnesses. There is a risk of mold growth due to improper storage of products like seeds, which can facilitate the growth of mold that may produce harmful mycotoxins, unlike the intentional mold in blue seeds, which has some commercial value.

3. Excessive Consumption

 Digestive Issues Overconsumption of fermented foods can cause bloating, gas, and diarrhea due to high probiotic and fiber levels.	 Nutrient Imbalance Excess intake, especially of dairy-based ferments, can lead to nutrient imbalances, including increased saturated fat and calories.
 Alcohol Content Some ferments, like kombucha, contain small amounts of alcohol, posing risks for those sensitive to alcohol or avoid it for religious or health reasons.	 Probiotic Overdose Excessive probiotics may overwhelm the gut, causing issues in immunocompromised individuals or disrupting gut balance.

Then there may be off flavors and textures resulting from the fermentation process or due to contamination. Spoilage alters taste and texture. For example, yogurt can become too sour, and sauerkraut may turn mushy if not properly fermented. Inadequate pH reduction during fermentation may allow spoilage organisms to thrive, such as in kimchi lacking sufficient acidity. And then storage is very, very important.

4. Food Spoilage

 Microbial Contamination Improper preparation or storage of fermented foods can allow harmful bacteria like Salmonella or E. coli to grow, causing foodborne illnesses.	 Off-Flavors & Textures Spoilage alters taste and texture; for example, yogurt can become too sour, and sauerkraut may turn mushy if not properly fermented.
 Mold Growth Improper storage of products like cheese can lead to mold, which may produce harmful mycotoxins, unlike the intentional mold in blue cheese.	 pH Changes Inadequate pH reduction during fermentation may allow spoilage organisms to thrive, such as in kimchi lacking sufficient acidity.
 Storage Conditions Inconsistent temperatures, like overheating kombucha, can cause uncontrolled fermentation and spoilage.	

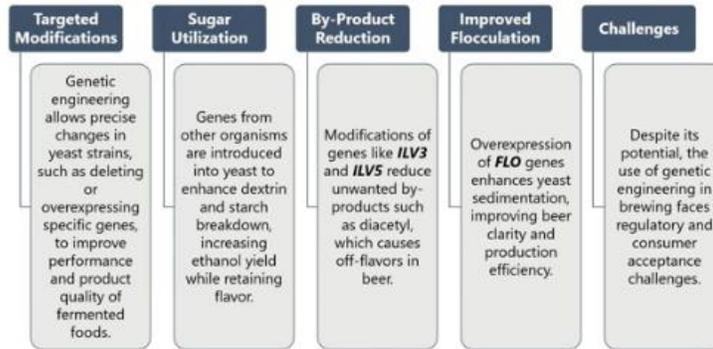
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Inconsistent temperatures, like overheating kombucha, can affect and control fermentation and spoilage. Let us now discuss some modern biotechnology advances in fermentation. Let us start with genetic engineering, particularly the genetic improvement of yeast, where we undergo targeted modifications that allow precise changes in yeast strains, such as deleting or overexpressing specific genes to improve performance and product quality of fermented foods. Then there are certain cases where genes from other organisms are introduced into yeast to enhance dextrin and starch breakdown, increasing ethanol yield while retaining flavor. We also modify genes like ILV3 and ILV5 to reduce unwanted byproducts such as diacetyl, which causes off flavors in beer.

Then we also want to improve the flocculation of yeast in the final product. This is done by overexpressing flocculation genes, which enhances yeast sedimentation, improving beer clarity and production efficiency. However, despite the vast potential, the use of genetic engineering in brewing faces regulatory and other challenges like consumer acceptance. So, much research remains to be done on different fronts. Let us discuss the genetic improvement of bacteria, which particularly focuses on bioconservation.

GENETIC ENGINEERING: Aims and Perspective

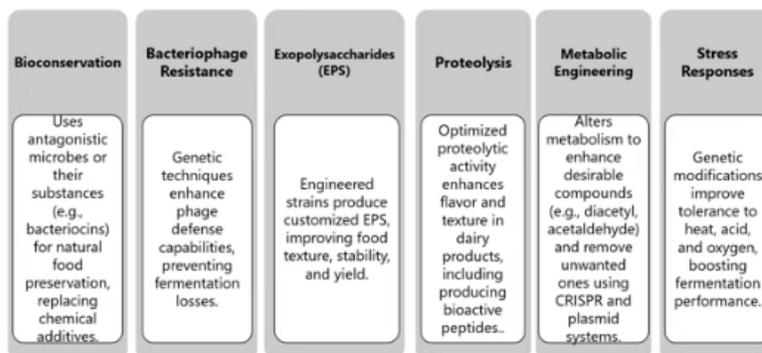
Genetic improvement of Yeast



We use antagonistic microbes or their substances, for example, bacteriocins for natural food preservation, replacing chemical additives. We can also build up bacteriophage resistance using genetic techniques to enhance phage defense capabilities, preventing fermentation losses. The engineered strains can produce customized exopolysaccharides, improving food texture, stability, and yield. Optimized proteolytic activity enhances flavor and texture in dairy products, including the production of bioactive peptides. We also have metabolic engineering approaches.

We alter the metabolism to enhance desirable compounds, such as diacetyl and acetaldehyde, and remove unwanted ones using CRISPR and plasmid systems. We also try to address challenges like stress by using genetic modifications. We improve tolerance to heat, acid, and oxygen, boosting fermentation performance, etc. Some examples of genetic improvement of bacteria include *Lactococcus lactis*, which is well-characterized for different milk fermentations. Some of the focus is on genetic engineering to enhance production and control.

Genetic Improvement of Bacteria



Then we have *Lactobacillus* species, which are diverse and used in various fermentations. Genome sequencing has aided in better exploitation for traditional and novel uses. Then we have *Streptococcus thermophilus*, which is used in yogurt and cheese production. Genomic research is improving strains for large-scale processes. Then we have *Leuconostoc* species, essential in vegetable fermentation, where genomic insights have enhanced flavor production in industrial applications.

Then we have *Pediococcus* species used in meat and vegetable fermentation, plus gene analysis and genetic studies for strain improvement. Then there is another example of *Oenococcus oeni*, which is key for malolactic fermentation in wine. Genome sequencing has been undertaken to refine fermentation control and improve wine quality. One of the important works that has been done is in the strain improvement of lactic acid bacteria, particularly in dairy fermentation. LAB is known for its safe use in milk fermentation and health-promoting properties, as it is a key component of human gut microbiota and widely used as a probiotic.

Genetic Improvement of Bacteria: examples



Lactococcus lactis: Well-characterized for different milk fermentation; ongoing genetic engineering to enhance production and control.

Lactobacillus spp.: Diverse species used in various fermentations; genome sequencing aids in better exploitation for traditional and novel uses.

Streptococcus thermophilus: Used in yogurt and cheese production; genomic research to improve strains for large-scale processes.

Leuconostoc spp.: Essential in vegetable fermentation; genomic insights to enhance flavor production and industrial applications.

Pediococcus spp.: Used in meat and vegetable fermentation; plasmid analysis and genetic studies for strain improvement.

Oenococcus oeni: Key for malolactic fermentation in wine; genome sequencing to refine fermentation control and improve wine quality.

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Due to their safe and beneficial properties, LAB are suitable candidates for genetic modification in food, industrial, and pharmaceutical applications. So, here we have two approaches. One is without genetic modification. And another is genetic modification. So, in the first case, we use random mutagenesis, or we go for adaptive laboratory evolution and also food-grade plasmid transfer through conjugation.

This can be done with the help of starter cultures, probiotics, and research tools, and of course, these act as cell factories. Then, in the GMO (genetically modified organism) approach, we go for plasmid-based homologous recombination, recombineering, and use modern tools like CRISPR-Cas genome engineering. This is an overview of the genomic modification tools and their applications in improving the properties of dairy fermentation.

So, what we do in adaptive laboratory evolution is a technique for evolving strains with improved traits by long-term culture under specific selection pressure.

Strain Improvement of Lactic Acid Bacteria (LAB).

Lactic Acid Bacteria (LAB) in Dairy Fermentation:

LAB, known for their safe use in milk fermentation and health-promoting properties, are key components of human gut microbiota and widely used as probiotics.

Due to their safety and benefits, LAB are suitable candidates for genetic modification in food, industrial, and pharmaceutical applications

(Xie et al., 2024)

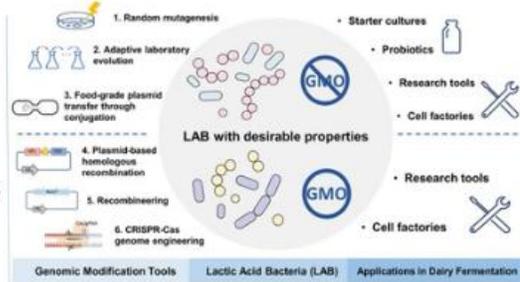


Fig- Overview of genomic modification tools and their applications in improving LAB properties for dairy fermentation

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It is widely used to enhance strains particularly lab strains for fermented dairy products without being classified as genetically modified and so there is the advantage of not getting rejected by consumers who are little bit conservative about GMO foods. And beyond strain improvement, ALE aids in understanding gene function regulation and bacterial population evolution. So, some of the applications of ALE is thermotolerant *Lactococcus lactis* mutant for reduced seeds ripening time. So, we have more cut down in the cost in terms of time and investment. Then we have the lactic acid bacillus, rhamnosus GG mutants with enhanced freeze-thaw viability and acid-tolerant lactic acid bacillus casein mutants which can survive lethal pH conditions.

Adaptive Laboratory Evolution (ALE)

ALE is a technique for evolving strains with improved traits by long-term culture under specific selection pressures. It is widely used to enhance LAB strains for fermented dairy products without being classified as genetically modified. Beyond strain improvement, ALE also aids in understanding gene function, regulation, and bacterial population evolution.



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Applications:

- i. Thermotolerant *Lactococcus lactis* mutants for reduced cheese ripening time (Dorau et al., 2021).
- ii. *Lactocaseibacillus rhamnosus* GG mutants with enhanced freeze-thaw viability (Kwon et al., 2018).

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In rhamnomutagenesis, We introduce random genome mutations, characterizing numerous variants and selecting mutants with desirable traits. This method widely used in fermented dairy production avoids classification as genetically modified microorganisms. Some of

the examples are the use of UV for mutagenesis, lactobacillus, Helveticus mutans was produced and found to have improved yogurt texture and reduced post acidification. Then we can go for mild mutagenesis with high throughput screening combined with microfluidic droplet technology.

Lactococcus lactis variants were identified tripling riboflavin production during milk fermentation compared to wild type strains. And we can also go for heavy ion mutagenesis. In this case, generated lactobacillus thermophilus mutans with high lactic acid production was obtained. So briefly, from the application point of view, precision fermentation supports the sustainable production of enzymes, lipids, vitamins and flavouring agents, often more efficiently than traditional methods. Then another approach is food-grade plasmid transfer through conjugation.

Random Mutagenesis

Random mutagenesis involves introducing random genome mutations, characterizing numerous variants, and selecting mutants with desirable traits. This method, widely used in fermented dairy production, avoids classification as genetically modified microorganisms (GMM). Examples include:



- **UV Mutagenesis:** A *Lactobacillus helveticus* mutant improved yogurt texture and reduced post-acidification (Guan et al., 2021).
- **Mild Mutagenesis with HTS:** Combined with microfluidic droplet technology, *Lactococcus lactis* variants were identified, tripling riboflavin production during milk fermentation compared to wild-type strains (Chen et al., 2017).
- **Heavy-Ion Mutagenesis:** Generated *Lactobacillus thermophilus* mutants with high lactic acid production (Jiang et al., 2018).

Applications: Precision fermentation supports the sustainable production of enzymes, lipids, vitamins, and flavoring agents, often more efficiently than traditional methods.

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Conjugation is a natural DNA transfer process where plasmids move from a donor to a recipient cell via cell-to-cell contact through a pili. In lab, particularly lactococcus starters, conjugative plasmids carry transfer genes, tra genes, and encode traits like lactose metabolism, bacteriophage resistance, and bacteriocin production. And when it comes to the applications in the dairy industry, some examples include the transfer of PMRC-01 encoding the bacteriocin-lactocin-3147 improved starter strains by preventing non-starter lab outgrowth during cheddar seeds ripening and inhibiting *Listeria monocytogenes* in cottage seeds. Then, there is the stacking multiple bacteriocins in starter systems which enhances food safety. While most applications focus on lactococcus conjugation has shown potential for transferring traits to other lab species such as from lactococcus lactis to lactobacillus reuteri GG.

Food-Grade Plasmid Transfer Through Conjugation

Conjugation is a natural DNA transfer process where plasmids move from a donor to a recipient cell via cell-to-cell contact through a pilus. In LAB, particularly *Lactococcus* starters, conjugative plasmids carry transfer genes (*tra* genes) and encode traits like lactose metabolism, bacteriophage resistance, and bacteriocin production.

Applications in the Dairy Industry:

- Transfer of *pMRC01* encoding the bacteriocin lactacin 3147 improved starter strains by preventing non-starter LAB outgrowth during Cheddar cheese ripening (Ryan et al., 1996) and inhibiting *Listeria monocytogenes* in cottage cheese (McAuliffe et al., 1999).
- Stacking multiple bacteriocins in starter systems enhances food safety (Mills et al., 2017).
- While most applications focus on *Lactococcus*, conjugation has shown potential for transferring traits to other LAB species, such as from *Lactococcus lactis* to *Lacticaseibacillus rhamnosus* GG (Hussain et al., 2021).

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Here in this plasmid-based homologous recombination, we have the integrative suicide vectors which contain an antibiotic marker and two contiguous homologous regions HR1 and HR2 that allow integration in the bacterial chromosome having homologous regions. These vectors cannot replicate in the recipient and therefore must integrate to maintain antibiotic resistance. After transformation, a single crossover event occurs. By the first homologous region, a second crossover then excises the plasmid resulting in either a wild type or mutant mutation genotype depending on which homologous region is involved.

Non-replicative vectors can efficiently modify the genome in this manner. However, it suffers from certain limitations like low efficiency and it is also a very, very time consuming process and often we end up with high pulse positive rates and there are issues with foreign DNA in the food grade. Let us now discuss the next approach which is recombineering. Recombineering developed for *E. coli* enables precise genome editing using fast derived recombination systems. It integrates oligonucleotides into genomes with single-stranded DNA or double-stranded DNA templates.

Plasmid-Based Homologous Recombination

Integrative suicide vectors contain an antibiotic marker (AB_p) and two non-contiguous homologous regions (HR1 and HR2) that allow integration into the bacterial chromosome. These vectors cannot replicate in the recipient and must integrate to maintain antibiotic resistance. After transformation, a single crossover event occurs via the first homologous region (HR1). A second crossover then excises the plasmid, resulting in either a wild-type or mutant genotype, depending on which homologous region is involved. Non-replicative vectors can efficiently modify the genome in this manner.

Limitations: Low efficiency, time-consuming process, and high false-positive rates.

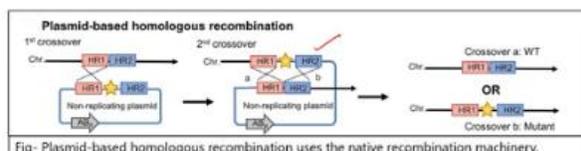


Fig- Plasmid-based homologous recombination uses the native recombination machinery.

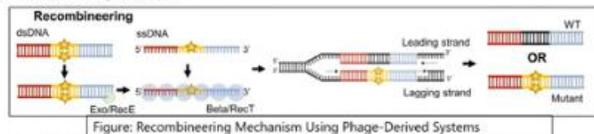
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Key systems include lambda-red beta-gam-exo and rac-prophase-derived rac-e-oblique-t, which facilitates DNA annealing and protects introduced DNA from degradation. Some of the applications with respect to lab is in *Lactococcus lactis* and *Limosilactobacillus reuteri*, Using recti-mediated SS-DNA recombination, mutagenesis has been achieved with efficiencies up to 19%. And then in DS, double-stranded DNA recombining, we have found that large deletions are possible up to 6 kB.

And gene insertions demonstrated in *Lactiplantibacillus plantarum* and Lactic Acid Bacillus Cassi has been reported. The next most interesting application is that of CRISPR-Cas9 based genome engineering in lab. The CRISPR-Cas system is basically an adaptive bacterial immunity mechanism which has become a powerful tool for genome engineering. The cast-end nucleus guided by RNA, as you can see in this picture, targets and cleaves DNA at specific sequences.

Recombineering

Recombineering, developed for *E. coli*, enables precise genome editing using phage-derived recombination systems. It integrates oligonucleotides into genomes with single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) templates. Key systems include λ -Red (Beta, Gam, Exo) and Rac prophage-derived RecE/T, which facilitate DNA annealing and protect introduced DNA from degradation.



Applications in LAB:

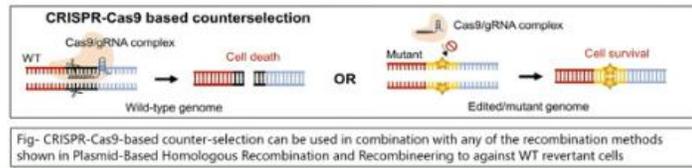
- Achieved in *Lactococcus lactis* and *Limosilactobacillus reuteri* using RecT-mediated ssDNA recombination, with mutagenesis efficiencies up to 19% (van Pijkeren and Britton, 2012).
- dsDNA recombineering enables large deletions (up to 6 kb) and gene insertions, demonstrated in *Lactiplantibacillus plantarum* (Yang et al., 2015) and *Lactocaseibacillus casei* (Xin et al., 2018).

These are basically flanked by the photospecial adjacent motif sequences. These result in the double strand break as you can see over here which are actually lethal and will kill the cell in the absence of a repair system. In many lab strains, the repair system required for DNA repair are absent or inactivated, making CRISPR-Cas9 system an effective counter-selection tool as wild-type cells are killed by Cas9 cleavage. However, in the presence of a repair system, the cell will survive. So, CRISPR-Cas9-assisted recombinant requires two plasmids, one for the recombinase machinery and another for Cas9 and single-guide RNA.

CRISPR-Cas9-Based Genome Engineering in LAB

The CRISPR-Cas system, an adaptive bacterial immunity mechanism, has become a powerful tool for genome engineering. The Cas9 nuclease, guided by RNA, targets and cleaves specific DNA sequences flanked by a protospacer adjacent motif (PAM). This results in double-strand DNA breaks, which are lethal in the absence of a repair system. In many LAB strains, the repair systems required for DNA repair are absent or inactivated, making the CRISPR-Cas9 system an effective counter selection tool, as wild-type cells are killed by Cas9 cleavage.

CRISPR-Cas9-assisted recombineering requires two plasmids: one for recombinase machinery and another for Cas9 and sgRNA. The recombineering template can be ssDNA, dsDNA, or plasmid-based. The dual-step procedure is more efficient than the single-step method. This system has been successfully used in various bacteria, achieving high recombination efficiency.

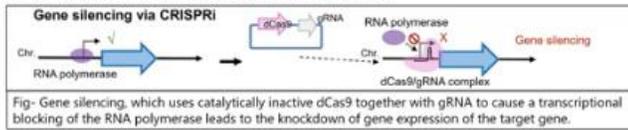


The recombineering template can be single-stranded DNA, double-stranded DNA, or plasmid-based. The dual-step procedure is more efficient than the single-step method. This system has been successfully used in various bacteria, achieving high recombination efficiency and resulting in edited mutant genomes. Some of the applications of CRISPR-Cas9 in the lab include recombineering, where CRISPR-Cas9-assisted recombineering improves efficiency for single-stranded and double-stranded DNA editing, achieving up to 100% success in *LIMO*, *Silylactobacillus roteri*, and *Lactiplantibacillus plantarum*. There are also cases of base editing where catalytically impaired Cas9 variants coupled with cytidine deaminase allow C-to-T substitutions, which is used in *Bifidobacterium* and *Lactobacillus*.

Some of the applications in the lab include CRISPR interference (CRISPRi). This catalytically inactive Cas9 silences genes via transcriptional blocking. It has been demonstrated in *Lactobacillus* and *Lactiplantibacillus plantarum*. Here, in this figure, you can see gene silencing, which uses catalytically inactive dCas9 together with guide RNA to cause transcriptional blocking of RNA polymerase, leading to the knockdown of target gene expression. CRISPR-Cas9 has revolutionized genome editing in the lab with precise modifications but faces challenges like plasmid instability and varying efficiencies.

Applications in LAB:

- **CRISPR Interference (CRISPRi):** Catalytically inactive Cas9 (dCas9) silences genes via transcriptional blocking, demonstrated in *Lactococcus lactis* and *Lactiplantibacillus plantarum* (Berlec et al., 2018; Myrbråten et al., 2019).



CRISPR-Cas9 revolutionizes genome editing in LAB with precise modifications but faces challenges like plasmid instability and varying efficiencies. **Nickase variants (nCas9)** reduce lethality and boost recombination, while **endogenous CRISPR-Cas systems** (types I and II) simplify editing and lower cytotoxicity, though requiring thorough characterization. These advancements enhance genome engineering for research and industrial applications.

Nicky's variants reduce lethality and boost recombination, while endogenous CRISPR-Cas systems, both type 1 and type 2, simplify editing and lower cytotoxicity, though they require thorough characterization. These advancements enhance genome engineering for research and industrial applications. In this table, we have summarized the comparison of lab genomic modification tools from the perspectives of advantages and disadvantages. Some advantages of random mutagenesis include no recombinant DNA requirement and applicability to all lab strains and species. This is not considered a GMO.

But the disadvantage is that it is untargeted and introduces unintended mutations randomly, requires high-throughput screening, and is limited by existing genetic potential. In the case of adaptive laboratory evolution, the advantage we have is that it is not considered a GMO. It also does not require recombinant DNA applicable to all labs. It can help us achieve directed evolution. But the disadvantage is that, like random mutagenesis, it is untargeted and co-selects undesired traits, such as contamination risk, long evolution times, and being limited by genetic potential.

Table- Summary and Comparison of LAB Genomic Modification Tools: Advantages and Disadvantages

Tool	Advantages	Disadvantages
Random Mutagenesis	- No recombinant DNA required. - Applicable to all LAB. - Not considered GMO.	- Untargeted and introduces unintended mutations. - Requires high-throughput screening. - Limited by existing genetic potential.
Adaptive Laboratory Evolution	- No recombinant DNA required. - Applicable to all LAB. - Directed evolution. - Not considered GMO.	- Untargeted and co-selects undesired changes. - Contamination risk with long evolution times. - Limited by genetic potential.
Food-Grade Plasmid Transfer	- Targeted modification. - Natural method. - Not considered GMO.	- Limited host range. - Relies on food-grade conjugative plasmids. - Risk of unintended gene transfer.
Plasmid-Based Homologous Recombination	- Targeted modification. - Widely used in LAB. - Conditionally replicating vectors improve efficiency.	- Low efficiency, time-consuming. - High false-positive rates. - Considered GMO.
Recombineering	- Targeted modification. - Avoids cloning of homologous regions. - Suitable for high-efficiency genome editing.	- Requires recombinase optimization. - Limited plasmid stability. - Considered GMO.
CRISPR-Cas Genome Engineering	- Versatile: gene deletion, insertion, and silencing. - High precision and efficiency. - No genome "scars".	- Risk of off-target mutations. - Requires replicable plasmids. - Considered GMO. - Limited application across LAB strains.

In the case of food-grade plasmid transfer, we can achieve targeted modification. It's a natural method, also not considered a GMO, but it has a limited host range, relies on food-grade conjugative plasmids, and risks unintended gene transfer. In the case of plasmid-based homologous recombination, we can achieve targeted modification. This is widely used in labs. Conditionally replicating vectors improve efficiency.

However, it has low efficiency and is very time-consuming. We have high false-positive rates, and it is considered a GMO. In fact, recombineering and CRISPR-9 engineering also suffer from the same GMO tag. So, in recombineering, we can achieve targeted modification. It avoids cloning homologous regions, is suitable for high-efficiency genome editing.

But it requires recombinase optimization, limited plasmid stability, and considered as a GMO. And CRISPR-Cas9 engineering, it's a versatile technique for gene deletion, insertion, and silencing. High precision and efficiency, no genome scars is required. I have seen RICS off-target mutations requires replicable plasmids considered as GMO limited applications across the lab strains. Now let us look into the potential applications of genetically modified lab in dairy fermentation.

Table- Summary and Comparison of LAB Genomic Modification Tools: Advantages and Disadvantages

Tool	Advantages	Disadvantages
Random Mutagenesis	<ul style="list-style-type: none"> - No recombinant DNA required. - Applicable to all LAB. - Not considered GMO. 	<ul style="list-style-type: none"> - Untargeted and introduces unintended mutations. - Requires high-throughput screening. - Limited by existing genetic potential.
Adaptive Laboratory Evolution	<ul style="list-style-type: none"> - No recombinant DNA required. - Applicable to all LAB. - Directed evolution. - Not considered GMO. 	<ul style="list-style-type: none"> - Untargeted and co-selects undesired changes. - Contamination risk with long evolution times. - Limited by genetic potential.
Food-Grade Plasmid Transfer	<ul style="list-style-type: none"> - Targeted modification. - Natural method. - Not considered GMO. 	<ul style="list-style-type: none"> - Limited host range. - Relies on food-grade conjugative plasmids. - Risk of unintended gene transfer.
Plasmid-Based Homologous Recombination	<ul style="list-style-type: none"> - Targeted modification. - Widely used in LAB. - Conditionally replicating vectors improve efficiency. 	<ul style="list-style-type: none"> - Low efficiency, time-consuming. - High false-positive rates. - Considered GMO.
Recombineering	<ul style="list-style-type: none"> - Targeted modification. - Avoids cloning of homologous regions. - Suitable for high-efficiency genome editing. 	<ul style="list-style-type: none"> - Requires recombinase optimization. - Limited plasmid stability. - Considered GMO.
CRISPR-Cas Genome Engineering	<ul style="list-style-type: none"> - Versatile: gene deletion, insertion, and silencing. - High precision and efficiency. - No genome "scars". 	<ul style="list-style-type: none"> - Risk of off-target mutations.²⁰ - Requires replicable plasmids. - Considered GMO. - Limited application across LAB strains.

For example, if we look at them from the perspective of various traits and applications and some of the examples of these strains, when it comes to safety, the application of these labs, particularly lactococcus lactis and L. plantarum, is antimicrobial peptide production and degradation of harmful substances. In the case of quality, we have lower post-fermentation acidification like L. plantarum than degradation of indigestible polysaccharides like L. rhamnosus. And from a sensory point of view, flavor aroma improvement by autolysis and production of aroma compounds possible in the case of lacto-coccus lactis and helveticus. Then degradation of undesirable flavors like L-Casey,

then enhanced sweetness like streptococcus thermophiles, then improved texture L-Casey and L-Helveticus. Then we have these stress tolerance, particularly growth tolerance to stress like temperature, oxygen, pH, bile.

We have L-Gassery, L-Acidophilus and L-Casey. Then we have certain valuable products like the production of micronutrients, sweeteners and chemicals by L-rotary, L-lactase and L-halviticus. Let us now discuss briefly about the exploration of synthetic biology in food fermentation, which can help us in engineering the microbes. Synthetic biology enables modifying microbes to enhance fermentation by expressing genes that produce specific flavors. For example, heme for meaty tastes and nutrients, for example, polyunsaturated fatty acids for health benefits.

Table: Potential Applications of Genetically Modified LAB in Dairy Fermentation

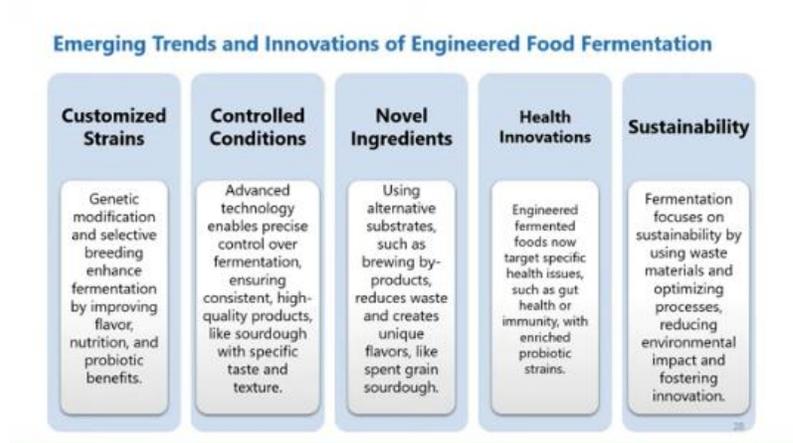
Trait	Application	Example LAB Strains
Safety	Antimicrobial peptides production	<i>Lactococcus lactis</i> , <i>L. plantarum</i>
	Degradation of harmful substances	<i>Limosilactobacillus reuteri</i> , <i>L. plantarum</i>
Quality	Lower postfermentation acidification	<i>L. plantarum</i> , <i>L. helveticus</i>
	Degradation of indigestible polysaccharides	<i>L. rhamnosus</i>
Sensory	Flavor/aroma improvement (autolysis, aroma compounds)	<i>Lactococcus lactis</i> , <i>L. helveticus</i>
	Degradation of undesirable flavors	<i>L. casei</i>
	Enhanced sweetness	<i>Streptococcus thermophiles</i>
Stress Tolerance	Improved texture	<i>L. casei</i> , <i>L. helveticus</i>
	Growth tolerance to stress (temp., oxygen, pH, bile)	<i>L. gasseri</i> , <i>L. acidophilus</i> , <i>L. Casei</i>
Valuable Products	Production of micronutrients, sweeteners, and chemicals	<i>L. reuteri</i> , <i>L. lactis</i> , <i>L. helveticus</i>

L = *Lactobacillus* 26

It can also help us improve the nutritional value. Ingenious microbes can address nutritional deficiencies by producing essential fatty acids and other vital nutrients. It can help us enhance yield and quality both. Optimizing metabolic pathways in microbial cell factories increases the yield and quality of fermented products, improving consumer appeal. And it can help us achieve sustainability since synthetic biology reduces reliance on traditional agriculture, lowering land use, greenhouse gas emissions, and environmental impact while meeting food demands.

So, some of the emerging trends and innovations in engineered food fermentation are the development of customized strains, genetic modification, and selective breeding to enhance fermentation by improving flavor, nutrition, and probiotic benefits. Then the optimization of control conditions like advanced technology enables precise control over fermentation, ensuring consistent high-quality products like sourdough with specific taste and texture. It can also help us obtain novel ingredients using alternative substrates such as brewing byproducts, reducing waste and creating unique flavors like spent grain

sourdough. And then it can help us achieve health innovations like For example, engineered fermented food now targets specific health issues such as gut health or immunity with enriched probiotic strains.



And it can help us achieve sustainability. Fermentation focuses on sustainability by using waste materials and optimizing processes, reducing environmental impact and fostering innovations. Let us now move to section 2, where we discuss precision fermentation. We will discuss the technological approaches for precision fermentation. First, let us begin with the definition and overview of precision fermentation.

Precision fermentation involves optimized fermentation processes using specially designed microbial hosts as cell factories to produce high-value functional food ingredients with high yields and purity. Basically, it utilizes microbial metabolism for the chemical transformation of organic matter, and the aim is to create products with enhanced health benefits and improved organoleptic profiles. It involves techniques such as high-throughput screening and CRISPR-Cas9 for strain engineering. Some of the applications include precision fermentation, which supports the sustainable production of enzymes, lipids, vitamins, and flavoring agents, often more efficiently than existing traditional methods. So now, if we make a comparison between traditional fermentation and precision fermentation, and we look into the aspect of process control.

DEFINITION AND OVERVIEW OF PRECISION FERMENTATION

Definition: Precision fermentation involves optimized fermentation processes using specially designed microbial hosts as "cell factories" to produce high-value functional food ingredients with high yields and purity.

- Utilizes microbial metabolism for the chemical transformation of organic matter.
- Aims to create products with enhanced health benefits and improved organoleptic profiles.
- Involves techniques such as high-throughput screening and CRISPR-Cas9 for strain engineering.

Applications: Precision fermentation supports the sustainable production of enzymes, lipids, vitamins, and flavoring agents, often more efficiently than traditional methods.

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Traditional fermentation is often artisanal, spontaneous, and undefined. We rely on natural microbial populations and backslopping techniques. Precision fermentation employs optimized methods, including high-throughput screening and genetic engineering, to control and enhance the fermentation process. With regards to product consistency, traditional fermentation can lead to variability in product quality and flavor due to uncontrolled fermentation conditions. Precision fermentation aims for consistent and improved product quality and safety through precise microbial manipulation.

Table - Distinction Between Traditional and Precision Fermentation

Aspect	Traditional Fermentation	Precision Fermentation
Process Control	Often artisanal, spontaneous, and undefined; relies on natural microbial populations and back slopping techniques.	Employs optimized methods, including high-throughput screening and genetic engineering, to control and enhance fermentation processes.
Product Consistency	Can lead to variability in product quality and flavor due to uncontrolled fermentation conditions.	Aims for consistent and improved product yields, quality, and safety through precise microbial manipulation.
Technological Integration	Typically lacks advanced technologies and relies on historical practices.	Integrates modern techniques like CRISPR-Cas9 and multi-omics to enhance fermentation efficiency and product characteristics.

(Chai et al. 2022)

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And with regards to technological integration, traditional fermentation typically lacks advanced technologies and relies on historical cultural practices. Precision fermentation integrates modern techniques like CRISPR-Cas9 and multi-omics to enhance fermentation efficiency and product characteristics. So, the various techniques and technologies, for example, here we can have an overview of the use of precision methods in fungal fermentation, which uses CRISPR-Cas9, high-throughput screening, next-generation sequencing, molecular cloning, and multi-omics. So, some of the advantages are it increases process efficiency, cuts down costs, increases yields, enhances safety, the

nutritional value is also enhanced, and the organoleptic profile is also enhanced. Now, CRISPR and genetic engineering for precision fermentation are essential for precision fermentation, enabling high-end accuracy modifications of microbial genomes to develop strains that efficiently produce desired food ingredients.

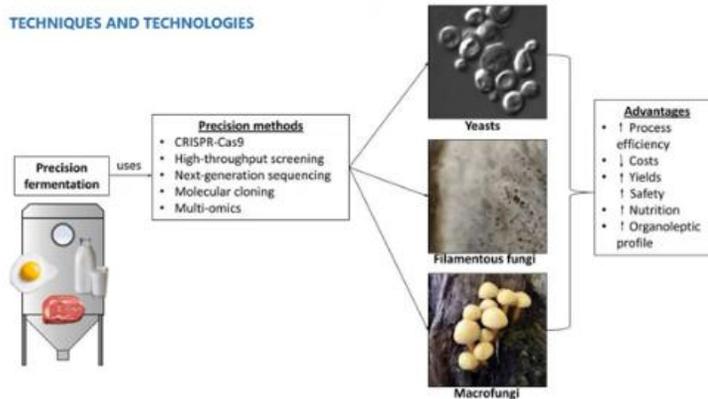


Fig.- An overview of the use of precision methods on **fungal fermentation**. (Adapted from Chai et al. 2022)

Then we have the strain engineering which involves selecting and modifying specific microorganisms such as grass, those are generally recognized as safe, bacteria and yeast both to enhance their production capabilities under optimal fermentation conditions. Then we have the application in these microbial strains. Engineered strains can produce alternative proteins like those from PCA pastries used to create meat analogs, showcasing how precision fermentation can offer sustainable food processes while mimicking traditional ingredients. The CRISPR-CAS10 tool has been utilized to create e-strains that enhance express recombinant enzymes from other organisms, such as rhizobus oryzae, to enhance sour beer production with unique flavor profiles. So, this table shows conventional fermented food beverages prepared using precision methods.

CRISPR and Genetic Engineering for Precision Fermentation

CRISPR and Genetic Engineering: These techniques are essential for precision fermentation, enabling high-accuracy modifications of microbial genomes to develop strains that efficiently produce desired food ingredients.

Strain Engineering: This involves selecting and modifying specific microorganisms, such as GRAS (Generally Recognized As Safe) bacteria and yeasts, to enhance their production capabilities under optimal fermentation conditions.

Application in Microbial Strains:

- Engineered strains can produce alternative proteins, like those from *Pichia pastoris*, used to create meat analogues, showcasing how precision fermentation can offer sustainable food sources while mimicking traditional ingredients. (further elaborated in M9L5)
- The CRISPR-Cas9 tool has been utilized to create yeast strains that express recombinant enzymes from other organisms, such as *Rhizopus oryzae*, to enhance sour beer production with unique flavor profiles.

In the case of bread dough, while *Saccharomyces cerevisiae* strain from a traditional Korean microbial fermentation starter Noorook was used. And CRISPR-Cas9 genome editing was deployed, disrupting the RGT2 and SNF3 genes, which elevated glucose repression, resulting in increased carbon dioxide production during bread dough fermentation and improved strain livening compared to the parental strain. Then we have the wheat-based, bread and potato-based chips. Wild *saccharomyces cerevisiae* is strained from a traditional Korean microbial fermentation starter, similar to the earlier one. Here, again, we used CRISPR-Cas9 genome editing.

For overexpressing ASP3 and deleting URE2 or GZF3 significantly for increased asperase in consumption leading to reduced acrylamide content in the baked bread. And in the case of wine, *Saccharomyces cerevisiae* lalvin strain EC1118 was genetically engineered using CRISPR-Cas9 editing technique for disrupting the ECM33 gene in these strain, which improved fermentation efficiency by 20% in nitrogen limited and 13% in nitrogen sufficient media under continuous shaking. The mutated yeast also settled faster resulting in clearer fermentation media and larger yeast sediments. Let us now discuss about the importance of fermentation monitoring and control where we use sensors which are basically electric noses and tongues. These multi-sensor systems are effective for monitoring fermentation processes allowing for the detection of volatile organic compounds that indicate fermentation quality.

Table - Conventional fermented foods/beverages prepared using precision methods.

Food/beverage	Parental strain	Precision method used	Observations on the fermentation process and/or product
Bread dough	Wild <i>S. cerevisiae</i> strain from a traditional Korean microbial fermentation starter (Nuruk)	CRISPR-Cas9 genome editing	Disrupting the RGT2 and SNF3 genes alleviated glucose repression, resulting in increased CO ₂ production during bread dough fermentation and improved leavening compared to the parental strain.
Wheat-based bread and Potato-based chips	Wild <i>S. cerevisiae</i> strain from a traditional Korean microbial fermentation starter (Nuruk)	CRISPR-Cas9 genome editing	Overexpressing ASP3 and deleting URE2 or GZF3 significantly increased asparagine consumption, leading to reduced acrylamide content in baked bread.
Wine	<i>S. cerevisiae</i> Lalvin EC1118	CRISPR-Cas9 genome editing	Disrupting the ECM33 gene in EC1118 yeast improved fermentation efficiency by 20% in nitrogen-limited and 13% in nitrogen-sufficient media under continuous shaking. The mutated yeast also settled faster, resulting in clearer fermentation media and larger yeast sediments.

(Chai et al. 2022)

And we get rapid responses. The use of these sensors enables quick identification of deviations in fermentation conditions, which is essential for manufacturing, maintaining product quality, and preventing resource waste. And automation is largely beneficial. Integrating automation with sensor technology facilitates precise control over fermentation parameters, reducing the need for manual intervention and minimizing downtime. Real-

time expert systems like GENSIMS-02 are increasingly being applied to fermentation processes, as these systems integrate data from advanced sensors, utilize improved signal analysis and control algorithms, and apply artificial intelligence techniques.

This approach enhances our understanding of fermentation behavior and enables more precise monitoring and control of bioreactors. Let us discuss the applications in functional food production of functional ingredients, particularly bioactive peptides, vitamins, antioxidants, and others. In the case of bioactive peptides, engineered *Saccharomyces cerevisiae* produces bioactive peptides that boost immune function and lower blood pressure. Then, *Aspergillus oryzae* is used in precision fermentation to produce essential vitamins like riboflavin (B2) for functional foods. Then, in the case of *Xanthophyllomyces dendrorhous*, it generates astaxanthin, a potent antioxidant for incorporation into health-focused food products.

Fermentation Monitoring and Control

Use of Sensors-

- **Electronic Noses and Tongues:** These multi-sensor systems are effective for monitoring fermentation processes, allowing for the detection of volatile organic compounds (VOCs) that indicate fermentation quality.
- **Rapid Response:** The use of these sensors enables quick identification of deviations in fermentation conditions, which is essential for maintaining product quality and preventing resource waste.
- **Automation Benefits:** Integrating automation with sensor technology facilitates precise control over fermentation parameters, reducing the need for manual intervention and minimizing downtime.

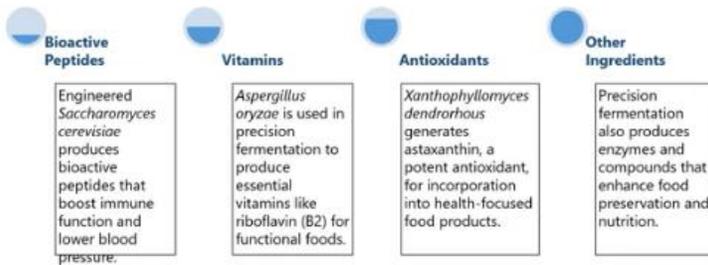
Real-time expert systems, like **Gensym's 02**, are increasingly being applied to fermentation processes as these systems integrate data from advanced sensors, utilize improved signal analysis and control algorithms, and apply AI techniques. This approach enhances our understanding of fermentation behavior and enables more precise monitoring and control of bioreactors.

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Besides this, precision fermentation also produces enzymes and compounds that enhance food preservation and nutrition. Tailoring fermented foods for specific health benefits today is a reality. It helps us in enhanced nutrition and targeted health benefits, flavor improvement, and long-term sustainability. Precision fermentation engineers microbial strains to produce nutrients like vitamins and bioactive peptides, as we have discussed in the earlier slides, which boosts the health benefits of fermented foods.

APPLICATIONS IN FUNCTIONAL FOODS

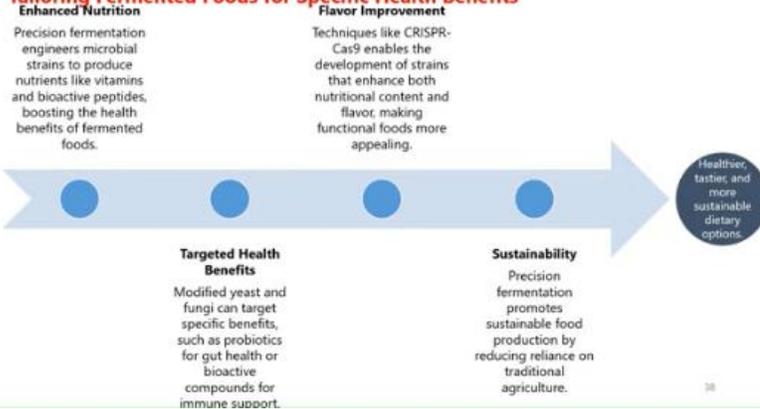
Production of Functional Ingredients



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Modified yeast and fungi can target specific benefits such as probiotics for gut health or bioactive compounds for immune support. And CRISPR-Cas9 techniques enable the development of strains that enhance both nutritional content and flavor, making functional foods more appealing. Precision fermentation promotes sustainable food production by reducing reliance on traditional agriculture and all of these in brief provides us healthier, tastier and more sustainable dietary options. So, now there are many companies which are involved in fermentation or production of food ingredients. In the table you can see companies like Argeta, Peptotec, Richcore Licenses, Remilk,

Tailoring Fermented Foods for Specific Health Benefits



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So, on and also they are classified into companies which some of them produce strains and recombinant proteins, some of them produce only proteins, some of them are focused on production of lipids and companies like Dupin also produces probiotics for food, beverages and dietary supplements. So, for example, RJR goes for designer fermentation strains for ingredients. Then you have the PEPROTECT, which is focused on recombinant proteins, including growth factors for cultivated meat. And similarly, Rich Core Life Sciences also

has similar focus areas. Then you have biosyntha, which uses mycelium as a platform for animal muscle proteins in meat alternatives.

Then you have free milk, where dairy proteins through microbial fermentation is being done. And then you have final foods, whey proteins produced by yeast for seeds. Then better dairy produces dairy proteins using precision fermentation and foamy ingredients trying for egg replacement ingredient produced by microbial fermentation. Then we have companies like Melt and Marble that focuses on recreating animal fats using precision fermentation. And Sains Foods are trying to produce fats by fermentation for dairy products.

Table- List of companies involved in fermentation for production of food ingredients.

Company	Interest - Ingredient /Product
Strains/Recombinant Protein	
Arzeda	Designer fermentation strains for ingredients
Peptotech	Recombinant proteins including growth factors for cultivated meat
Richcore Lifesciences	Recombinant proteins including growth factors for cultivated meat
Proteins	
Biosyntha	Mycelium as platform for animal muscle proteins in meat alternatives
Remilk	Dairy proteins through microbial fermentation
Final Foods	Whey proteins produced by yeast for cheese
Better Dairy	Production of dairy proteins using precision fermentation
Fumi Ingredients	Egg replacement ingredient produced by microbial fermentation
Lipids	
Melt and Marble	Re-creating animal fats using precision fermentation
Change Foods	Producing fats by fermentation for dairy products
Yali-bio	Precision fermentation-derived fats for animal-free products
Other Ingredients	
DuPont	Probiotics for food, beverage and dietary supplements

("Innovation in Precision Fermentation for Food Ingredients" 2024)

Yelly Bio focuses on precision fermentation-derived fats for animal-free products. So this table looks very, very—I mean, imaginary—but many of these are now becoming possible. So, in the future, we may be able to reduce our dependence on limited resources like land and water if many of these things become scalable and also accepted by consumers. So, monitoring and artificial intelligence applications in fermentation are among the recent advances.

Here, techniques like high-performance liquid chromatography and infrared spectroscopy improve process control by accurately measuring product and substrate concentrations. AI is getting integrated; machine learning methods such as XGBoost and Random Forest are used to predict optimal fermentation conditions. That can be analyzed in Periscope Opt. Periscope Opt is a machine learning-based platform for predicting optimal fermentation conditions and yields for recombinant periplasmic protein expressed in Escherichia coli, enhancing efficiency and scalability in fermentation technology. So, what are the key considerations for introducing precision fermentation-derived ingredients into the market?

Know-how is also crucial because it is never mentioned in scientific papers or even in patents. Finally, of course, fermentation will be carried out by microbes. Strain selection and engineering, the choice of feedstock, the fermentation process, and downstream processes—all of which contribute to the final product—along with scaling up the entire process, will be very important considerations. Then, its application in food—particularly ingredient specifications, physical functionality, nutritional properties, and safety—will determine whether it is fit for consumption. This plays a critical role, especially for precision fermentation-derived food ingredients.

With that, we come to the end of today's lecture. Thank you for your kind attention. Amen.