MICROBIAL BIOTECHNOLOGY

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Lecture-31

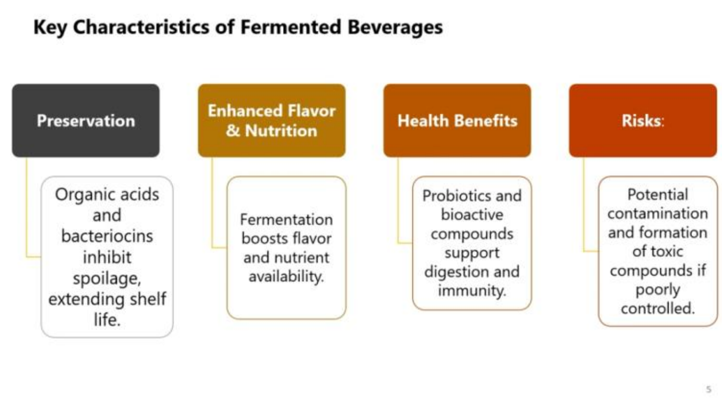
Lec 31: Fermented Beverages: Microbial Processes and Biotechnological Innovations

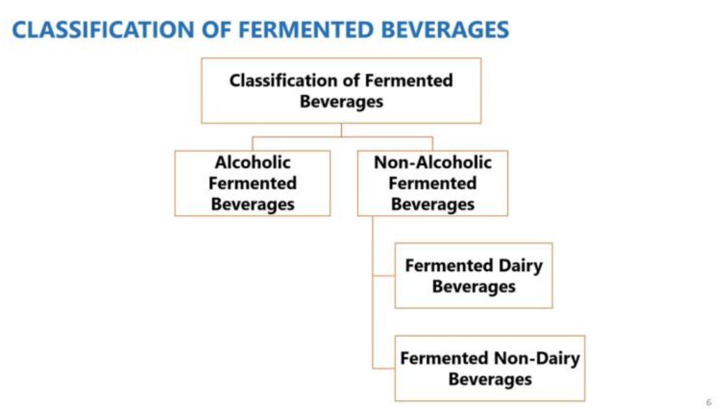
Welcome to my course on microbial biotechnology. We are in module 9, discussing food production involving microorganisms and their products. Today, we will discuss fermented beverages, trying to understand the microbial processes and various biotechnological innovations involved in producing them. This lecture is broadly divided into two sections. In the first section, we have an introduction to fermented beverages and the role of microbes in producing them.

In section two, we will study the biochemical and biotechnological aspects of beverage fermentation. Let's begin with section one, where we introduce fermented beverages. We start with definitions and key characteristics of fermented beverages, then the classification of fermented beverages, and we discuss various microorganisms used in fermentation to produce beverages, like yeast, bacteria, and molds. Fermented beverages are a diverse category of drinks. Produced through the metabolic activity of microorganisms, which may be yeast, bacteria, or molds, these beverages can be broadly classified into two main types. Number one: alcoholic fermented beverages and non-alcoholic fermented beverages. So, here you can see a can of kombucha, about which we will have a detailed discussion later.

Some of the key characteristics, advantages, and even disadvantages of fermented beverages can be as follows. This helps us in preservation. Organic acids and bacteriocins inhibit spoilage, extending shelf life. Then, we have enhanced flavor and nutrition. Fermentation boosts flavor and nutrient availability.

They also have a lot of health benefits to offer, such as probiotics and bioactive compounds, support digestion and immunity. However, there are certain disadvantages like potential contamination and formation of toxic compounds if poorly controlled or managed. Let us now look into the various types of fermented beverages. We can broadly classify them into two as already discussed in the starting slide into alcoholic fermented beverages and non-alcoholic fermented beverages which can be further classified into fermented dairy beverages and fermented non-dairy beverages. So, let us look into the broad diversity of the alcoholic beverages which can be wines, beer, cider, spirits, mead, sake and michelinous and under non-alcoholic we may have alcohol filled beer, kombucha, kefir, iron, was and vinegar.

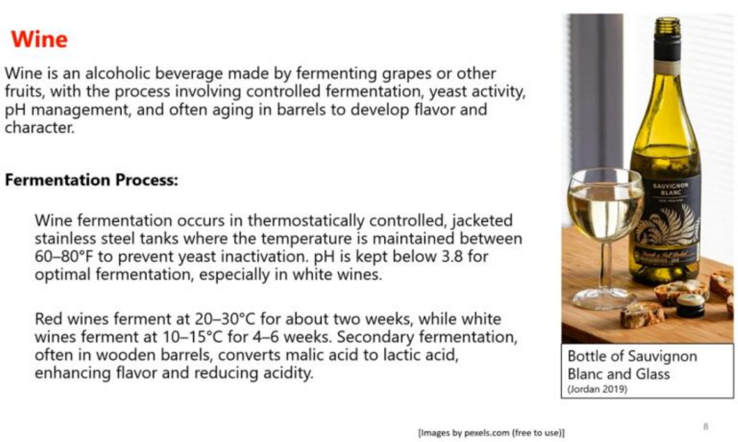




Alcoholic fermented beverages are drinks containing ethanol produced by fermenting sugars. With yeast, these beverages have an alcohol volume between 0.55% and 76%. So, some of the examples we have already discussed over here, wine, beer, cider and so on. And then we have traditional drinks like sake and the maximum Palkyu and South American Chika. They are made either through fermentation alone or through a combination of fermentation and distillation.

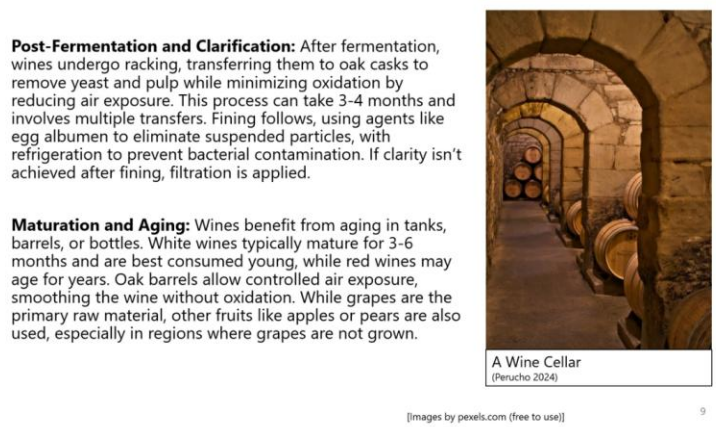
Few of these are explained in the next few slides. So, this is a bottle of Sauvignon blank and glass. So, here is a bottle of this very unique wine. Wine is an alcoholic beverage made by fermenting grapes or other fruits with the process involving controlled fermentation. Yeast activity, pH-min, is often aging in barrels to develop flavor and character.

The fermentation process occurs in thermostatically controlled, jacketed stainless steel tanks, where the temperature is maintained between 60 and 80 degrees Fahrenheit to prevent yeast inactivation. The pH is kept below 3.8 for optimal fermentation, especially in the case of white wines. Then we have red wines, which ferment at 20 to 30 degrees Celsius for about two weeks, while white wines ferment at 10 to 15 degrees Celsius for four to six weeks. Secondary fermentation, often in wooden barrels, converts malic acid to lactic acid, enhancing flavor and reducing acidity. So, here you can see a wine cellar where we have these wine barrels stacked one upon another.



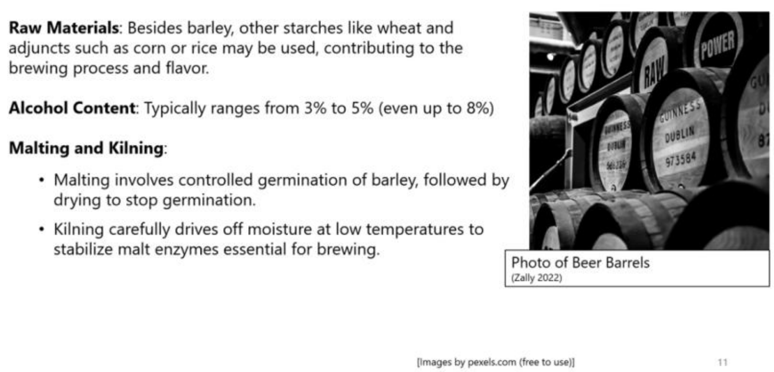
This is basically a storage structure. So, the post-fermentation operations, which include clarification, are a very important phase in wine production. After wine fermentation, the wine undergoes racking, transferring it to oak casks to remove yeast pulp while minimizing oxidation by reducing exposure. This process can take three to four months and involves multiple transfers. Fining follows, using agents like albumin to eliminate suspended particles, with refrigeration to prevent bacterial contamination.

If clarity isn't achieved after fining, filtration is also applied. Maturation and aging are also important for improving the quality of wines. Wines benefit from aging in tanks, barrels, or bottles. White wines typically mature for three to six months and are best consumed when fresh or young, while red wines may age for years. Oak barrels allow controlled air exposure, smoothing the wine without oxidation.

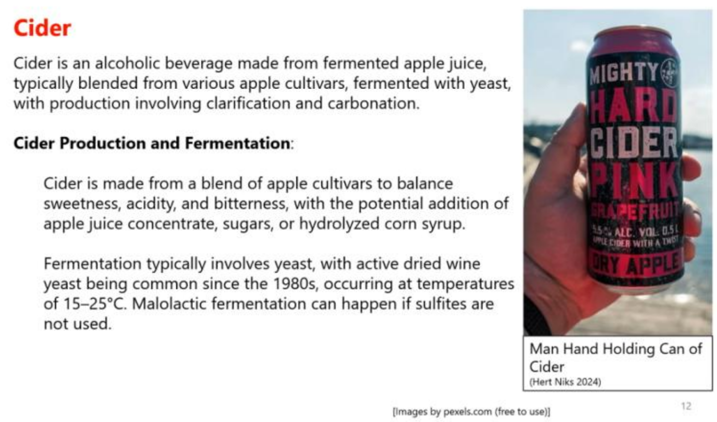


While grapes are the primary raw material, other fruits like apples or pears are also used, especially in regions where grapes are not grown. Another important alcoholic beverage is beer, which is consumed globally. Made from malted barley starch, it is the oldest and most produced alcoholic beverage worldwide, ranking third in popularity after water and tea. The various ingredients are basically barley starch, which is saccharified to extract sugars and fermented by Saccharomyces cerevisiae, producing ethanol and carbon dioxide. The resulting extract, known as wort, is created during the brewing process, which also involves hops for flavor and preservation.

Besides barley, other starches like wheat and adjuncts such as corn or rice may be used, contributing to the brewing process and flavor. Typically, beer has 3-5% or even up to 8% alcoholic content. Malting and kilning are important. Malting involves controlled germination of barley, followed by drying to stop germination. Kilning carefully drives off moisture at low temperatures to stabilize malt enzymes essential for brewing.

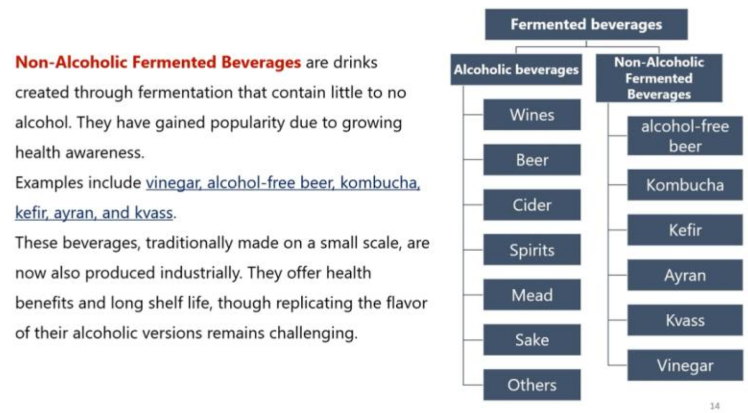


Another important alcoholic beverage is cider, which is made from fermented apple juice, typically blended from various apple cultivars, fermented with yeasts, with production involving clarification and carbonation. Cider is made from a blend of apple cultivars to balance sweetness, acidity, and bitterness, with the potential addition of apple juice concentrate, sugars, or hydrolyzed corn syrup. Fermentation typically involves yeast, with active dried wine yeast being common since the 1980s, occurring at temperatures of 15 to 25 degrees centigrade. Malolactic fermentation can happen if sulfides are not used. For color and flavor development, the color of cider arises from the oxidation of polyphenols, which can be controlled by sulfide addition.



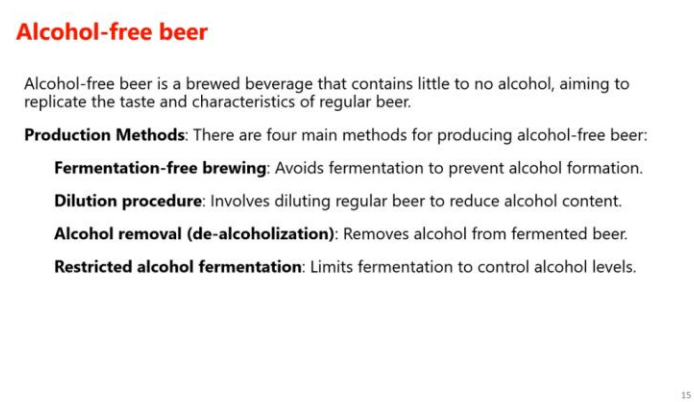
Bitterness and astringency are influenced by procyanidins. Yeast produces volatile compounds that contribute to flavor. Basically, those, for example, come from esters. Cider clarification is achieved through natural settling, centrifugation, or fining agents. After racking, filtration, and blending, ciders are usually pasteurized and carbonated before packaging, ensuring quality and consistency.

Let us now discuss non-alcoholic fermented beverages, which are produced through fermentation and contain little or no alcohol. They have gained popularity due to growing health awareness. Examples include vinegar, alcohol-free beer, kombucha, kefir, and carbonated drinks. These beverages, traditionally made on a small scale, are now also produced industrially at large scales. They offer health benefits and a long shelf life, though replicating the flavor of alcoholic versions remains challenging.

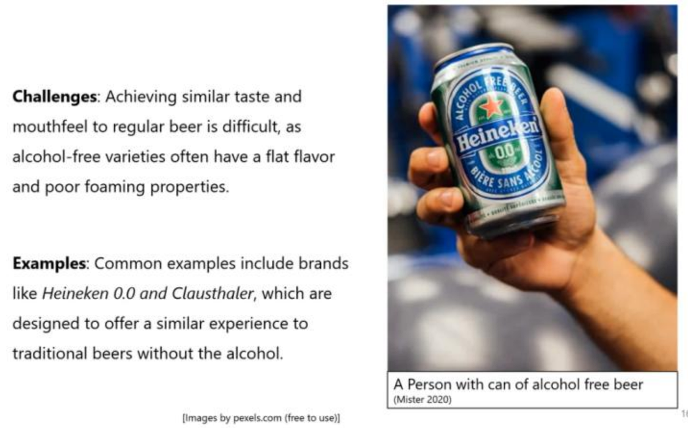


One popular drink is alcohol-free beer, which is a brewed beverage that contains little or no alcohol, aiming to replicate the taste and characteristics of regular beer. It is produced by four main methods. Fermentation-free brewing avoids fermentation to prevent alcohol production. The dilution procedure involves diluting regular beer to reduce alcohol content. Alcohol removal or de-alcoholization removes alcohol from fermented beer.

Then, restricted alcohol fermentation limits fermentation to control alcohol levels. However, there are certain challenges in such beer production. Achieving similar taste and mouthfeel to regular beer is difficult, as alcohol-free varieties often have a flat flavor and poor foaming properties. So, here are some examples given from the academic point of view and not for any commercial promotion.



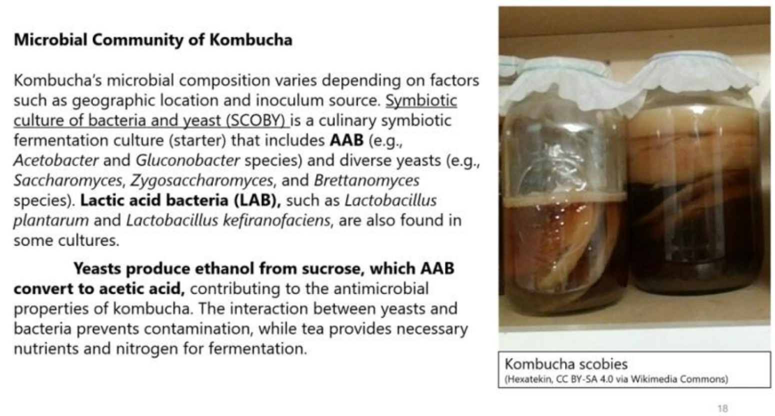
This is Heineken 0.0, which shows it has no alcohol. There is also another brand, Klosterhaler, designed to offer a similar experience to traditional beers without alcohol, thereby leading to health benefits. Then we have Kombucha, a fermented tea made by fermenting sweetened black or green tea with a symbiotic culture of acetic acid bacteria and yeast, which forms a cellulose-like pellicle on the surface, originating in China thousands of years ago. It was first consumed for its health benefits. It spread to Russia, Germany, and Europe, becoming popular in France in the 1950s.



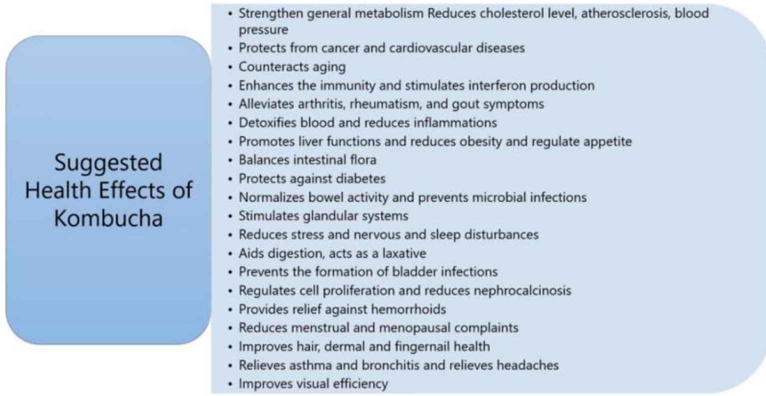
Kombucha became popular in the US for its revitalizing and therapeutic effects, with many claiming it promotes longevity by reversing aging. It is now commercially produced worldwide in various flavors. You can see here lemon, lime, and mint, and this is ginger lemon. So, there are many different varieties. So, what is the microbial community that helps in the production of kombucha?

It varies depending on factors such as geographic location, nucleolus source, and symbiotic culture of bacteria and yeast. SCOBY is a culinary symbiotic fermentation culture starter that includes acetic acid bacteria, e.g., Acetobacter and Gluconobacter species, and diverse yeasts, e.g., Saccharomyces, Zygosaccharomyces, and Brettanomyces species.

Lactic acid bacteria, such as Lactobacillus plantarum and Lactobacillus casei, are also found in some cultures. Yeasts produce ethanol from sucrose, which acetic acid bacteria convert to acetic acid, contributing to the antimicrobial properties of kombucha. The interaction between yeast and bacteria prevents contamination, while tea provides necessary nutrients and nitrogen for fermentation. You can see here the kombucha SCOBY. Some of the health effects of kombucha, as revealed or suggested by researchers,



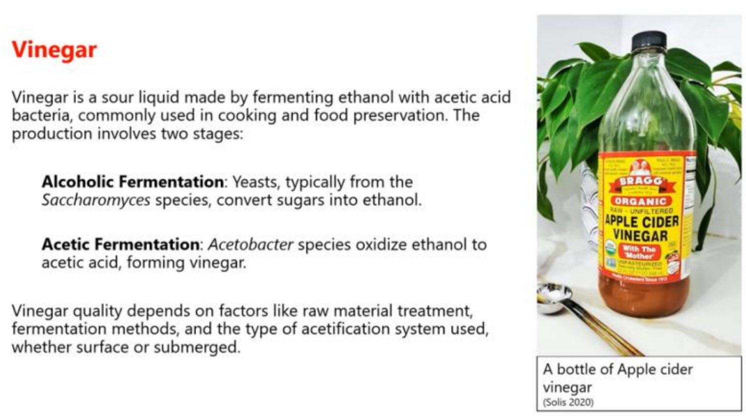
are not all verified, so please consider them only from an academic perspective, which may require further research. There are claims that it strengthens general metabolism, reduces cholesterol levels, atherosclerosis, and blood pressure. It may protect against cancer and cardiovascular diseases, counteract aging as an anti-aging agent, enhance immunity, and stimulate interferon production. It alleviates arthritis, rheumatism, and gout symptoms; detoxifies blood; reduces inflammation; promotes liver function; reduces obesity; regulates appetite; balances intestinal flora; protects against diabetes; normalizes bowel activity; prevents microbial infections; stimulates glandular systems; reduces stress, nervousness, and sleep disturbances; aids digestion; and acts as a laxative. It prevents bladder infections, regulates cell proliferation, reduces nephrocalcinosis, relieves hemorrhoids, reduces menstrual and menopausal complaints, improves hair, skin, and fingernail health, relieves asthma and bronchitis, alleviates headaches, and improves visual efficiency.



Fermented Beverages. 2019. *Elsevier EBooks*. https://doi.org/10.1016/c2017-0-02379-0. [19]

So, these are some of the things suggested about the health effects of kombucha. Now let us go to a very popular beverage or fermented beverage which is found in the kitchen of every home. Vinegar, which is basically a sour liquid made by fermenting ethanol with acetic acid bacteria, is commonly used in cooking and food preservation. The production involves two stages. The first stage is alcoholic fermentation, where yeast, typically from Saccharomyces species, converts sugars into alcohol.

Then comes the acetic acid fermentation stage, where Acetobacter species oxidize the ethanol to acetic acid, forming the vinegar. The quality of vinegar depends on factors like raw material treatment, fermentation methods, and the type of acidification systems used, whether surface or submerged. Acetobacter and Gluconobacter are primarily essential for producing vinegar by converting ethanol to acetic acid. Despite their industrial significance in vinegar production, bacterial cellulose manufacturing, and sorbose production, biochemical and genetic studies on these bacteria are limited due to genetic instability from spontaneous mutations. The instability complicates strain identification and industrial use. Advances in



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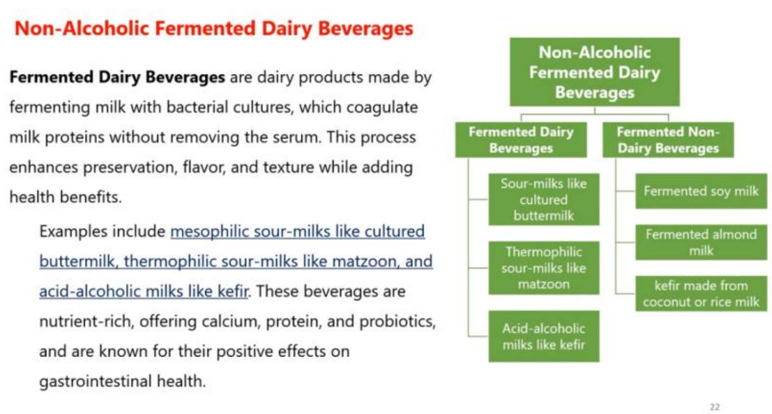
recombinant DNA technology offer new opportunities for improving these bacteria. To enhance their industrial potential, further research in gene expression, protein engineering, and process development is needed. Let us now discuss another kind of non-alcoholic fermented beverage, which is basically dairy beverages. Fermented dairy beverages are products made by fermenting milk with bacterial cultures, which coagulate milk proteins without removing the serum. This process enhances preservation, flavor, and texture while adding health benefits.

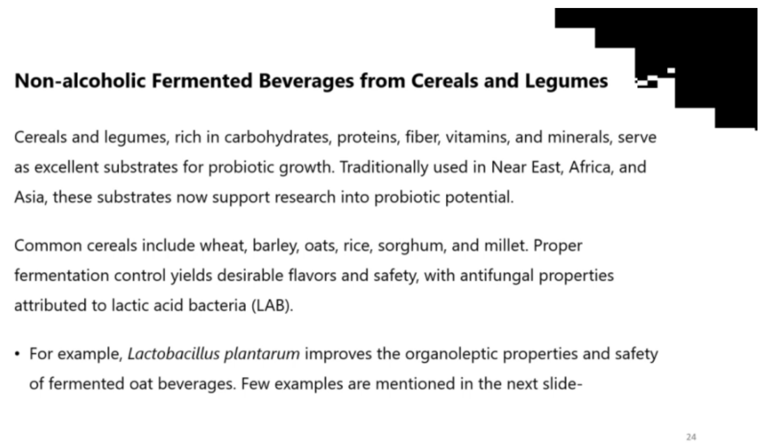
Some of the examples include mesophilic sour milks like cultured buttermilk, thermophilic sour milks like marjoram and acid alcoholic milk like kefir. These beverages are nutrient rich, offering calcium, protein and probiotics and are known for their positive effects on gastrointestinal health. So, we can look into non-alcoholic fermented beverages as two broad types. One which is actually produced with milk and one which is not produced with animal milk but from other type of milk obtained from vegetable sources. So, we have the actual fermented dairy beverages which are sour milks like cultured buttermilk and thermophilic sour milks like Majoon and acid alcoholic milks like Kefir.



Then we have fermented soy milk basically the milk obtained from soy bean is fermented and then we have fermented almond milk and also kefir made from coconut or rice milk so here these fermented non dairy beverages are plant-based drinks produced by fermenting grains fruits or vegetables these beverages are ideal for health conscious consumers offering a lactose-free, low-fat option, rich in bioactive compounds, vitamins, minerals, antioxidants, and dietary fiber. They also serve as excellent carriers for probiotics. Popular examples include fermented soy milk, almond milk, and kefir made from the coconut or rice milk, which I already pointed out.

As interest in non-diarrheal alternatives grows, research continues to focus on their safety, nutritional benefit, and potential for large-scale production. Let us now discuss about fermented beverages from cereals and legumes. Cereals and legumes rich in carbohydrates, proteins, fiber, vitamins and minerals serve as an excellent substrate for probiotic growth. Traditionally used in Near East Africa and Asia, these substrates now support research into probiotic potential. Common cereals include wheat, barley, oats, rice, sorghum and millet.

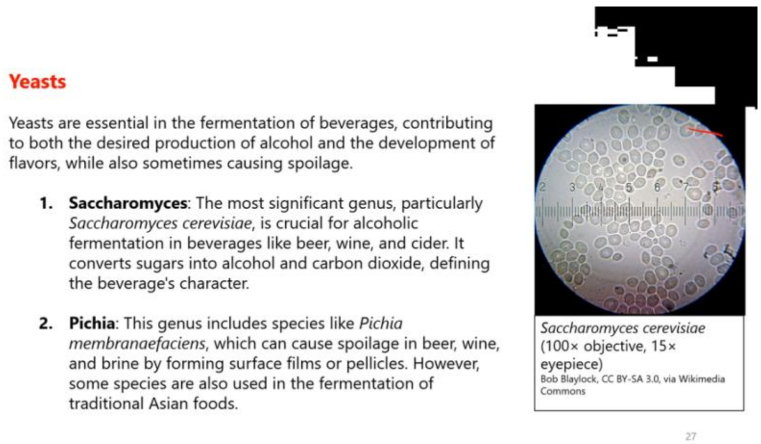
Proper fermentation control yields desirable flavors and safety with antifungal properties attributed to lactic acid bacteria. For example, Lactobacillus plantarum improves the organoleptic properties and safety of fermented oat beverages. Few examples are Boja from Bulgaria and Turkey, a slightly alcoholic brew from mixed cereals fermented by bacteria and yeast. Then we have Amazake from Japan, a sweet, low-alcohol rice



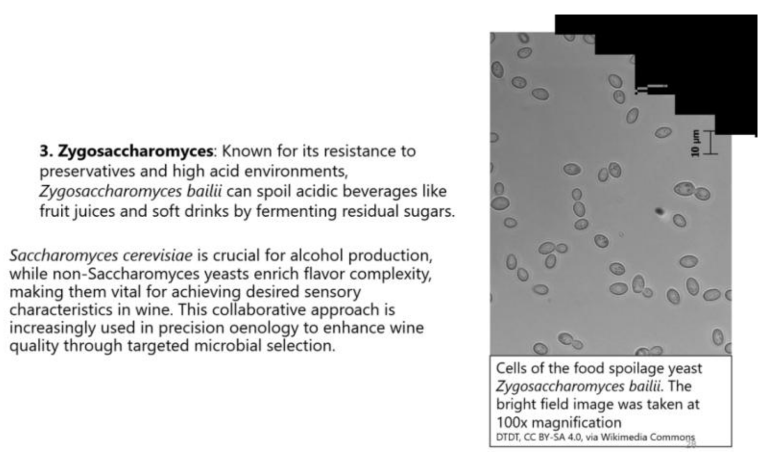
beverage with antioxidant potential. Then we have Siang from India, a mild alcoholic barley-millet drink known for its nutritional benefits, produced with mixed cultures of yeast, LAB, and molds. Then we have Bushera from Uganda, an active fermentation sorghum or millet drink produced with strains like Lactobacillus fermentum. So, this is a glass of Boja wheat cinnamon from Turkey. Now, let us discuss the various microorganisms involved in beverage fermentation.

Let's start with yeast, which is the most popular one. Yeasts are essential in the fermentation of beverages, contributing to both the desired production of alcohol and the development of flavors, while also sometimes causing spoilage. So, this is Saccharomyces cerevisiae, 100x objective, 15x IPs. The most significant genus of Saccharomyces is Saccharomyces cerevisiae. It plays a crucial role in alcoholic fermentation in beverages like beer, wine, and cider.

It converts sugar into alcohol and carbon dioxide, defining the beverage's character. Then we have the piscia, which includes species like piscia membranofacients, which can cause spoilage in beer, wine, and brine by forming surface films or pellicles. However, some species are also used in the fermentation of traditional Asian foods. Then we have Gigosaccharomyces, known for its resistance to preservatives and high-acid environments. Gigosaccharomyces bilei can spoil acidic beverages like fruit juices and soft drinks by fermenting residual sugars.

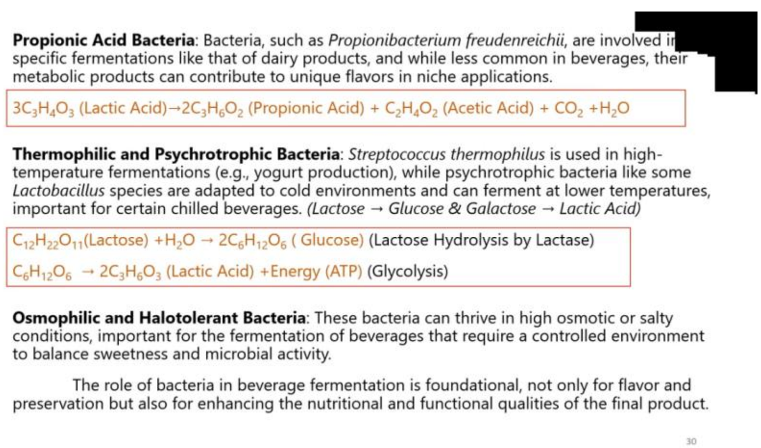


So, here in this picture, you can see cells of the food spoilage yeast Gigosaccharomyces bilei. The bright-field image was taken at 100x magnification. So, we have Saccharomyces cerevisiae, which is crucial for alcohol production, while non-Saccharomyces yeasts enrich flavor complexity, making them vital for achieving desired sensory characteristics in wine. This collaborative approach is increasingly used in precision oenology to enhance wine quality through targeted microbial selection. So, let us now discuss the role of bacteria in the fermentation of substrates to beverages and how they contribute to the development of flavors, preservation, and nutritional benefits.



Some of the key bacterial groups involved in this process are lactic acid bacteria, which include Lactobacillus, Pediococcus, and Leuconostoc. These are essential in fermenting beverages or producing beverages like kefir, contributing to acidity and flavor through lactic acid production. Then we have the acetic acid bacteria group, particularly Acetobacter species, for example, which are responsible for the production of acetic acid in vinegar. They contribute to the unique taste profiles in kombucha by converting ethanol into acetic acid. Then we have the propionic acid bacteria, like Propionibacterium freudenreichii, which are involved in specific fermentations.

like that of dairy products and while these are less common in beverages, their metabolic products can contribute to unique flavors in each applications. So, you have this lactic acid broken down into propionic acid and acetic acid with the release of carbon dioxide and water. Then we have the thermophilic and psychotropic bacteria like Streptococcus thermophilus, which is used in high temperature fermentations, example for yogurt production, while psychotropic bacteria like some lactobacillus species are adapted to cold environment and can ferment at lower temperatures and which are important for certain chilled beverages where lactose is converted to glucose and galactose and then finally lactic acid. Then we have this osmophilic and halotolerant bacteria.

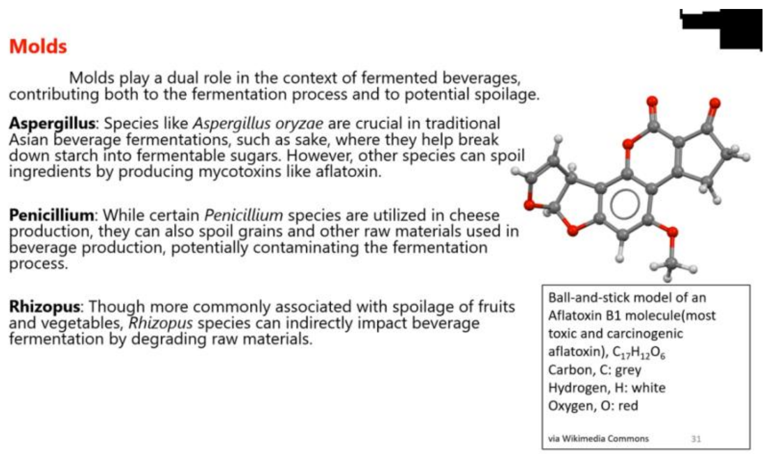


This bacteria can thrive in high osmotic or salty conditions important for the fermentation of beverages that can require a controlled environment to balance sweetness and microbial activity. The role of bacteria in beverage fermentation is foundational or basic, not only for flavor and preservation, but also for enhancing the nutritional and functional qualities of the final product. Then we have another microorganism, the moles, that play a dual role in the context of fermentation. of beverages contributing to both fermentation process and to potential spoilage. Aspergillus, for example, aspergillus oryzae are crucial in traditional

ASEAN beverage fermentation such as sake, where they help break down starch into fermentable sugars.

However, other species of aspergillus can spoil ingredients by producing mycotoxins like alpha toxin. So, this is a ball and stick model of an aflatoxin B1 molecule. This is the most toxic and carcinogenic aflatoxin which is having 17 carbon, 12 hydrogen and 6 oxygen. Then we have these penicillium, certain species of penicillium are utilized in seeds production However, they can spoil grains and other raw materials used in beverage production, potentially contaminating the fermentation process.

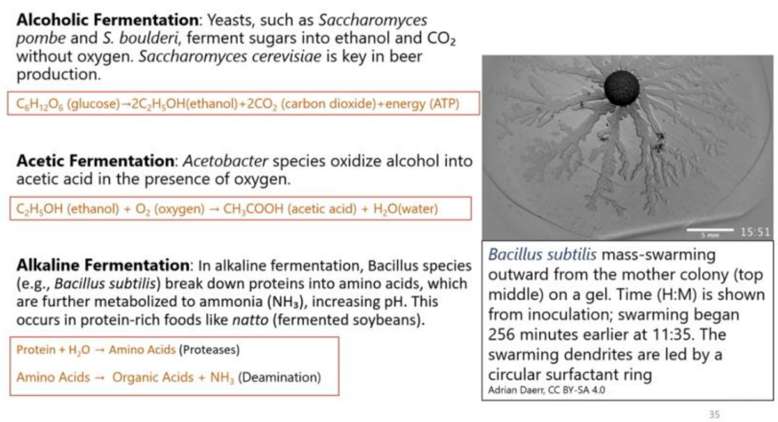
Then we have the Rhizopus. These are most commonly associated with the spoilage of fruits and vegetables. Rhizopus species can indirectly impact beverage fermentation by degrading raw materials. Molds are significant in both enhancing and compromising the quality of fermented beverages, making them essential in production. Let us now move to Section 2, where we will discuss the biochemical processes in fermented beverages and microbial biotechnology in various fermentations.



Let us start with the biochemical processes in fermented beverages. Fermentation processes can be classified based on the microbes involved. For example, we can classify them as lactic acid fermentation. Here, lactic acid bacteria convert carbohydrates to lactic acid without oxygen. There are two pathways. One is the homofermentative

LAB like Lactococcus, Pediococcus, and some Lactobacilli use the glycolytic pathway to primarily produce lactic acid. Then we have the heterofermentative pathway, where LAB like Leuconostoc, Weissella, and some Lactobacilli use the 6-phosphogluconate pathway to produce lactic acid, acetic acid, ethanol, and carbon dioxide. Then we have alcoholic fermentation, such as Saccharomyces pombe, Saccharomyces boulardii, which ferment sugars into ethanol and carbon dioxide without oxygen. Saccharomyces cerevisiae is key in beer production.

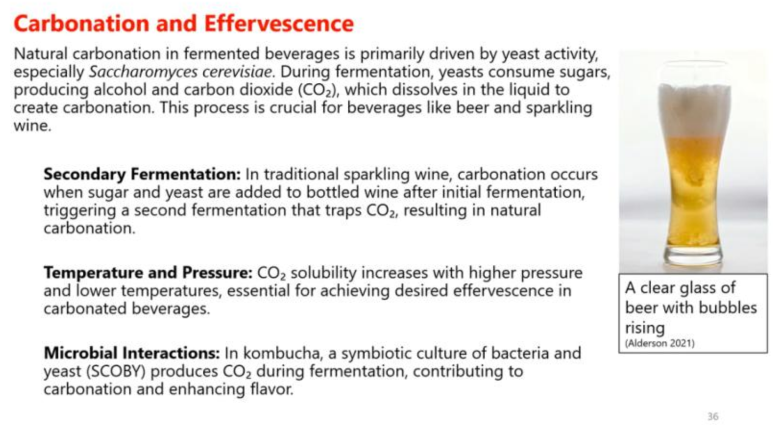
So, you can see here glucose getting converted to ethanol and carbon dioxide with the release of energy. Then we have acidic fermentation where Acetobacter species oxidize alcohol into acetic acid in the presence of oxygen. And then the alkaline fermentation where Bacillus species like Bacillus subtilis break down proteins into amino acids, which are further metabolized to ammonia, increasing pH. This occurs in protein-rich foods like natto. Then here in this picture, you can see Bacillus subtilis mass swarming outward from the mother colony



on a gel. This time in hours per minute is shown from inoculation. Swarming began 2.56 minutes earlier at 11:55. The swarming dendrites are led by a circular surfactant ring. So, let us now discuss one of the important aspects of fermentation in beverages, that is carbonation and effervescence.

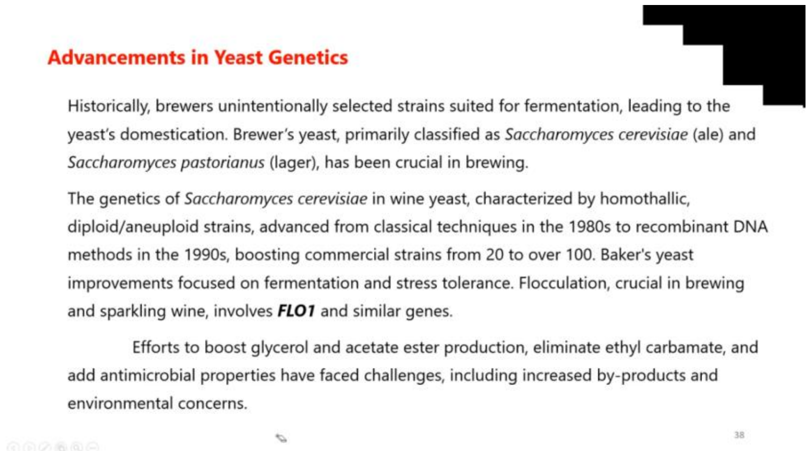
Natural carbonation in fermented beverages is primarily driven by yeast activity, especially Saccharomyces cerevisiae. During fermentation, yeasts consume sugars, producing alcohol and carbon dioxide, which dissolves in the liquid to create carbonation. This process is crucial for beverages like beer and sparkling wine. So, here we can see a clear glass of beer with bubbles slowly rising.

Now, there are secondary fermentations in traditional sparkling wine. Carbonation occurs when sugar and yeast are added to bottled wine. After initial fermentation, a second fermentation is triggered, trapping carbon dioxide and resulting in natural carbonation. Then, we have the importance of temperature and pressure. Carbon dioxide solubility increases with higher pressure and lower temperatures, essential for achieving desired effervescence in carbonated beverages. Microbial interactions, for example in kombucha, involve a symbiotic culture of bacteria and yeast (SCOBY), which produces carbon dioxide during fermentation, contributing to carbonation and enhancing flavor.



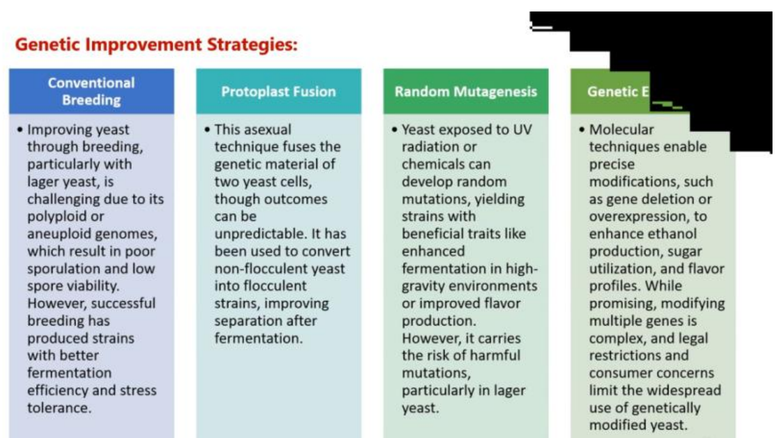
So, let us now have a discussion on microbial biotechnology in beverage fermentation. Historically, brewers unintentionally selected strains suited for fermentation, leading to the domestication of yeasts. Brewers' yeasts, primarily classified as Saccharomyces cerevisiae and Saccharomyces pastorianus—the latter producing lager beer and the former producing ale beer—have been crucial in brewing. The genetics of Saccharomyces cerevisiae in wine yeast, characterized by homothallic diploid-aneuploid strains, advanced from classical techniques in the 1980s to recombinant DNA methods in the 1990s, boosting commercial strains from 20 to over 100. Baker's yeast improvements focused on fermentation, stress tolerance, and flocculation.

Crucellin brewing and sparkling wine involve fluine and similar genes. Efforts to boost glycerol and acetate ester production, eliminate ethyl carbamate, and add antimicrobial properties have faced challenges, including increased byproducts and environmental concerns. So, some of the genetic improvement strategies are conventional breeding, which has been in practice for a long time. Improving yeast through breeding, particularly with lager yeast, is challenging due to its polyploid or aneuploid genomes, which result in poor sporulation and low spore viability. However, successful breeding has produced strains with better fermentation efficiency and stress tolerance.

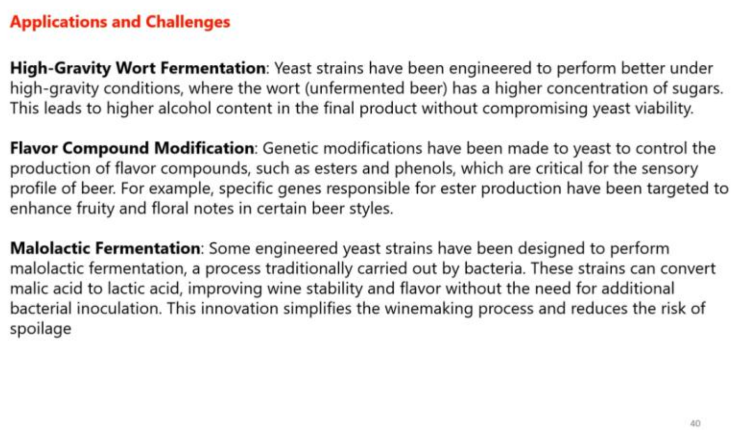


Another approach is protoplast fusion. This asexual technique fuses the genetic material of two yeast cells, though outcomes can be unpredictable. It has been used to convert non-flocculent yeast into flocculent strains, improving separation after fermentation. Then we have random mutagenesis, where yeasts are exposed to UV radiation or chemicals and can develop random mutations, yielding strains with beneficial traits like enhanced fermentation in high-gravity environments or improved flavor production. However, it carries the risk of harmful mutations, particularly in lager yeasts.

Then we have genetic engineering strategies, where molecular techniques enable precise modifications such as gene deletion or overexpression to enhance alcohol production, sugar utilization, and flavor profiles. While promising, modifying multiple genes is complex, and legal restrictions and consumer concerns limit the widespread use of genetically modified yeast for alcoholic beverage production. So, what are the various applications and the challenges? There is high-gravity wort fermentation.



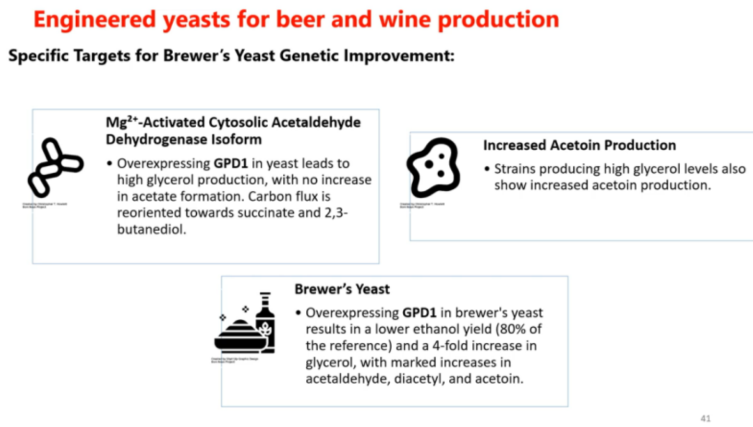
Yeast strains have been engineered to perform better under high-gravity conditions, where the wort and unfermented beer have a high concentration of sugars. This leads to higher alcohol content in the final product without compromising yeast viability. Then we have flavor compound modifications. Genetic modifications have been made to yeast to control the production of flavor compounds such as esters and phenols, which are critical for the sensory profile of beer. For example, specific genes responsible for yeast metabolism have been targeted to enhance fruity and floral notes in certain beer styles.



Then we have the malolactic fermentation. Some yeasts have been designed to perform the malolactic fermentation process traditionally carried out by bacteria. These strains can convert malic acid to lactic acid, improving wine stability and flavor without the need for additional bacterial inoculation. This innovation simplifies the winemaking process and reduces the risk of spoilage. Apart from these applications, let us also discuss the challenges.

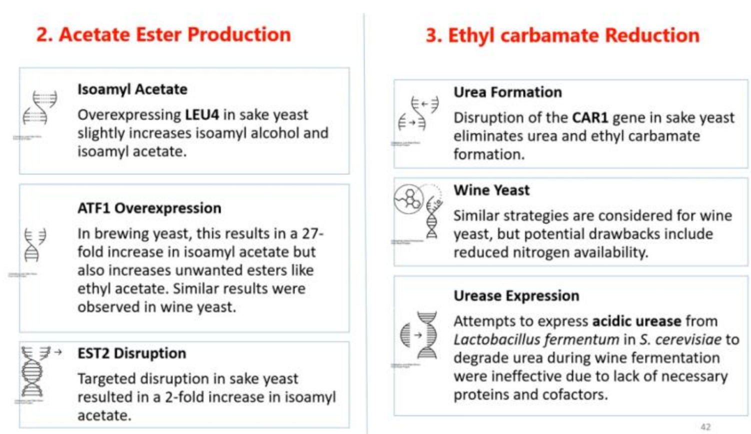
While genetic engineering offers precise improvements, its application in brewing is limited by regulatory and consumer resistance, as we discussed in the earlier slide. However, research continues to refine its techniques, aiming to enhance brewing efficiency and product quality. So, what are some of the specific targets for brewer's yeast genetic improvement, particularly concerning beer and wine production? So, here, in the first case, we have the magnesium-activated cytosolic acetaldehyde dehydrogenase isoform. Overexpressing GPD-1 in yeast leads to high glycerol production

with no increase in acetate formation. Carbon flux is redirected toward succinate and 2,3-butanediol. Then we have increased acetone production, where strains producing high glycerol levels also show increased acetone production. Then, overexpressing GPD-1 in brewers results in a lower ethanol yield (80% of the reference) and a four-fold increase in glycerol, with a marked increase in acetaldehyde, diacetyl, and acetone. So, next, let us discuss acetate ester production, where isoamyl acetate is the target.



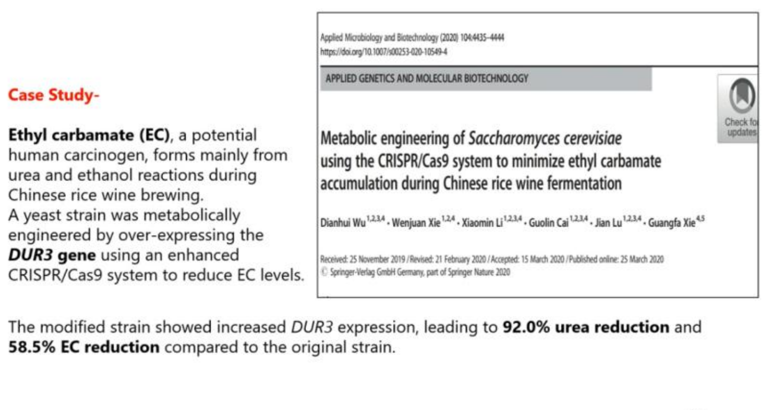
Overexpressing leupho in sake-ist Japanese wine slightly increases isoamyl alcohol and isoamyl acetate. ATF1 expression and overexpression in brewing-ist results in a 27-fold increase in isoamyl acetate. but also increases unwanted esters like ethyl acetate. Similar results were observed in wine yeast. Then there is an EST2 disruption.

This targeted disruption in sake results in a two-fold increase in isoamyl acetate. With regards to ethyl carbamate reduction, the disruption of carbon genes in sake yeast eliminates urea and ethyl carbamate formation, thereby addressing urea formation. Then we have wine yeast; similar strategies are considered for wine yeast. But potential drawbacks include reduced nitrogen availability. Then we have attempts to express acidic urease from Lactobacillus fermentum in S. cerevisiae to degrade urea during wine fermentation, which were ineffective due to the lack of necessary proteins and cofactors.



So, this paper discusses the work done by Wu et al. on metabolic engineering of Saccharomyces cerevisiae using the CRISPR-Cas9 gene editing system to minimize ethyl carbamate accumulation during Chinese rice wine fermentation. Here, ethyl carbamate, a potential human carcinogen, forms mainly from urea and ethanol reactions during Chinese rice wine brewing. This strain was metabolically engineered by overexpressing the dietary gene using an enhanced CRISPR-Cas9 system to reduce ethyl carbamate levels. The modified strain increased dietary expression, leading to a 92% urea reduction and 58.5%

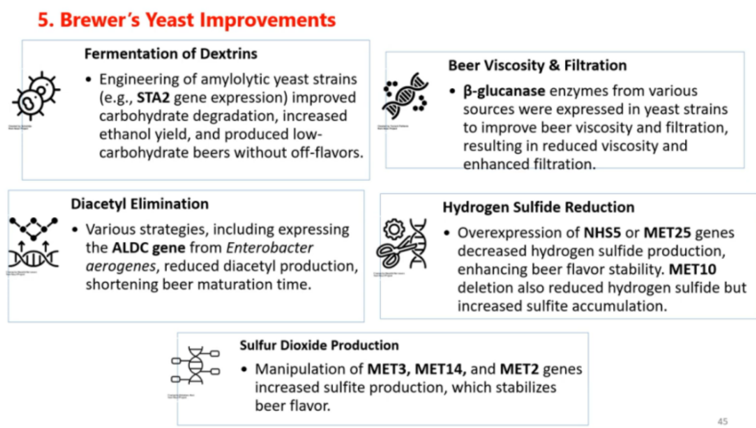
EC reaction compared to the original strain. So here, some of the important things are the antimicrobial properties. For example, in the case of gymosine production, integrating K1-DSRNA into K29 yeast grant resistance against wild yeast strains, Then it also have certain you know bactericidal yeast focus like genes encoding pediocin and leucocin were expressed in Saccharomyces cerevisiae for potential use in reducing sulfur dioxide and other preservatives in wine. Then strains were also developed with both killer factor and antibacterial properties enhancing resistance to contamination thereby providing bioprotection.



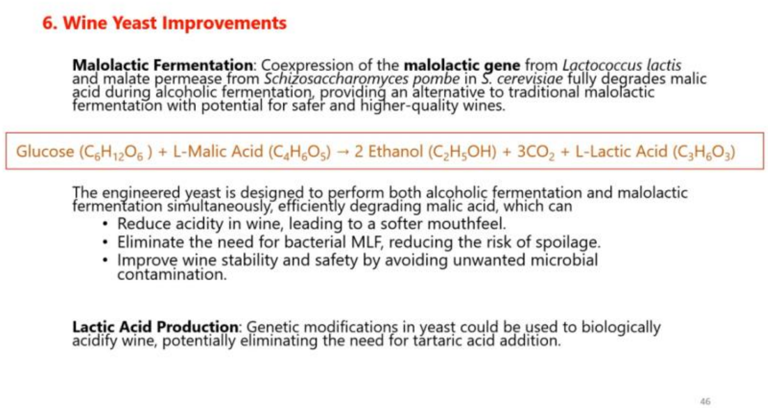
Some of the improvements in brewer's yeast are as follows. The fermentation of dextrins, engineering of amylolytic yeast strains, example star 2 gene expression, improved carbohydrate degradation, increased ethanol yield and produced low carbohydrate beers without off flavors. Then, the viscosity and filtration of beer, for example, beta-glucanase enzyme from various sources were expressed in e-strains to improve beer viscosity and filtration, resulting in reduced viscosity and enhanced filtration. Then there were attempts for diacetyl elimination, various strategies including expressing the ALDC gene from enterobacter aerogens, reduced diacetyl production, shortening beer maturation time. And then attempts were there for hydrogen sulfide reduction by overexpression of NHS5 or

MAT25 genes, which decreased hydrogen sulfide production, enhancing beer flavor stability.

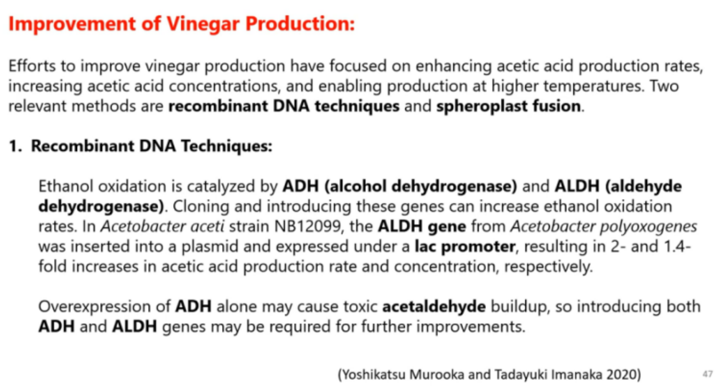
MAT10 deletion also reduced hydrogen sulfide but increased sulfide accumulation. Then there have been attempts for sulfur dioxide production, manipulation of MAT3, MAT14 and MAT2 genes increased sulfide production which stabilizes beer flavor. Some of the improvements in wine yeast like malolactic fermentation, co-expression of the malolactic genes from Lactococcus lactis and Malate Pamias from Caesosaccharomyces pombii in Saccharomyces cerevisiae fully degrades malic acid during alcoholic fermentation providing an alternative to traditional malolactic fermentation with potential for safer and higher quality wines. So, here you can see the engineered yeast is designed to perform both alcoholic fermentation and malolactic fermentation simultaneously, efficiently degrading malic acid which can reduce acidity in wine leading to a softer mouthfeel. Then eliminate the need for bacterial MLF, reducing the risk of spoilage.



Then improve wine stability and safety by avoiding unwanted microbial contamination. Then we have lactic acid production, where genetically modified yeast could be used to biologically acidify wine, potentially eliminating the need for tartaric acid addition. Efforts to improve vinegar production have also been made and have focused on enhancing acetic acid production rates, increasing acetic acid concentrations, and enabling production at higher temperatures. Two relevant methods are recombinant DNA techniques and spheroplast fusion for the improvement of vinegar production. So, let us discuss the recombinant DNA technique.

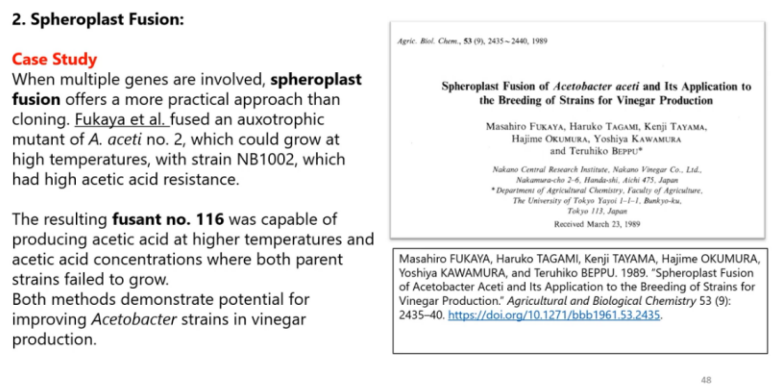


Ethanol oxidation is catalyzed by ADH (alcohol dehydrogenase) and ALDH. Cloning and introducing these genes can increase ethanol oxidation rates. In Acetobacter aceti strain NB 12099, the ALDS gene from Acetobacter polyoxogenes was inserted into a plasmid and expressed under a leg promoter, resulting in 2- and 1.4-fold increases in acetic acid production rate and concentration, respectively. Overexpression of ADH alone may cause toxic acetaldehyde buildup. So, introducing both ADH and ALDS genes may be required for further improvements.



So, let's discuss the work by Fukaya. They have tried spheroplast fusion of Acetobacter aceti and its application to the breeding of strains for vinegar production. When multiple genes are involved, spheroplast fusion offers a more practical approach than cloning. This group, led by Beppu with Fukaya as the lead author, attempted to fuse an oxotropic mutant of Acetobacter aceti (number 2), which could grow at high temperatures, with strain NB1002, which had high acetic acid resistance. The resulting fusion, number 116, was capable of

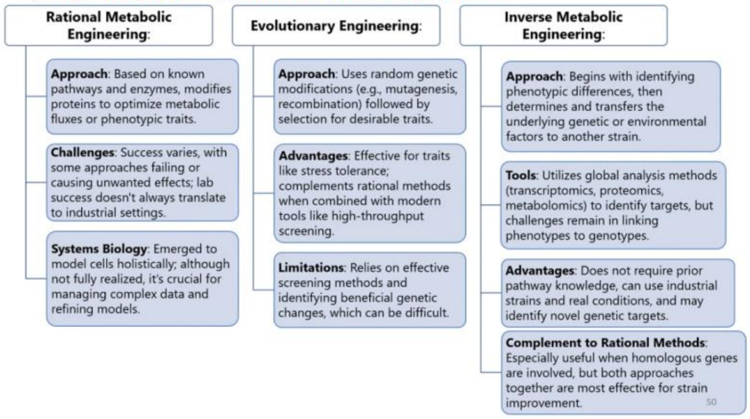
Producing acetic acid at high temperatures and acetic acid concentrations where both parent strains fail to grow. So, this is quite an achievement where the fusion is able to survive in a situation where both the parents are unable to exist. Both methods demonstrate potential for improving Acetobacter strains in vinegar production, particularly through this technique: spheroplast fusion. So, another approach is metabolic engineering. This was introduced by Bailey in 1991 and involves improving cellular activities via manipulation of enzymes, transport, and regulatory functions using recombinant DNA technology.



This requires a holistic approach, iterative process, engineering, analysis, modeling, and integration with other disciplines like systems biology and evolutionary engineering. The goal of metabolic engineering is to modify metabolic fluxes to improve the production of natural or novel metabolites and utilize atypical substrates. There is some overlap with genetic engineering, as both involve targeted specific genetic modifications. Metabolic engineering focuses on improving cellular activities,

which may include stress tolerance. Microbial strain improvement involves rational engineering to modify known pathways, inverse engineering to identify and transfer genetic traits from phenotypes, and evolutionary engineering using random mutagenesis to enhance multigenic traits—all without needing prior pathway knowledge. So, these are some of the engineering approaches for microbial strain improvement in a nutshell. We have the rational metabolic engineering approach, which is based on pathways and enzymes, modifying proteins to optimize metabolic fluxes or phenotypic traits. However, some of the challenges are the variation in success obtained, with some approaches failing or causing unwanted effects. Lab success doesn't always translate to industrial settings, so viability is also a challenge or a question mark many times.

Fig- **Engineering approaches for microbial strain improvement**



Now, from the point of view of systems biology, this has emerged to model cells holistically. Although not fully realized, it's crucial for managing complex data and refining the models. The next approach is evolutionary engineering, which uses random genetic modifications like mutagenesis and recombination, followed by selection for desirable traits. Some of the advantages of evolutionary engineering are that it is effective for traits like stress tolerance and complements rational methods when combined with modern tools like high-throughput screening. However, it also suffers from certain limitations, as it relies on effective screening methods, and identifying beneficial genetic changes can be difficult.

Then we have the inverse metabolic engineering approach, which begins with identifying phenotypic differences, then determines and translates the underlying genetic or environmental factors to another strain. Some of the tools here utilize global analysis methods like transcriptomics, proteomics, and metabolomics to identify targets, but challenges remain in linking phenotypes to genotypes. However, some advantages are that it does not require prior pathway knowledge, can use industrial strains in real conditions, and may identify novel genetic targets. So, this is a complement to rational methods, especially useful when homologous genes are involved.

But both approaches together are most effective for strain improvement. So, with this, we come to the end of today's lecture.