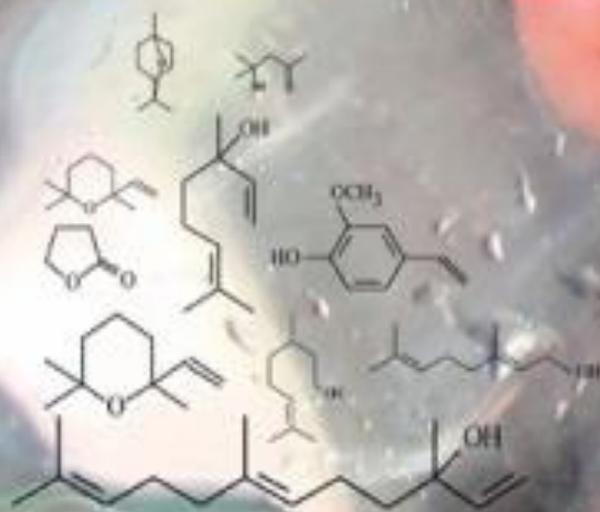


Trends in Biotechnology



Yeast
diversity
and
flavor complexity

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In food biotechnology the ‘flavor phenotype’ is considered an extremely important property when developing yeast screening methods. Grape and wine microbiology research has contributed significantly to our understanding of how native flora can increase flavor complexity and the final sensory quality of wines. In pages 148–154 of this issue, Carrau and colleagues discuss opportunities for improving the aroma and flavor of fermented beverages by harnessing the wide natural diversity of yeasts.

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Yeast diversity and native vigor for flavor phenotypes

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***Saccharomyces cerevisiae*, the yeast used widely for beer, bread, cider, and wine production, is the most resourceful eukaryotic model used for genetic engineering. A typical concern about using engineered yeasts for food production might be negative consumer perception of genetically modified organisms. However, we believe the true pitfall of using genetically modified yeasts is their limited capacity to either refine or improve the sensory properties of fermented foods under real production conditions. Alternatively, yeast diversity screening to improve the aroma and flavors could offer groundbreaking opportunities in food biotechnology. We propose a ‘Yeast Flavor Diversity Screening’ strategy which integrates knowledge from sensory analysis and natural whole-genome evolution with information about flavor metabolic networks and their regulation.**

Food fermentation and consumer preference

Fermented foods were originally developed by our ancestors as a biological way to preserve different fresh agricultural products such as fruit juices, milk, or meat. The challenge in those times was to extend shelf-life, freshness, flavor, and edibility of food after harvest. The objective was to conserve freshness without adding preservatives such as salt or vinegar, which can dramatically affect sensory characteristics. After many centuries of accumulating practical knowledge, mastery of fermentation enabled the management of what chemists of the 19th century called ‘enzyme activities.’ This involved decreasing pH or generating compounds such as ethyl alcohol in fermented food to prevent microbial food spoilage. Interestingly, the discovery of the biological basis of yeast fermentation was first accomplished in industrial brewing settings [1,2]. The role of yeasts in the fermentation of sugars to alcohol and carbon dioxide has been known for almost two centuries. However, well over a half of the 19th century elapsed before the role of yeast strains in the production of different wines was published by Pasteur in 1866 [3].

Today, food fermentation is all about increasing the sensory quality for the consumer, and obtaining unique

signature flavors that help to distinguish a product from others on the market [4]. Consumer flavor sensations, not other benefits such as increased shelf-life or nutritional value, may in fact be the key factor that defines a successful food product. The increasing preference for designer wine, beer, or cheese among consumers is an excellent illustration of the power of flavor. This increased interest in specialty foods will continue to grow among affluent consumers (see Glossary) as they refine, improve, and discover their tasting abilities all around the world. These discerning consumers select products based on taste, preferring to pay more for a refined sensation, rather than less for quantity [5].

Gene manipulation and flavors

The aroma profile of fermented foods and beverages comprises hundreds of compounds, many of which have

Glossary

Affluent consumers: in marketing and financial services, consumers whose wealth or income is above the average. Luxury consumers look for products of outstanding quality and high performance that are well worth the price. In food products these consumers are considered as ‘enthusiastic’ or more knowledgeable for enjoying flavors and premium foods.

Ecotilling: the mutation detection technology used in ‘Tilling’ (targeting induced local lesions in genomes) was adapted to the discovery of polymorphisms in natural populations. This technology could help to find native proteins with improved functional designs. The technology is applicable to any organism including those that are heterozygous and polyploid, as is the case for many native types of yeast.

Evolutionary engineering: continuous evolution procedures based on the application of an artificial selection pressure to obtain a desired phenotype.

Flavor complexity: multisensory experience through which the human beings perceive a complex mixture of volatile and non-volatile molecules in foods.

Globalizing quality: the term is employed in the context of assuring a basic uniform quality of fermented beverages using a pure and standard ferment inoculum. This lack of differentiation between the final products does not favor consumer attention in a huge competitive market.

Natural biodiversity: the degree of variation of life in terms of genetic variation, ecosystem variation, or species variation (number of species) within a given area.

Polyphenolic maturity: during red grape maturation in the vineyard, sugars increase concentration and the phenolic compounds, mainly present in seeds and skin, are usually considered mature once sugar maturity is reached. After fermentation, higher alcohols are obtained, and mature phenolics are softer and not astringent as they can be if the grapes are still green.

Sensory analysis: scientific discipline that applies principles of experimental design and statistical analysis to the use of human senses (sight, smell, taste, touch, and hearing) for the purpose of evaluating consumer products.

Yeast native vigor: genetic variation present in particular native yeast strains that can outperform laboratory strains for specific traits such as speed of fermentation, temperature-tolerance, or the production of compounds that may influence the aroma of the fermented food product. These strains are expected to be better adapted to industrial conditions in non-sterile fermentation media.

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sensory thresholds corresponding to very low concentrations ($\mu\text{g/L}$). An aroma profile interacts with hundreds of olfactory receptors triggering the transduction and integration of diverse and complex signals within the human brain [6,7]. Current yeast genetic engineering (GE) approaches designed to improve fermented beverage flavor take one or very few aroma compounds as the subject of study and manipulation. Examples of these approaches are scarce [8–10] and, to the best of our knowledge, subsequent formal sensory analysis and evaluation of the final product is exceptional [10]. Besides negative consumer perception of genetically modified organisms, the current limitation to the use of these strains under real winemaking conditions is the lack of stability or cell vigor of these strains in industrial settings [11]. Other breeding strategies or generation of interspecific wine yeast hybrids have successfully improved wine flavor by reducing off-flavor production and enhancing volatile thiol release in *Saccharomyces* [12,13].

From a different perspective, genetic manipulation to improve other traits can affect flavor-compound levels indirectly as was seen in several attempts to decrease ethanol yield in wines. In many wine regions the optimal polyphenolic maturity of grapes is obtained with excess sugar content, but this results in the undesired byproduct of high ethanol-containing wines [14]. The strategy to

resolve this issue was to decrease ethanol yield, thus increasing glycerol formation from sugars during wine fermentation [15–20]. Overexpression of the *GPD1* gene, which encodes glycerol-3-phosphate dehydrogenase, in combination with the deletion of *ALD6*, encoding acetaldehyde dehydrogenase, yields the desired changes in metabolite accumulation (Box 1). These two alterations resulted in increased glycerol production and reduced acetate formation [17,18]. Yeast strains were successfully engineered, yielding the desired levels of glycerol, ethanol, and acetate, but the wines presented unacceptable aroma characteristics. This undesired outcome was the result of significant changes in the aroma flavor profiles (higher alcohols, acids, esters, etc.) due to redox balance-dependent compensatory regulation of NAD^+/NADH pools in the modified strains (Box 1).

Primary metabolism is resilient to changes in flux distributions that are optimal for cell growth [21]. Genetic alterations that target primary metabolism (affecting compound concentrations by g/L) generate unpredicted redistributions of micrometabolic fluxes, profoundly affecting the concentration of compounds that are within the $\mu\text{g/L}$ range [21,22]. These changes are mainly due to compensatory regulation of redox balance within the cell, which can affect many flavor pathways [23]. Recently it was demonstrated in cheese, yogurt, and wine that slight changes of a few mV in

Box 1. Carbon metabolism and intracellular redox balance significantly affect wine flavor phenotype

In a scheme (Figure 1) based on experiments reported by Eglinton *et al.* and Cambon *et al.* [17,18], the aim was to construct strains that produce wine with increased glycerol and decreased ethanol contents. Manipulation of primary metabolism alters NAD^+/NADH balance, and concomitant reinstallation of redox homeostasis largely impacts on small-scale ($\mu\text{g/L}$) byproduct yields. For

example, the formation of higher alcohols increases NAD^+ , and biosynthesis of isoacids increases NADH . Both families of compounds could be considered as undesired aromas. Flavor metabolic pathways had a significant susceptibility to genetic modifications that affect redox and also ATP/ADP balances during fermentation.

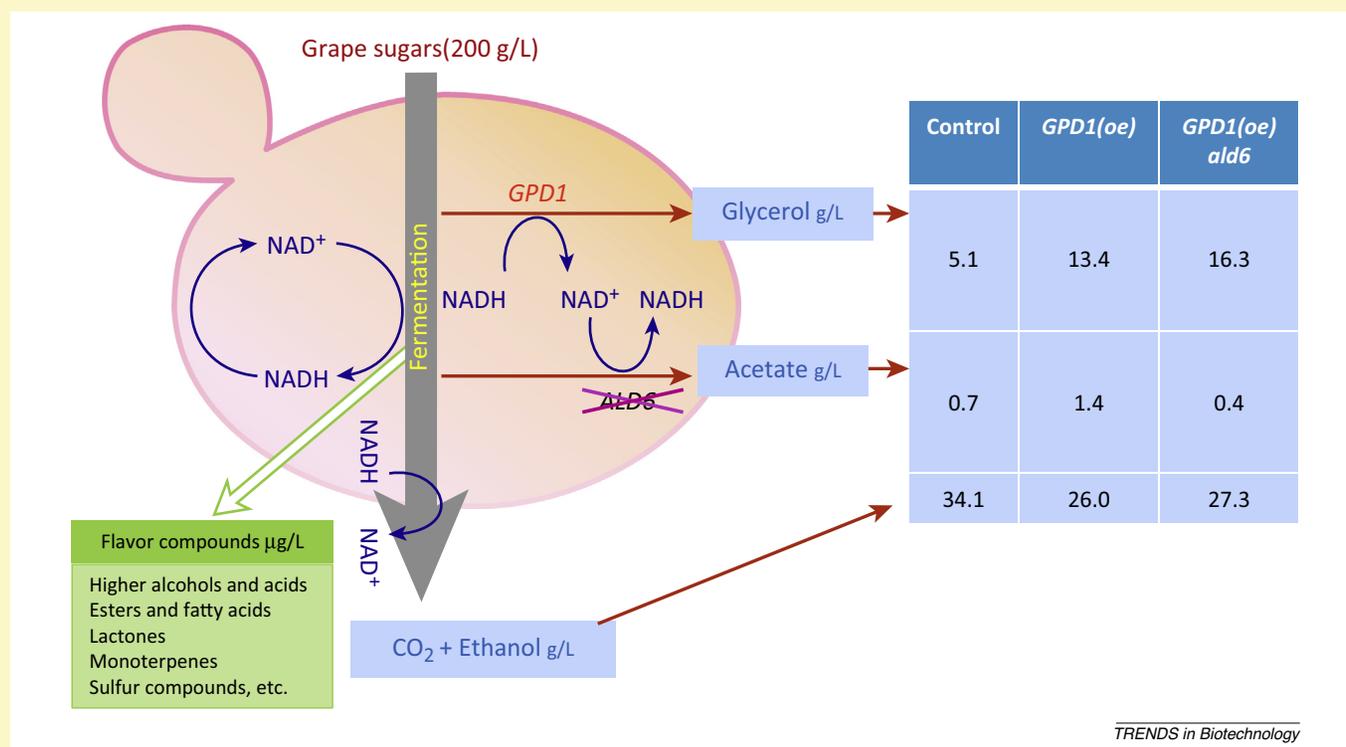


Figure 1. Carbon phenotypes and wine flavor. Abbreviations: ALD6, aldehyde dehydrogenase; GPD1, glycerol-3-phosphate dehydrogenase; oe, overexpression.

the redox potential during fermentation greatly affect the aroma profiles of the final product [24–26]. This suggests that single-gene modifications affecting cell redox balances might modify diverse fermentation pathways, which will certainly impact on the flavor-compound matrix. Other approaches in addition to GE have been used to pursue the goal of obtaining wines with lower ethanol levels [14,19,27,28]. Whole-genome evolutionary engineering approaches are currently aided by adaptive evolution strategies customized to select organisms with desired phenotypes [22]. Although there have been many experiments using adaptive evolution strategies to improve yeast carbon fluxes such as enhanced maltose or xylose fermentation or ethanol resistance [29], there are limited examples of applications to aroma quality improvement of fermented beverages [30]. The question of whether evolutionary engineering approaches could be useful for improving yeast primary metabolism pathways to enhance the flavor phenotype quality remains unanswered. Various metabolic engineering strategies for reducing glycerol production and increasing ethanol content have been successful in the biofuel industry because, unlike the food industry, flavor phenotypes are unimportant.

Increased biodiversity and flavor complexity

There was a time when the key differences between wine and beer producers and their brands were the levels of artistry and knowledge of their winemakers or beermasters. The ability to manage spontaneous microbial fermentation before Pasteur's time was achieved through a patient and knowledgeable ability to control the process at an industrial level. One hundred years ago [31] there was great enthusiasm for fermentation with 'pure yeast' using new microbiological techniques. It was expected that having pure strains would globalize quality in production of fermented beverages. However, today some winemakers or beermasters are, in fact, rediscovering the value of using mixed cultures or spontaneous fermentation to increase flavor complexity [32–35]. In wine, non-*Saccharomyces* yeast strains that account for more than 99% of the grape native flora have a well-recognized genetic diversity. However, understanding their impact on flavor richness of wines is still poorly explored [8,36,37].

Biodiversity is generally defined as a function of species number, abundance, and spatial distribution, as well as the genotype interactions, functional types, and traits that are present in a specific system [38]. It is widely believed that increased biodiversity in a given ecosystem increases its stability [39]. This idea is also demonstrated in microbial fermentation ecosystems [40]. Today some yeast microbiologists contend that the limited number of commercial yeast strains used for winemaking throughout the world contributes to the production of wines with relatively uniform style. This leads to the compromise of potential diversity in wine character in a highly competitive market [5]. In addition, vineyards close to a winery can be affected by commercial *S. cerevisiae* strains used nearby, and the frequency of commercial strains can increase from 0 to 10% within the natural grape microflora after a few vintages [41]. Regional and site-specific environments, native flora, fauna, and grape variety can also shape the fungal consortia inhabiting wine grapes [42]. Interestingly, the social

wasp gut is a key environmental niche for the evolution of natural *S. cerevisiae* population diversity during winter when fruits are not available [43]. All of these factors contribute to shape the unique microbial input to regional variation in wine grape quality. The presence of commercial yeast strains at a winery could affect region-specific wine characteristics [41], risking a decrease in wine quality and complexity. French producers refer to this effect as losing wine 'terroir' differentiation [44,45].

Yeast diversity and foods: flavor traits matter most

In metabolomics, functional traits matter most in a given ecosystem. However, in food biotechnology the 'flavor phenotype' can be considered to be an extremely important property when developing yeast selection methods [46]. More than 2500 volatile compounds are found in foods, and the human olfactory system has over 400 receptors, which work in a combinatorial fashion [6,7]. In addition, compounds that affect flavor are more affected by polygenic features than are traditional enological traits such as alcohol tolerance, low acetic acidity, or good fermentation rate at low temperature [27,47–49].

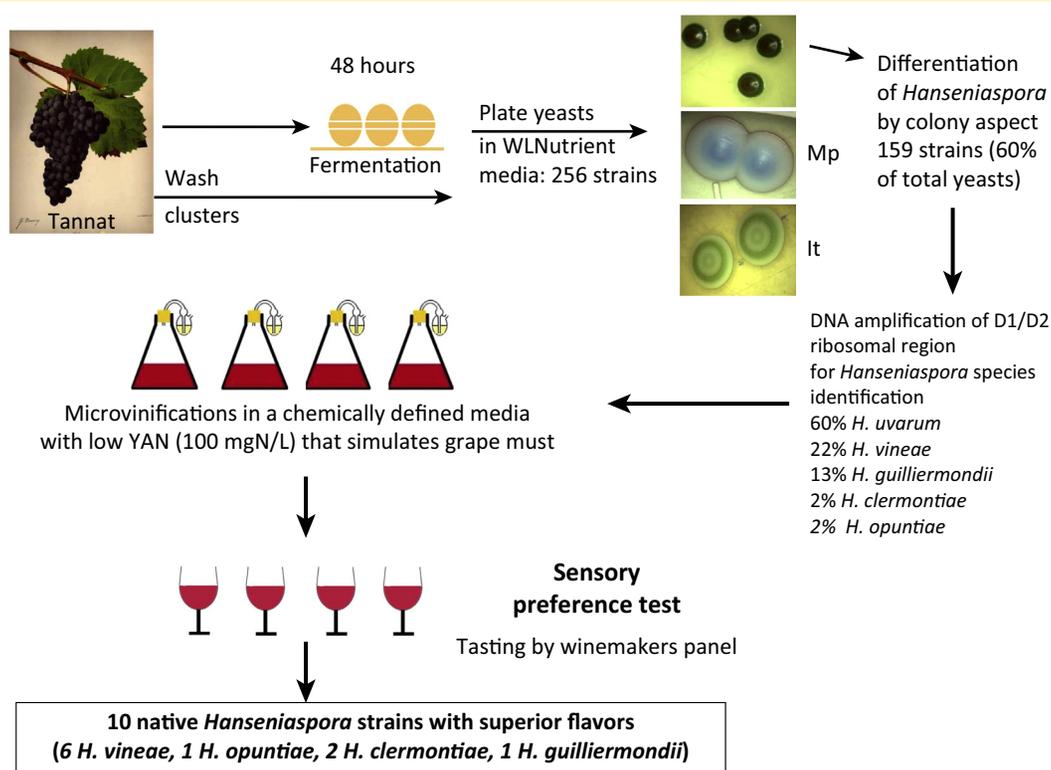
Because flavor traits are not necessarily essential for energy-yielding metabolism or cell survival, we can speculate that underlying genetic pathways are less affected by the positive selection pressures that contribute to increased whole-genome variability during microbial evolution [47,50]. In the same way, dynamic interactions between cells and fermentation media and microbial competition during food processes increase the complexity of the system, resulting in hard-to-predict phenotype–genotype interactions [50]. Furthermore, compounds that are normally considered to be plant metabolites, such as resveratrol or monoterpenes, can also be produced by endophytic fungi during growth within the plant [51,52], as well as during yeast grape-must fermentation from sugars [53].

Grape and wine microbiology research has contributed significantly to understanding how complex natural flora can affect the fermentation behavior of a commercial yeast inoculum, as well as how to achieve spontaneous fermentation [54,55]. Spontaneous fermentation or co-inoculation with a non-*Saccharomyces* yeast, *Hanseniaspora vineae*, increased strain diversity and sensory wine quality, in comparison to conventional commercial yeast fermentation alone for producing white Chardonnay wine under real winemaking conditions [55]. Increased yeast diversity was correlated with production of more stable wines because complete exhaustion of key nutrients and growth factors occurred after fermentation [56]. This has the potential effect of preventing further contamination. The *H. vineae* strain used had been previously identified along with other native grape yeasts, representing species capable of producing 'good flavor' wine. A collection of 800 native grape yeasts was screened primarily by sensory analysis to identify these 'good flavor' strains. The process utilized for screening the main yeast group, *Hanseniaspora* genus, provides a specific example of the general strategy (Box 2). The complexity of mixed culture fermentations and the impact of yeast diversity on flavor phenotypes indicate that the development of integrated research approaches is required for success in generating new yeast

Box 2. Screening methods for superior flavor phenotypes

The challenge today is the development of screening methods to identify strains that improve wine quality from the great, unexplored diversity of natural grape yeasts. An example of such a screen is a method utilized to identify yeast strains from Tannat grapes that produce wines with superior flavors (Figure 1). Yeasts from the *Hanseniaspora* genus (apiculate yeasts) were predominant, but two other examples of yeasts are also shown: *Issatchenkia terricola* (It) and *Metschnikowia pulcherrima* (Mp). Less than 10% of the isolated *Hanseniaspora* strains resulted in controlled microvinification with

relevant aroma characteristics [32]. Interestingly, although *H. uvarum* was the most abundant species isolated (60%), not even one *H. uvarum* strain showed good flavor quality. If this is confirmed by further studies, *H. uvarum* strains could be discarded in the final steps of screening, reducing microvinification and sensory analysis tests. This strategy is based on identifying non-*Saccharomyces* strains that will improve overall flavor by adding more complexity and including all aspects of flavor perception. To achieve this goal we propose to use the most sensitive instrument: the human noses of a trained panel.



TRENDS in Biotechnology

Figure 1. Methods for identifying yeast from Tannat grapes with superior flavor phenotypes.

strains adapted to the food industry. The use of non-*Saccharomyces* yeasts and mixed cultures with *Saccharomyces* to increase flavor complexity in fermented beverages will offer novel opportunities to the food industry.

Native strain identification and screening methods

It is very likely that only a small percentage of the total native yeast diversity has been cultivated, and thus only a small fraction of the metabolites these yeasts can produce during fermentation have been identified [8,57,58]. Thanks to the development of molecular techniques, great advances have been made in strain identification and differentiation within *Saccharomyces* [47,59]. These methods were of fundamental importance for strain differentiation within *S. cerevisiae*, and new methods are being used to discriminate strains of other yeast species at a subspecies level [60]. This technology could help yeast breeders to identify new native yeasts of interest from among complex native microbial consortia.

Yeast metabolic footprinting is an analytical method for determining levels and identities of extracellular metabolites consumed or produced by a strain during

fermentation. Metabolic footprinting is considered to be more effective in discriminating strains of a given species than are genetic, transcriptomic, or proteomic methods [61]. Protocols for standardization of growth medium composition, inoculum size, fermentation vessels, temperature, etc. have been crucial to developing data models that enable discrimination of industrial yeast strains. For example, simply changing the inoculum size in single-strain *Saccharomyces cerevisiae* fermentations significantly affects aroma compound synthesis [62]. Thus, these protocols will allow new and improved metabolic footprinting methods for detecting aroma compounds produced during fermentation [63]. Then one can ask: how will synthesis of aroma compounds be affected if we increase diversity in this particular system by changing the number and strain proportions in mixed culture fermentations?

Interestingly, a new concept known as Functional Biodiversity Research (FBR) aims to identify mechanisms and effects of biodiversity changes on ecosystem functions [64]. Refinement of this idea may allow the design of strategies that address microbial complexity and flavor

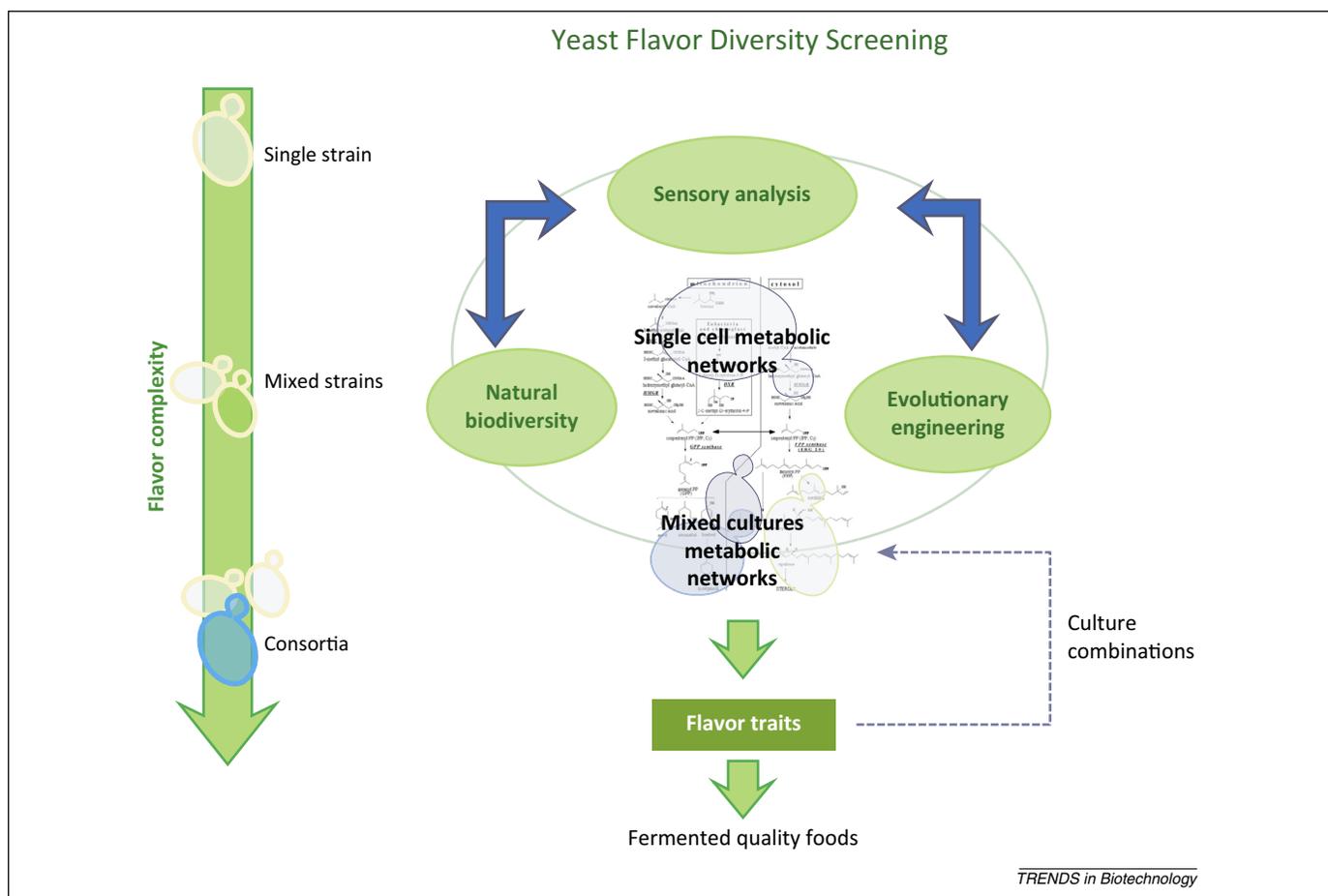


Figure 1. Scheme illustrating the Yeast Flavor Diversity Screening concept for food biotechnology. Sensory analysis screening methods will allow the identification of strains with preferred flavor phenotypes from among the natural diversity of yeast. After a novel native strain is identified, improvement techniques based on a combination of evolutionary engineering and mixed culture fermentations will increase flavor diversity and complexity.

traits in fermented foods, with the objective of increasing sensory quality.

We noted that food biotechnology and fermentation, from the consumer point of view, is all about sensory characteristics of the final product: flavors and the tactile sense of texture. Accordingly, wine fermentation is one of the best fermentation model systems to study and learn about what we define here as a Yeast Flavor Diversity Screening strategy, in which different cell numbers, genotypes, species interactions, nutrient flavor precursors, etc. all dynamically coexist within the wine matrix (Figure 1).

We show here that diversity in native grape yeasts and the identification of strains with ‘native vigor’ can offer fermentation biotechnologists an excellent opportunity to improve the ‘flavor’ of fermented foods. Ecosystems such as fruits, flowers, insect guts, etc. are known natural sources of yeast biodiversity [65]. New screening methods will increase food biotechnologists’ knowledge of native yeast strains capable of conferring desirable aromas to fermented food while exhibiting vigorous adaptation to fermentation in an industrial setting. Searches for protein variants in genes of interest could be performed by adapting the ecotilling strategy defined by plant biologists [66]. Native strain improvement programs that integrate evolutionary engineering strategies, such as adaptive evolution, comparative population genomics, quantitative trait analysis,

or interspecific hybridization, may help to meet the challenge of improving the flavor phenotypes of industrial food yeasts. The identification of new yeast strains that are already adapted to a wild environment, or to complex mixed culture strategies that enhance the sensory complexity of fermented beverages, remains an important goal in food biotechnology (Figure 1).

Concluding remarks and future perspectives

We have discussed how the manipulation of primary metabolic pathways can negatively impact upon the flavors of fermented products. A quantitative understanding of metabolic network behavior during fermented beverages production, encompassing a wide range of metabolites identities and concentrations (from g/L to $\mu\text{g/L}$), will require new strategies. Alternatively, high-throughput sequencing and efficient genome-assembly technologies applied in non-model organisms have recently enabled the identification of many native genes related to novel phenotypes [67–69], aiding the development of desired flavors. Microbial diversity screening strategies, mimicking the principles of natural whole-genome evolution, will also have a real impact on traditionally fermented food quality.

In other industrial scenarios, such as biofuel production, effluent treatment, or biopharmaceutical production, conventional metabolic engineering strategies using

S. cerevisiae and other non-*Saccharomyces* strains can provide quality yeasts. However, the limitations and challenges of applying metabolic engineering to flavor phenotypes should be faced and contrasted with the potential and importance of natural biodiversity. We propose that future breakthroughs in the rational design of microbial flavor phenotypes will necessarily come from the integration of knowledge coming from three areas: natural yeast diversity, sensory analysis experience, and evolutionary engineering.

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