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Review

Yeast Hybrids in Brewing

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Abstract: Microbiology has long been a keystone in fermentation and the utilization of yeast biology reinforces molecular biotechnology as the pioneering frontier in brewing science. Consequently, modern understanding of the brewer's yeast has faced significant refinement over the last few decades. This publication presents a condensed summation of *Saccharomyces* species dynamics with an emphasis on the relationship between traditional ale yeast, *Saccharomyces cerevisiae,* and the interspecific hybrids used in lager beer production, *S. pastorianus*. Introgression from other *Saccharomyces* species is also touched on. The unique history of *Saccharomyces cerevisiae* and *Saccharomyces* hybrids are exemplified by recent genomic sequencing studies aimed at categorizing brewing strains through phylogeny and redefining *Saccharomyces* species boundaries. Phylogenetic investigations highlight the genomic diversity of *Saccharomyces cerevisiae* ale strains long known to brewers by their fermentation characteristics and phenotypes. Discoveries of genomic contributions from interspecific *Saccharomyces* species into the genome of *S. cerevisiae* strains is ever more apparent with increased investigations on the hybrid nature of modern industrial and historical fermentation yeast.

Keywords: hybrid, lager, yeast, introgression, interspecific, domestication, phylogeny, brewing, molecular, genomics

Species of *Saccharomyces*

Saccharomyces cerevisiae may be one of the oldest domesticated organisms known to humanity. Domestication events imposed on brewing strains of the budding yeast species, *S. cerevisiae,* resulted in unique strains similar to the diverse animal lineages of *Canis familiaris* breeds or plant lineage of *Brassica oleracea* foods. It has been suggested that *S. cerevisiae* is a synanthropic species following human settlements as a commensal organism residing in gardens, vineyards, and the like. When and where yeast originated has been the subject of much debate throughout time. Domesticated *Saccharomyces* brewing yeast feature flocculation capabilities, fast fermentation rates, malt sugar utilization, pleasant aromas, and sometimes phenolic (POF) negative [1,2]

Well over a century has passed since the first accessible description of the brewer's yeast in 1838 and its recognition in fermentation [3,4]. Recently, phylogenic research utilizing genomics and modern molecular biology techniques has shed some light on the historically messy nomenclature surrounding this budding yeast. The modern *Saccharomyces* genus is defined by eight species to include *S. cerevisiae, S. paradoxus (syn. S. cariocanus, S. cerevisiae var. tetraspora, S. cerevisiae var. terrestris, S. douglasii), S. uvarum (syn. S. bayanus var. uvarum), S. mikatae, S. kudriavzevii, S. arboricola (syn. S. arboricolus), S. eubayanus, and S. jurei* [3,5–19]. Moreover, two natural hybrids are recognized in the *Saccharomyces* clade as *S. pastorianus* (syn. *S. carlsbergensis*, *S. monacensis*) and *S. bayanus* [20–22]. Latest genomic analysis of the genomic nucleic acid sequence of *Saccharomyces* yeast has consolidated many variations into the eight individual species (Table 1).

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Table 1. Current *Saccharomyces* Yeast Species History.

Saccharomyces	Described	Substrate	Location	Reference
cerevisiae	1838	Beer	Germany	Meyen, 1838
uvarum	1898	*Ribes rubrum*, redcurrant juice	South Holland, Netherlands	Beijernck, 1898
paradoxus	1914	Tree sap	Russia	Batshinskaya, 1914
kudriavzevii	1991	Decayed leaf	Japan	Kaneko, 1991 Naumov, 200
mikatae	1993	Decayed leaf	Japan	Yamada, 1993 Naumov, 200
arboricola	2008	*Fagaceae* spp	West China	Wang, 2008
eubayanus	2011	*Nothofagus* spp & parasitic fungi *Cyttaria* spp	Andean, Patagonia	Libkind, 2011
jurei	2017	*Quercus robur*	Saint Auban, France	Naseeb, 2017

Hybrid Nature of Yeast

Interspecific hybrids are not unique to lager brewing, the livestock and agricultural industries commonly employ selective breeding to increase yields [23–25]. A time-honored showpiece of hybrid vigor is the mule, a great pack animal known for its hardiness and longevity. For over 4,000 years this hybrid is the progeny of a male donkey and a female horse. Since the early 1900s maize has been hybridized to increase yields and introduce biodiversity [26]. Hybrid yeast has been isolated from fermentations on numerous occasions (Figure 1). A hybrid between *S. cerevisiae* x *S. kudriavzevii* has been isolated from Belgian Trappist beers [27]. Popular in wine production, strain VIN7 is a hybrid of *S. cerevisiae* x *S. kudriavzevii* [28]. Additionally, other interspecific *S. cerevisiae* x *S. uvarum* hybrids are regularly used for production of wines [29,30]. Spontaneous fermentations have also yielded *Pichia apotheca*, a hybrid of *P. membranifaciens* and an unknown species [31]. Hybrid vigor, or heterosis, confers a competitive advantage by facilitating transgressive phenotypes in changing environments, and has been known to be a driver of fungal evolution and adaptation [32]. Importantly, during the many stages of fermentation and maturation, there lie opportunity for a microbially competitive environment in which rapid adaptation may be advantageous.

The mule of the brewing industry is the lager yeast *S. pastorianus,* an interspecific hybrid that produces the lions share, in volume, of the global beer production. Although its use is widespread, the biodiversity is limited by two main lineages, Saaz / group I (*syn. S. carlsbergensis,* (L31: Czechers, type strain CBS1513*)* and Frohberg/ group II (L13: Global, type strain - Weihenstephan 34/70). Saaz and Frohberg lineages vary in their genomic composition from each parent species, *S. eubayanus* and *S. cerevisiae,* which influence important fermentation characteristics. Application of genomic analysis found Saaz lineage to have a genomic composition of 1:2 *S. cerevisiae* to *S. eubayanus* sub genome and frohberg lineage to be 2:2 *S. cerevisiae* to *S. eubayanus* sub genome which supported the two traditional designations used by brewers [33–35]. Saaz lineage hybrids are very well adapted to cold fermentations but lack maltotriose utilization. The Frohberg hybrids contain more *S. cerevisiae* genomic content conferring greater attenuation, higher ethanol production, differing ester profiles, and higher typical viabilities. [36]. The composition of genetic material transferred and retained in the hybrid imparts important fermentation characteristics and phenotypes such as the POF (phenolic off flavor) trait, efficient fermentation of maltose and maltotriose, reduction of diacetyl, flocculation, production of unique volatile metabolite profiles that are low in off-aroma/flavors. Investigations into these hybrid lineages support bolstering of the fermentation capacity of *S. cerevisiae* by hybrid vigor from *S. eubayanus* incorporation and conveyance of a positive phenotype.

a/α – the two mating types of *S. cerevisiae* that enable a cell fusion response when a complimentary pheromone is detected. This mating type and sexual state is largely determined by the MAT locus on chromosome III and is expressed when the yeast cell contains one set of chromosomes (haploid, 1*n*), especially after sporulation.

Allele – A variant of a particular gene found at the same genomic location on a particular chromosome. The mating type of yeast is determined by which allele, MATa or MATα, is present at the MAT locus.

Alloploids – a hybrid organism or cell composed of two or more sets of chromosomes obtained from two separate species.

Auxotrophy – the loss of a gene and that function, especially in yeast genetics for controlling the growth and genetic content by amino acid metabolism.

Ascus – the saclike structure enclosing the four spores of *Saccharomyces* and is important in classification of the *Ascomycota* (sac fungi) yeast.

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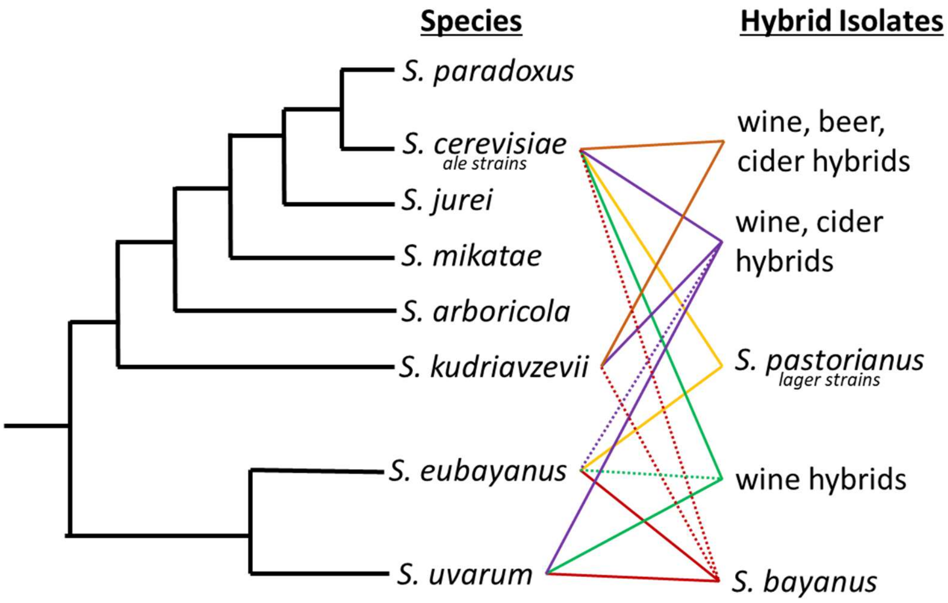


Figure 1. Saccharomyces Phylogenetic Tree with Industrially Important Hybrid. Industrial hybrids are listed to the right by the fermentation they have predominately been associated with. Solid lines between two species signifies the interspecific hybrids and dashed lines denote introgression from a 3rd or 4th species that may not always be present in each hybrid strain.

Brassica oleracea **-** Important food crop plant species that in the uncultivated form presents as wild cabbage, but has been cultivated over time to include broccoli, brussels sprouts, kale, cauliflower, cabbage, and collards.

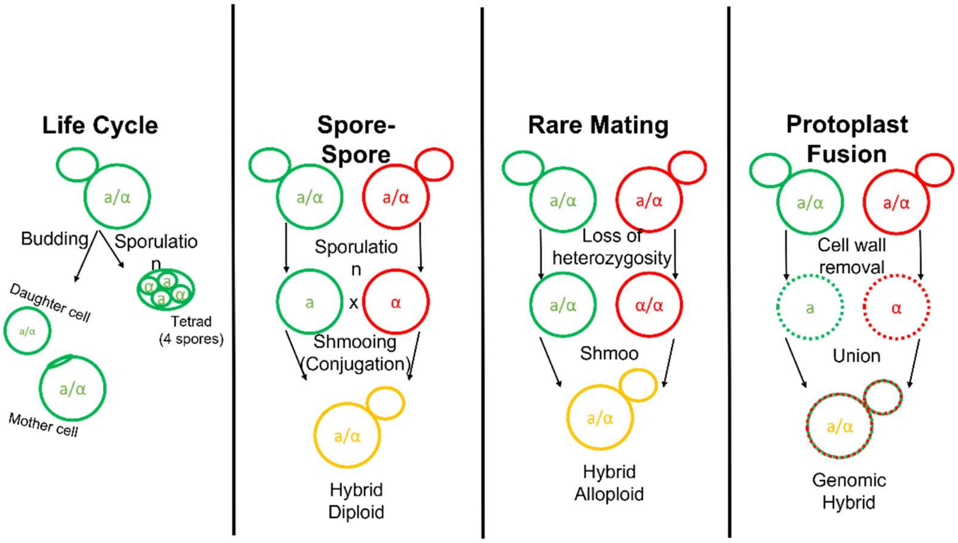
Crabtree Positive – microorganism that metabolizes sugar into ethanol in the presence of oxygen via fermentation, not cellular respiration.

Diauxic Shift – the shift from glycolytic fermentation producing ethanol to respiration catabolizing ethanol.

Diploid – organism with two complete genomes (2*n*), such as a human with a set of genomes from each parent.

Novel Hybrid Development

The first yeast breeding experiments were conducted by Ojvind Winge aimed at combining desirable traits of brewing strains during his tenure at the Carlsberg Laboratory in the 1930s [37]. Hybrid yeast development has been carried out for over half a century aimed predominantly at increasing attenuation and fermentation rates via intraspecific crosses with ale and lab strains [38–40]. Modern fermentations benefit from many innate and acquired hybrids that have been isolated or developed [17,27,41–53]. Early efforts in brewing science established the fundamentals necessary to explore the phylogeny, genomics, and strain development for *Saccharomyces* fermentation. During typical rich nutrient propagations of yeast in a brewing environment, mother cells reproduce asexually to bud off small daughter clones (Figure 2). Under poor nitrogen conditions, such as proline, yeast growth changes to a pseudohyphal form [54,55]. The complete absence of a nitrogen source and the presence of a non-fermentable carbon source, like acetate, will sporulate yeast cells [56]. Sporulation transforms the cell wall into the ascus, or sack, that holds four spores termed a tetrad. Analogous to human eggs and sperms, these spores are equally divided into mating types as either a or α [57]. When conditions improve for yeast growth, new haploid (1n) yeast can conjugate with opposite mating type yeast as they form a shmoo. Depending on the genomic make up of each parental strain or species, there is some genomic instability or rewiring that occurs during the following mitotic budding growth.



(**a**) (**b**) (c) (d)

Figure 2. Life and Mating of Saccharomyces Yeast. Diagram pertaining to the clonal growth typical of yeast fermentation cultures and the various known techniques employed to generate yeast hybrids. (a) Diploid yeast cells may bud and grow clonally to form a mother and daughter cell or undergo sporulation to form a tetrad. (b) Yeast hybridization may form by direct spore to spore mating. (c) Yeast hybridization may form by rare mating events in which diploid cells mate. (d) Yeast hybridization may also form by fusion of two separate yeast cell protoplasts with their cell wall removed.

Interspecific hybridization is seen as a valuable tool for yeast strain development that enables the combination and enhancement of characteristics from both parental strains or species [58]. Development of hybrids is executed via three primary methodologies: spore-spore mating, rare mating, and protoplast fusion (Figure 2). Briefly, these techniques differ in various ways. Spore to spore mating is most similar to what would be considered a natural mating and has been outlined in the previous paragraph. This approach has a high success rate, high genomic stability, and can avoid the aid of selection markers like drug resistance or autotrophies. Rare mating utilizes a described spontaneous loss of heterozygosity at the mating type locus. Normal diploid cells carry two sets of chromosomes with both the MAT-a and MAT-α genetic alleles and do not respond to sex pheromones for mating purposes. The spontaneous loss of either sex allele tolerates yeast mating to a yeast cell of complimentary sex. This results in yeast with high chromosome counts, influencing gene dosage during cellular processes and partially explains the outperformance over a diploid yeast of the same background [58]. Rare mating, as the name implies, is not common and selection markers are needed to perform this technique. This procedure is beneficial in overcoming poor sporulation but provides hybrids prone to high genomic instability. Lastly, protoplast fusion is performed by removing the cell wall and fusing the protoplasts of two cells together before the cell wall is repaired. This technique generates cells with high chromosome copy number and higher genomic instability but overcomes low sporulation and can be used to mate yeast from outside the *saccharomyces* genera and otherwise incompatible cells. Recent select investigations into *Saccharomyces* hybrid application in beverage fermentations are listed in table format (Table 2).

***Fagaceae* spp** – Family of *Angiosperms* (flowering plants) that include the beech and oak trees.

Frohberg – one of two primary lineages of modern lager yeast known to brewers originally used in Germany.

Gene – a sequence of DNA characterized, most commonly, by the presence of a start sequence, segment translatable into protein of function, and a stop sequence. Classical genetic dogma dictates that one gene can translate into an organisms characteristic trait, such as in Mendel's peas.

Genomics - The study of complete sequences of genetic materials composed of DNA (deoxyribonucleic acid).

Haploid – organism with one complete genome (*1n*).

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Table 2. Interspecific Yeast Hybrids in Fermentation

History	Parents	Reference
Isolated	*S. cerevisiae x S. eubayanus*	Libkind, 2011
Isolated	*S. cerevisiae x S. eubayanus x S. uvarum*	Perez-Traves, 2014
Isolated	*S. cerevisiae x S. kudriavzevii*	Langdon, 2019 Gallone, 2019 Gonzalez, 2008
Isolated	*S. uvarum x S. eubayanus*	Langdon, 2019 Gallone, 2019
Developed	*S. cerevisiae x S. eubayanus*	Mertens, 2015 Magalhaes, 2017 Krogerus, 2015 Krogerus, 2016 Hebly, 2015
Developed	*S. cerevisiae x S. mikatae*	Nikulin, 2018 Bellon, 2013
Developed	*S. cerevisiae x S. kudriavzevii x S. paradoxus*	Bellon, 2011
Developed	*S. cerevisiae x S. kudriavzevii*	Bizaj, 2012
Developed	*S. cerevisiae x S. arboricola*	Winans, 2020 Nikulin, 2018

Hybrid Vigor (*syn.* Heterosis) – The tendency for the hybrid progeny between two species to outperform their parents in strength, size, or ethanol production.

Interspecific – arising or existing between separate species.

Intraspecific – arising or existing within a species or individuals from the same species.**Locus** – A specific fixed position on an individual chromosome where specified genetic content is present.

Make Accumulate Consume – ecological strategy used by S. cerevisiae yeast involving the ability to make ethanol from saccharides, the survival in accumulated levels of the toxins, and the consumption of ethanol for energy post fermentation.

Evolutionary Influence

The fermentation of sugars into ethanol and carbon dioxide is effectively utilized by brewers, vintners, and bakers alike to prepare food or drink for human consumption. The metabolic capacity to ferment sugars instead of aerobic respiration in the presence of oxygen is known as the Crabtree effect, a critical factor for modern fermentation of beer production. Researchers estimate this form of metabolism emerged around 125 million years ago [59]. The timeline conveniently placed this emergence in the Cretaceous period when modern fruiting plants also appeared [60]. Fungi and plants are thought to share a co-evolutionary history [61,62]. Hence, Crabtree positive yeast, such as *S. cerevisiae,* secured their niche ecological advantage via association with high sugar environments in the timeline described. Current yeast metabolic knowledge depicts a make-accumulate-consume strategy [63]. By converting sugar into ethanol, other microbes are outcompeted for the limited food source via inhibitory concentrations of alcohol. This tactic enable yeast to compete with faster growing bacteria. Later, the yeast may catabolize the ethanol for cellular energy once the fermentable carbon sources have been depleted. This process is termed the diauxic shift [59,64].

Outside of industrial fermentations and laboratory settings, *Saccharomyces* yeast are readily obtained from oak and beech tree bark which exudes tree sap seasonally as part of the *Fagaceae* Family. This fluctuation of available carbon, nutrition, and favorable climate influences the state of yeast cells and therefore cells spend much of their time in a non-dividing state termed quiescence [64]. Brewer's yeast is a unicellular fungi and is not considered mobile, in natural settings they have relied on passive transportation facilitated by mobile vectors such as insects [65–68]. Studies have found that the aromatic ester profile of some yeast has been more successful in attracting flies to their scents [65]. The production of fruity esters may be an advantageous evolutionary trait developed to allow passage of the yeast from one rich environment to the next either via insect or brewer. One theory suggests insect vectors' intestines act as a vessel for facilitating natural yeast hybridization events [66,69]. Unfortunately, the microscopic nature of yeast limit scientists

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to infer retrospectively on yeast mating modes and frequency by analyzing genomic data sets [70,71].

Increasing research in this field suggests that the exchange of DNA crosses fungal species barriers. Interspecific hybridizations facilitate exchange of DNA which intertwine lineages and blur traditional species boundaries [32]. As genomic sequencing becomes more ubiquitous, many eukaryotic loci are being found to originate from other species [72,73]. In yeast, the newly developed hybrids can rapidly adapt by filtering their diverse chromosomes and retaining the advantageous portions via loss of heterozygosity [74–76]. Cells experiencing a stressor, such as a drug or environmental condition, appear to prioritize genomic filtering early in growth demonstrated by *Saccharomyces* interspecific hybrids subjected to various temperatures. *S. cerevisiae* x *S. uvarum* hybrids retained the cryotolerant subgenome of *S. uvarum* during cold adaptation, but retained the *S. cerevisiae* subgenome during warm environmental growth [76,77]. The dynamics between interspecific hybridization, adaptation, and evolution in *Saccharomyces* is not fully understood, but recent evidence suggests that this exchange of DNA components has and will continue to play a pivotal role as new yeast hybrids are described.

Conclusions

The quest to gain diverse and novel fermentation characteristics from a pure culture remains an overarching goal for brewing molecular biologists. Flavor attributes and temperature phenotypes have been investigated by many research groups, yet there remain gaps of knowledge in the yeast fermentation and evolution field. Although not an exhaustive list, the following questions are unresolved. Are interspecific hybrids naturally abundant under the correct environmental conditions or are they rare success stories? To what extent are yeast present in various natural climates and substrates, what vectors are involved in mobility, what interactions occur with other microbes, and what is typical lifecycle timeline of wild yeast? Is the Out of Asia hypothesis accurate for the origin of Saccharomyces yeast without adequate sampling endeavors? Efforts large and small are being exerted worldwide to explore the awesome power of yeast genetics in brewing sciences, yet many questions still surround the budding fungus akin to the brewers and bakers of the globe. We report a few yeast phenotypes unexplored by target hybridization to include formation of antioxidants that increase flavor stability, formation of glycerol for better mouthfeel in low alcohol beverages, and reduced off-flavor production.

Many recent interspecific hybrids developed have largely focused on reinventing the lager yeast, *S. pastorianus*, by crossing *S. cerevisiae* with *S. eubayanus*. As research continues, understudied *Saccharomyces* species may serve as a reservoir for diverse genomic contributions. Natural isolates of *Saccharomyces* hybrids suggest that interspecific mating is not as uncommon as once thought and recent investigations continue to redefine species whilst uncovering genetic exchange events. The ability of yeast to participate in interspecific hybridization and not be hindered by large genetic distances between parent species is promising and revealing of the introgression likely yet to be discovered in many preserved isolates. While there are many yeast strains favored for production of quality fermented foods and beverages, their status as species or hybrids may be adjusted over time because of the continual refinement of phylogenetics, the advancement in genome sequencing technology, and increased accessibility to genomic sequencing capabilities. By increasing yeast sampling, focusing on metabolic specifics, and facilitating collaboration, the yeast of tomorrow will be driven by scientific innovation in the laboratory today.

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Maltotriose – prominent trisaccharide typical of beer wort that is enzymatically derived from starch. It is composed of three glucose molecules linked together by α-1,4 glycosidic bonds.

Nomenclature – The terminology and language used to categorize and communicate in various disciplines, but especially common in science.

Out of Asia/Silk Road Hypothesis – The idea that Saccharomyces yeast originate from an Asian geography because of the amount of biodiversity found in that region.

Phenotype – a characterized trait, best exemplified by measurable qualities.

Phylogeny - The lineage and evolution of relative organisms.

Progeny – the offspring or decendents of an organism.

Quiescence – inactivity, dormancy, or a period of idleness.

Saaz – one of two primary lineages of modern lager yeast known to brewers originally used in Bohemia.

Shmoo – the distinct physical form of two Saccharomyces yeast cells mating. The terminology comes from the similarity to an Al Cap cartoon character.

Synanthropic Species – an undomesticated organism that habitually exists together with human population benefiting from non-natural environments.

Tetrad – four spores produced via meisis of *Ascomycota* yeast, specifically *S. cerevisiae*

Transgressive Phenotype – formation of extreme phenotypes that surpass the ability of parental lineages, often found in hybrids and can be positive or negative for fitness of the individual.

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