

## MINIREVIEW

## Packing a punch: understanding how flavours are produced in lager fermentations

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**One sentence summary:** Beer is one of the most popular beverages in the world. This review takes a wholistic approach to examine how flavours are produced in the brewing process, focusing particularly on the contribution of the raw ingredients and the yeasts to the final flavour profiles of lager beers.

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### ABSTRACT

Beer is one of the most popular beverages in the world and it has an irreplaceable place in culture. Although invented later than ale, lager beers dominate the current market. Many factors relating to the appearance (colour, clarity and foam stability) and sensory characters (flavour, taste and aroma) of beer, and other psychological determinants affect consumers' perception of the product and defines its drinkability. This review takes a wholistic approach to scrutinise flavour generation in the brewing process, focusing particularly on the contribution of the raw ingredients and the yeasts to the final flavour profiles of lager beers. In addition, we examine current developments to improve lager beer flavour profiles for the modern consumers.

**Keywords:** *S. pastorianus*; lager beer; flavours; genetics; biochemistry

### INTRODUCTION

Beer is one of the most popular alcoholic beverages in the world and it has long been documented in the history of civilisation (Hill and Stewart 2019). Brewing practices vary worldwide

but develop upon the basic guidelines formulated in Germany in 1516 to regulate commercial brewing – *Reinheitsgebot* (Purity Law). Under the Purity Law, beer consists of three main ingredients: water, barley malt and hops; additionally, yeasts were

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introduced later after J. H. van den Broek and Louis Pasteur independently affirmed the importance of yeasts in alcoholic fermentation (Barnett 2000). Each of these ingredients influences the quality and sensory properties of the resulting beer (Wunderlich and Back 2009). The water quality is strictly controlled and ancillary factors such as pH values and mineral concentrations (water hardness) influence the type of beer that can be produced. Barley malt is considered the main raw material in brewing and its liquid extract is called wort. Cereals other than barley malt (adjuncts) are used outside of Germany, particularly in tropical and subtropical regions to reduce production cost (Taylor, Dlamini and Kruger 2013). Hops are added during wort boiling, adding bitterness to beer and assisting beer microbial stability (Almaguer et al. 2014). Finally, yeasts convert wort fermentable sugars to ethanol and carbon dioxide, and through their complex biochemical pathways produce secondary metabolites that characterise the final aromatic profiles of beers (Hazelwood et al. 2008; Pires et al. 2014).

Among the myriad of beer types in the world, fermentation style separates beer into two categories: top fermented beer – ale, and bottom fermented beer – lager. Ale is usually produced at temperatures between 15–26°C by *Saccharomyces cerevisiae*. Higher temperature renders this beer type fruitier than lagers, attributed to more ester formation by yeasts. In history, ale consumption predominated Europe until the 16th century, around when lager was invented in Bavarian monasteries (Pavslar and Buiatti 2009). The introduction of the *Reinheitsgebot* restricted brewing to the period between Michaelmas, the feast of St Michael on September 29th and the feast of St George, April 23rd (Monerawela and Bond 2017a). As a result, brewing was conducted in the colder months of the year. In order to preserve the beer for consumption throughout the year, monks introduced the practice of storing and maturing the fermented beer in cool dark caves, hence the use of the term, Lager – derived from the German word 'lagern', to store (Pavslar and Buiatti 2009). The introduction of these laws and new customs contributed to the emergence and domestication of a new interspecies hybrid yeast, *Saccharomyces pastorianus* (Gallone et al. 2016; Monerawela and Bond 2017a). The hybrid yeasts contain genetic materials from at least two different strains of *S. cerevisiae* and the cryotolerant strain *S. eubayanus* (Dunn and Sherlock 2008; Okuno et al. 2016). *S. eubayanus* is a cold-adapted strain first isolated in Patagonia, South America but later found in other wild environments in South and North America, Australasia and Asia, but only interspecific hybrids with *S. cerevisiae* have been found in Europe (Peris et al. 2016). The combination of inherited traits, such as high fermentative capacity from *S. cerevisiae* and cryotolerance from *S. eubayanus* has resulted in a novel yeast better adapted to fermentation in a cool climate at temperatures between 8 and 14°C. Compared to ale, lager beer enjoys a clean and crisp flavour persona and accounts for 84% of global consumption [<https://www.thebusinessresearchcompany.com/report/beer-global-market-report>].

A quintessential quality of beer is its sensory properties. These can vary from brand to brand, and consumers will have their favourites. This review focusses on the means of producing various flavour compounds in lager with a specific focus on the contribution of raw materials (barley malt and hops), and yeasts during fermentation. We discuss the range of these compounds, consider approaches that are currently undertaken to improve the intensity and the variety of aromas and flavours in lager beers, and compare analytical methods to study these flavour compounds.

## Brewing

### Barley malt and hops

Barley malt contains starch, cellulose and hemicellulose, proteins, lipids, polyphenol, vitamins and minerals. Starch resides in the endosperm and makes up for 63% dry weight of the grain. It appears in semi-crystalline granules and consists of amylose (20–25%) and amylopectin (75–80%; Wunderlich and Back 2009). Amylose and amylopectin are polymers formed by glucose via  $\alpha(1 \rightarrow 4)$  and  $\alpha(1 \rightarrow 6)$  glycosidic bond. Starch hydrolysis to dextrin and fermentable sugars during malting and mashing are mainly carried out by  $\alpha$ - and  $\beta$ -amylase. Another important nutrient in the endosperm is protein, which is about 8–13.5% grain dry weight and is essential in generating free amino nitrogen and sustaining foam and colloidal stability in the final product (Steiner, Gastl and Becker 2011). In addition to sugars and nitrogen sources, barley malt contains about 3% lipid that can impact foam stability and off-flavours related to beer aging (Vanderhaegen et al. 2006; Gordon et al. 2018). Polyphenols represent 0.1–0.3% grain dry weight and affect colour, foam and colloidal stability (Jongberga, Andersen and Lund 2020).

Hops (*Humulus lupulus*) is responsible for the distinctive bitterness and the microbial stability of beer. Hops contains several chemical components, but two are crucial to brewers: hop resins contributing to bitterness, and hop essential oils responsible for aromas (Almaguer et al. 2014). Hop resins contain  $\alpha$ -acids (humulones) and  $\beta$ -acids (lupulones; Hughes 2009). The  $\alpha$ -acids are the effective components that isomerise to iso- $\alpha$ -acids (cis- and trans-isohumulone) during wort boiling, producing the bitter taste. Hops (pellets) and hop products can be added during wort boiling, before filtration, or to flavour a beer after production (Wunderlich and Back 2009). Hop essential oils comprise mainly of terpene hydrocarbons and oxygenated compounds. Monoterpene myrcene, and the sesquiterpenes humulene and caryophyllene along with several other compounds are responsible for the pungent hoppy smell of beer (Hughes 2009).

### The process

The brewing process has been extensively reviewed in book chapters and many journal articles (Bamforth 2000; Briggs et al. 2004; Hughes 2009; Wunderlich and Back 2009; Alves et al. 2020). Briefly, brewing comprises of malting, milling, mashing, lautering, wort boiling (and whirlpooling), fermentation, maturation and storage (Fig. 1). Malting and mashing break down starch and protein respectively to fermentable sugars and digestible nitrogen sources such as free amino acids and short peptides to facilitate healthy fermentation. Malting is a similar process to germination (Fincher 2010), and consists of steeping, germination and kilning. Upon water uptake in steeping, the plant hormone gibberellic acid triggers the expression of hydrolytic enzymes in the aleurone layer to degrade storage proteins and starch in the endosperm. The most important hydrolytic enzymes are  $\alpha$ - and  $\beta$ -amylases, starch debranching enzymes and endo- and exo-peptidases (Fincher 2010). More than 40 endogenous endopeptidases have been identified (Jones and Budde 2005). Malting is conducted for 3–6 days at temperatures between 12 and 18°C (Briggs et al. 2004). The process is completed by kilning – drying germinated barley at temperatures up to 80°C for pale malt, to inactivate the enzymes, to reduce the grain moisture to between 4 and 6%, and to produce colours and aromas (Briggs et al. 2004). Milling is a physical process to break down starch and protein, facilitating downstream enzyme modification in mashing.

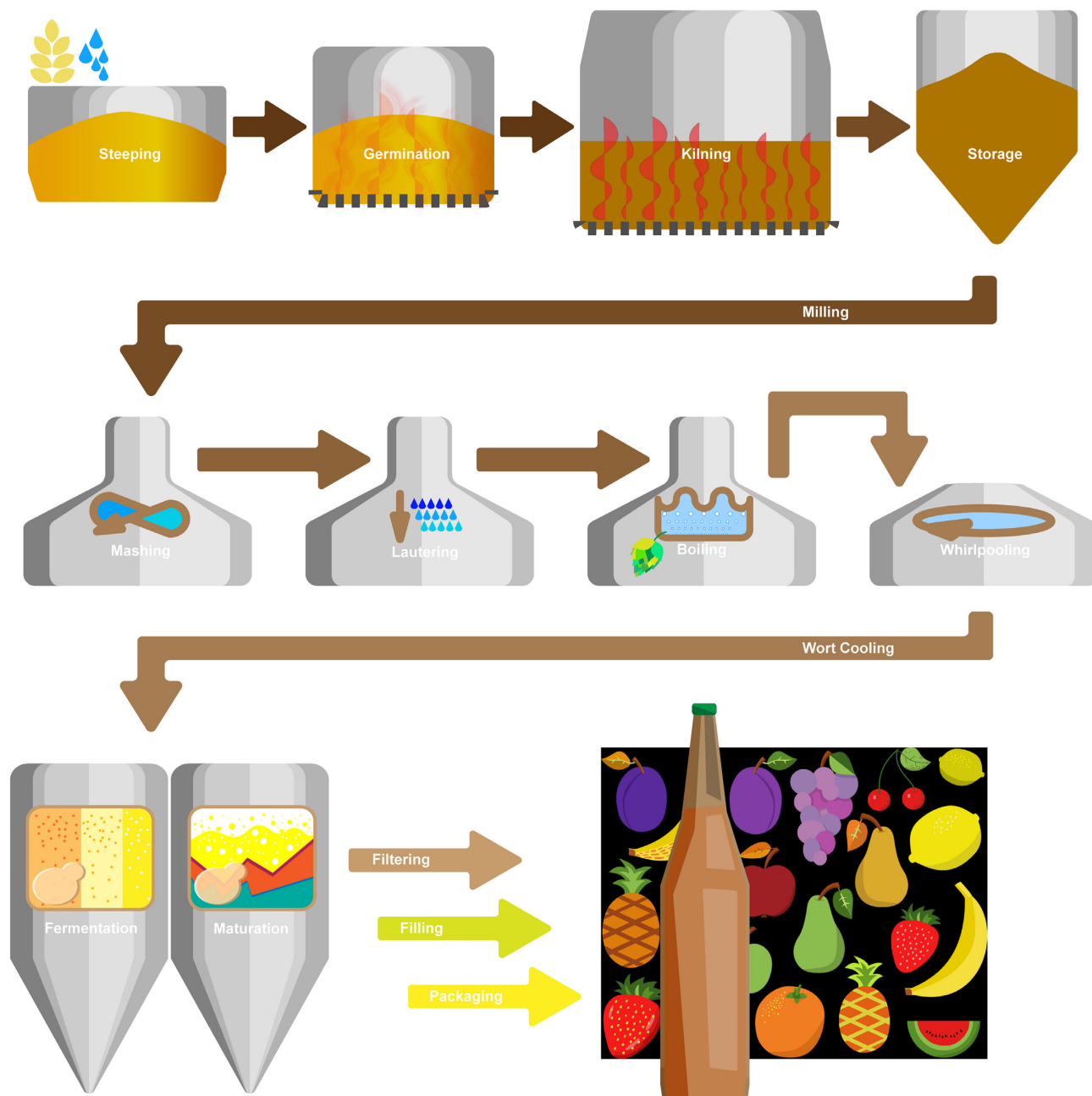


Figure 1. Major steps in the brewing process.

Mashing refers to a process of mixing ground malts (and other cereal grists) and water in a controlled ratio at a programmed temperature regime to extract sugars and nitrogen materials for fermentation (Briggs et al. 2004). Apart from sugars, nitrogenous materials in the wort are crucial for fermentation. Free amino nitrogen (FAN) mainly refers to amino acids, di- and tri-peptides and ammonia that can be taken up by the yeasts (Stewart, Hill and Lekkas 2013; Hill and Stewart 2019). Both malting and mashing contribute to the release of FAN; however, the percentage contribution by each process is still under debate (Osman et al. 2002; Jones and Budde 2005; Lekkas, Hill and Stewart 2014; Aldred, Kanauchi and Bamforth 2021). There are several mashing systems: traditional infusion mashing, decoction mashing, temperature-programmed infusion mashing and double mashing. Trends are turned towards

temperature-programmed infusion mashing (Briggs et al. 2004). Infusion mashing consists of a series of controlled temperature stands optimal for certain types of enzymes. Traditionally, a temperature between 40 and 50°C is considered optimal for proteases (Jones and Budde 2005), 60 and 65°C for  $\beta$ -amylase and 70 and 75°C for  $\alpha$ -amylase. A mash-off at 78°C at the end is applied to deactivate enzymes. FAN contribution during the protease rest depends on malt variety and quality. In modern brewing practice, brewers prefer to eliminate the 'protease rest' due to the concern of energy consumption and lipid oxidation contributing to aldehyde off-flavours in beer aging. On the other hand, exogenous proteases can be applied (outside of Germany) to increase FAN and to improve extract yield when poor malt quality is present or when high quantity of adjuncts is employed.

**Table 1.** Typical sugars and nitrogen materials in 12°P wort.

Selected components in wort	g/L
Glucose and fructose	9–12
Sucrose	4–5
Maltose	56–59
Maltotriose	14–17
Free amino nitrogen	0.10–0.25

Adapted from Briggs et al. 2004.

The Maillard reaction and the Strecker degradation in mashing contribute to beer flavours. The Maillard reaction is a reaction between reducing sugars and nitrogen materials containing an  $\alpha$ -amino groups, such as free amino acids, peptides and proteins (Ferreira and Guido 2018). This reaction, also referred to as non-enzymatic browning, is responsible for malt colour after kilning. The Maillard products can result in more higher alcohols production after fermentation (Dack et al. 2017). On the other hand, the Strecker degradation of amino acids leads to aldehydes contributing to off-flavours in beer (Hernandez-Artiga and Bellido-Milla 2009).

After mashing, the final wort is obtained by lautering – filtering and sparging through a grain bed and boiled with hops. Additionally, boiling evaporates dimethyl sulphide (DMS) – a product of S-methylmethionine from kilning (Briggs et al. 2004), deactivates enzymes, and sterilises the wort before fermentation. In summary, the final wort contains fermentable sugars including glucose, fructose, sucrose, maltose, maltotriose, free amino nitrogen and various nutrients for yeasts to function and propagate (Table 1). The concentration of mainly sugars from malt and other soluble content in wort can be measured by the Plato Gravity Scale, and expressed in Degree Plato (°P). The composition of FAN, particularly the amino acid profile in wort, directly affect flavour output after fermentation (He et al. 2014).

## The Yeasts: genetics and biochemistry of flavour compound production

### The hybrid genome

The heterogeneous *S. pastorianus* hybrids used for lager fermentations are classified into two distinct groups, the Saaz group (Hybrid Group I) and the Froberg group (Hybrid Group II). Both groups originated from hybridisation events between different strains of *S. cerevisiae* and *S. eubayanus* (Dunn and Sherlock 2008; Libkind et al. 2011). Group I strains are known to have a greater tolerance to lower temperatures and a poor utilisation of maltose and maltotriose, while Group II strains can use maltotriose as a carbon source (Gibson et al. 2013). The genomes of Group I strains have an approximate diploid *S. eubayanus* and a haploid *S. cerevisiae* DNA content. In contrast, Group II strains have an approximate 2n *S. cerevisiae* and 2n *S. eubayanus* DNA content. Furthermore, the chromosome copy number varies between strains of the same group with copy number of Group I strains ranging from 45 to 52 and Group II strains ranging from 42 to 84 (Monerawela and Bond 2018).

Next Generation Sequencing and genomic analysis showed that the resultant hybrid strains not only possess chromosomes of both parental strains but also hybrid chromosomes with known recombination breakpoints (Bond et al. 2004). Some of these breakpoints are located in coding regions, leading to a unique set of hybrid genes in the genus *Saccharomyces* (Nakao et al. 2009; Hewitt et al. 2014; Okuno et al. 2016; Monerawela and

Bond 2017b). In addition to hybrid chromosomes, translocations between chromosomes of the same sub-genome (Sc-Sc, Se-Se) and between chromosomes of different sub-genomes (Sc-Se, Se-Sc) are also observed.

Several scenarios have been proposed to account for the origin of *S. pastorianus* strains. Dunn and Sherlock originally hypothesised that both Group I and Group II arose from two independent hybridisation events with Group I emerging from a hybridisation of a haploid *S. cerevisiae* and a diploid *S. eubayanus*. Group II, in contrast, was hypothesised to have originated from a diploid *S. cerevisiae* and *S. eubayanus* strains (Dunn and Sherlock 2008). Later, as more genome sequences became available, it was discovered that Group II yeasts contain two different *S. cerevisiae* sub-genomes that can be distinguished by Single Nucleotide Polymorphisms (SNPs), with one sub-genome shared with Group I strains. This, together with information that both groups share some common recombination events between the parental chromosomes, led to the hypothesis that both groups arose from at least two different sequential hybridisation events between *S. cerevisiae* and *S. eubayanus*. The first hybridisation event may have occurred between a diploid *S. eubayanus* strain and a haploid *S. cerevisiae* 'Ale', strain (Monerawela et al. 2015; Monerawela and Bond 2017a). This progenitor hybrid gave rise to the Group I strains while Group II strains arose from a subsequent hybridisation with another *S. cerevisiae* strain. Both groups then evolved independently with Group I strains encountering a significant loss of the *S. cerevisiae* genome and both groups undergoing further recombination events between the sub-genomes (Okuno et al. 2016; Monerawela and Bond 2017a; Salazar et al. 2019).

The genome data is also consistent with a scenario in which both groups come from a single hybridisation event between a diploid *S. eubayanus* strain and a heterozygous *S. cerevisiae* diploid strain with Group I strains experiencing a selective loss of a significant proportion of the heterogeneous *S. cerevisiae* sub-genome due to different brewing conditions during the domestication process (Okuno et al. 2016; Salazar et al. 2019).

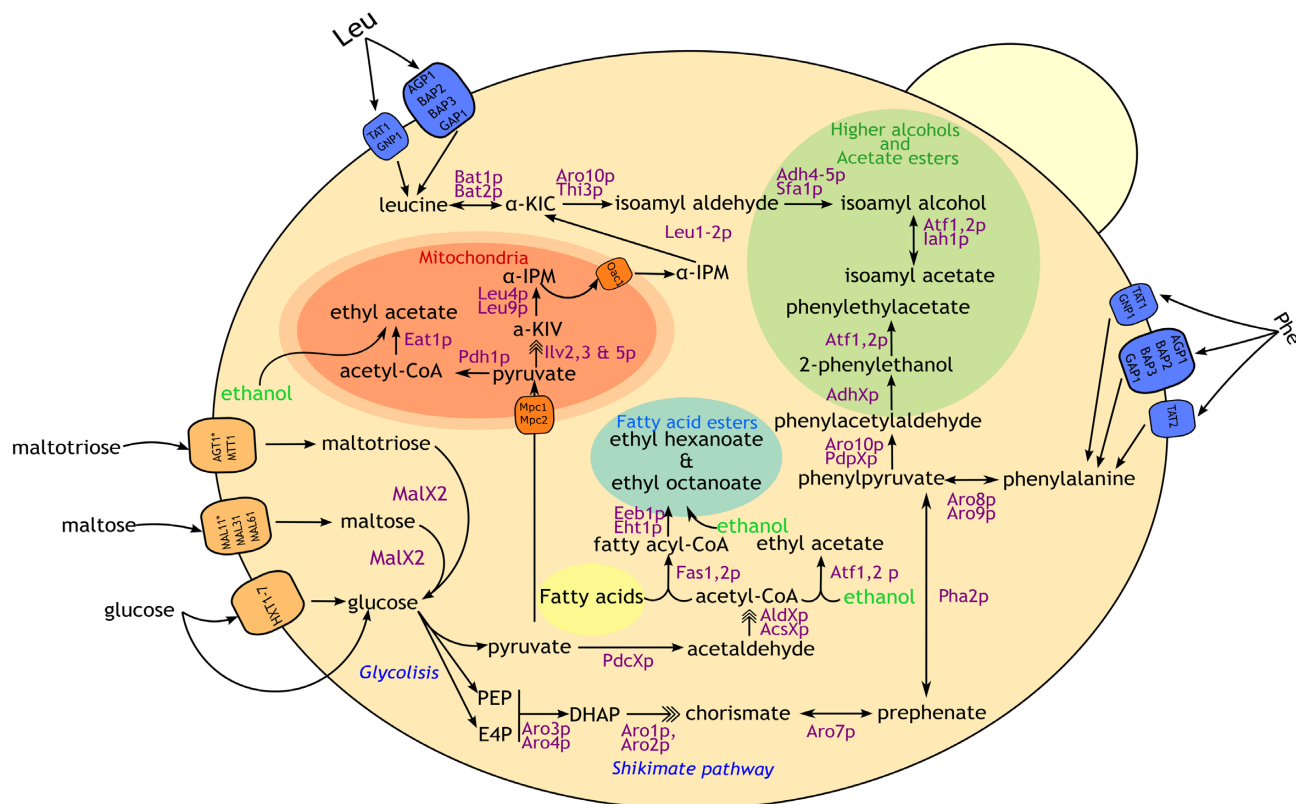
While great strides have been made to understanding the complexity of *S. pastorianus* genomes, much remains to be discovered, specifically in relation to flavour and aroma production during fermentation. The plasticity of the genomes and their aneuploidy may explain their adaptation and evolution in the brewing media (James et al. 2008; Rancati et al. 2008).

While the sequences of a large number of *S. pastorianus* genomes, 31 to date, are now deposited at the National Center for Biotechnology Information (NCBI <https://www.ncbi.nlm.nih.gov/genome/browse/#!/eukaryotes/342/>), providing researchers with a vast amount of information on the genomes, just one genome, the Group II strain CBS1483 is fully annotated and assembled into chromosomes (Salazar et al. 2019). To date, a fully assembled and annotated genome of Group I strains has yet to be deposited at NCBI. The recently developed open-source tool for functional annotation of hybrid aneuploid genomes, HybridMine, will greatly aid in the annotation of *S. pastorianus* strains (Timouma, Schwartz and Delneri 2020).

### Uptake of sugars and amino acids

The first sugars metabolised in wort are glucose and fructose. These sugars can be imported by facilitated diffusion via hexokinase or high-affinity transport via hexose transporters (Romano 1982; Bisson and Fraenkel 1983; Lang and Cirillo 1987; Lewis and Bisson 1991; Fig. 2). When hexoses are present in wort, the synthesis of maltose and maltotriose transporters are inhibited (Federoff, Eccleshall and Marmur 1983; Hu et al. 2000), but





**Figure 2.** Overview of the main metabolic pathways involved in the synthesis of higher alcohol, acetate esters and medium-chain fatty acid esters in *S. pastorianus*. The major biochemical pathways for the production of higher alcohols and esters from branched-chain and aromatic amino acids are shown. The enzymes required for each step are shown in purple. The triple arrows indicate that several steps are omitted. The higher alcohol, 2-phenylethanol, is produced from the degradation of the imported phenylalanine or the degradation of phenylpyruvate from the Shikimate pathway. Isoamyl alcohol is produced from the degradation of imported leucine or from the transformation of pyruvate in the mitochondria. The fatty acid esters ethyl hexanoate and ethyl octanoate are derived from transesterification of medium chain fatty acids. The major transporters for amino acids are shown as are the transporters for the major sugars found in wort. PEP: Phosphoenolpyruvate, E4P: Erythrose-4-Phosphate, DAHP: 3- deoxy-D-arabino-heptulosonate-7-phosphate,  $\alpha$ -KIV:  $\alpha$ -ketoisovalerate,  $\alpha$ -IPM:  $\alpha$ -isopropylmalate and  $\alpha$ -KIC:  $\alpha$ -ketoisocaproate.

once these sugars are consumed, maltose is the next sugar to be used. At least three maltose permeases have been identified, namely Mal, Agt1 and Mtt1/Mty1 (Jespersen *et al.* 1999; Salema-Oom *et al.* 2005; Vidgren, Ruohonen and Londesborough 2005), and are encoded by gene alleles from both parental strains. At least five Mal transporters are encoded by genes at five independent MAL loci (MAL1-4 and MAL6). Each locus, all located in subtelomeric regions, contains three genes that are required for maltose metabolism. MALX1 genes – X designating the locus number – encode for the maltose transporters (MalT). MALX2 genes encode for  $\alpha$ -glycosidases (MalS). MALX3 genes encode for transcriptional activators that regulate the previous two genes (MalR; Han *et al.* 1995). MAL11, also known as AGT1, is the only transporter of the 6 Mal loci that can import a wide range of sugars, including maltotriose. The *S. cerevisiae*-like copy of AGT1 is non-functional in *S. pastorianus* as it has an early stop codon within the open reading frame (Vidgren, Ruohonen and Londesborough 2005; Vidgren *et al.* 2009; Vidgren and Londesborough 2012). Mtt1 is another transporter found in *S. pastorianus* strains. It is 90% similar to MAL31 and 54% similar to AGT1 (Salema-Oom *et al.* 2005). Therefore, maltotriose is imported into *S. pastorianus* by Se Agt1 and Se and Sc Mtt1 and its uptake differs between Group I and Group II strains and also between strains from the same group (Vidgren *et al.* 2010; Magalhaes *et al.* 2016).

Amino acids uptake from wort involves at least 16 amino acid permeases, 12 of which are constitutively expressed, while four are regulated via catabolite repression (Boulton and Quain 2008;

James and Stahl 2014). The uptake occurs at the early stage of fermentation when ethanol level is low. The process depends on the type of permeases, their specificity, competition for binding by amino acids and feedback inhibition of certain permeases. Yeasts display preferences for different amino acids; based on their uptake rate, amino acids can be separated into four groups (Jones and Pierce 1964; Lekkas *et al.* 2007; Stewart, Hill and Lekkas 2013). In addition to importing amino acids from the wort, *S. pastorianus* can synthesise *de novo* all amino acids (Ljungdahl and Daignan-Fornier 2012).

#### Production of flavour compounds in *S. pastorianus*

Higher alcohols and their corresponding acetate esters are metabolites produced from the secondary metabolism of amino acids via the Ehrlich pathway (Hazelwood *et al.* 2008). It is the branched-chain (leucine, isoleucine and valine), aromatic (phenylalanine, tryptophan and tyrosine) and sulphur containing (methionine) amino acids that produce the major flavour compounds (Dickinson, Harrison and Hewlins 1998; Iraqi *et al.* 1998; Dickinson *et al.* 2000). Each higher alcohol and acetate ester produces a distinctive aroma or flavour. For example, 2-phenylethanol and 2-phenylethyl acetate impart rose and honey notes; whereas isoamyl alcohol and acetate impart banana and fruity notes (Table 3; Pires *et al.* 2014; Dzialo *et al.* 2017; Holt *et al.* 2019).

The Ehrlich pathway consists of three steps (Fig. 2; Hazelwood *et al.* 2008; Pires *et al.* 2014). Firstly, the  $\alpha$ -amino group of the

amino acid is transferred to  $\alpha$ -ketoglutarate in a transamination reaction, leading to an  $\alpha$ -ketoacid intermediate. This is carried out by the transaminases Aro8p/9p and Bat1p/2p: the former mainly acts on phenylalanine, tryptophan and tyrosine, while the latter acts on leucine, valine and isoleucine. Aro8p/Aro9p have broad substrate specificity and display activity towards leucine, methionine and  $\alpha$ -aminoadipate. Next, the  $\alpha$ -ketoacid is converted to a fusel aldehyde in an irreversible decarboxylation step by decarboxylases Pdc 1p,5p, 6p and Aro10p. Finally, the fusel aldehyde is reduced to a higher alcohol by two main classes of enzymes: alcohol dehydrogenases and aryl-alcohol dehydrogenases encoded by large multigene families (Nordling, Jörnvall and Persson 2002; Dickinson, Salgado and Hewlins 2003). The enzymes involved in the Ehrlich pathway vary in cellular localisation and substrate specificity (Fig. 2). The catabolism of aromatic and branched-chain amino acids is regulated by Nitrogen Catabolite Repression (NCR) and by negative feedback inhibition by the terminal amino acid (Hofman-Bang 1999; Cooper 2002).

Esters are an important class of aromas in beer. These molecules have a lower olfactive threshold than higher alcohols and generally have a better aromatic impact (Saison et al. 2009). There are two main groups of esters. Firstly, acetate esters are produced by the esterification of higher alcohols and ethanol with a molecule of coenzyme acetyl-CoA, catalysed by acetyltransferases. The reactions can occur in both the cytosol and mitochondria by Atf1p/Atf2p and Eat1p respectively (Fig. 2; Fujii et al. 1994; Nagasawa et al. 1998; Kruis et al. 2017). Secondly, medium-chain fatty acid esters are formed by Acyl-coenzymeA:ethanol O-acyltransferase, Eeb1p and Eht1p, whereby ethanol is added to medium chain fatty acids such as hexanoate and octanoate (Saerens et al. 2006).

The genes and proteins involved in the Ehrlich pathway have been well characterised in *S. cerevisiae* and it is known that other yeast species belonging to the *Saccharomyces sensu stricto* complex produce the same flavours but at different concentrations under the same fermentation conditions (Pérez et al. 2021). The complex genome of *S. pastorianus* with two sub-genomes from different *Saccharomyces* species raises an interesting question about the participation of the sub-genomes in flavour production. In total, two previously discovered *S. pastorianus* unique genes, LgATF1 and LgATF2, were later identified as being encoded by the *S. eubayanus* genome (Yoshimoto et al. 1999; Verstrepen et al. 2003). The high sequence identity between the sub-genomes in *S. pastorianus* leads to the possibility of trans-regulation of *S. cerevisiae* and *S. eubayanus* sub-genomes. Bolat et al. (2013) showed that in *S. pastorianus*, the deletion of the *S. eubayanus*-like copy of ARO80, the transcriptional activator that regulates amino acid biosynthetic genes, did not affect the expression of the *S. eubayanus* ARO10 gene, indicating that there is a trans-regulation between sub-genomes and that transcriptional factors may be interchangeable. Trans-regulation of sub-genomes has also been studied in an interspecific hybrid of *S. cerevisiae* and *S. paradoxus* (Tirosh et al. 2009). The interspecific hybrid showed that trans effects exhibited condition-dependent regulation. Also, this trans-regulation may be due to a new environmental sensing obtained from a new genomic combination, or new interactions between cis-trans regulators. Trans-regulation may also occur through the formation of hybrid protein complexes consisting of proteins encoded by each sub-genome, or through competition for substrates and co-factors. Several studies have shown the formation of chimeric protein complexes in interspecific hybrids (Piatkowska et al. 2013; Dandage et al. 2021). One such chimeric complex, Trp2p/Trp3p

**Table 2.** Average values of aromatic flavour compounds of Group I and Group II strains.

	Group I Average (mg/L)	Group II Average (mg/L)	Threshold (mg/L)
Acetaldehyde	13.62	10.04	10~25
Ethyl acetate	7.87**	20.13**	30
Ethyl propionate	0.34*	0.46*	-
Isobutyl acetate	0.02**	0.07**	1.6
Ethyl butyrate	0.12	0.10	0.4
1-Propanol	5.37**	7.87**	800
Isobutanol	7.92	8.86	200
Isoamyl acetate	0.52**	1.24**	1.2–1.6
1-Butanol	0.25	0.75	450
Isoamyl alcohol	36.74	34.65	70
Phenylethyl acetate	0.60**	1.05**	3.8
Phenyl ethanol	15.72	10.77	125
Ethanol (%)	4.78	5.40	-

Adapted from Mertens et al. 2015. P-value < 0.05\*, < 0.01\*\*. Threshold values adapted from Miller 2019. Hyphen, not calculated in beer.

that catalyses the first step in tryptophan biosynthesis produced a fitness advantage in growth medium lacking tryptophan. These findings show that the proteome of interspecific hybrids can lead to new combinations of protein complexes that may impart a better adaptation to specific conditions. Currently, our knowledge of the effects of hybrid protein complexes on flavour profiles in *S. pastorianus* is limited, however in a recent study the presence of hybrid complexes as well as sub-genome-specific complexes in *S. pastorianus* have been proposed (Timouma et al. manuscript submitted).

Several studies have measured the major flavour compounds produced by Group I and Group II lager yeasts under varying fermentation conditions in wort (Ekberg et al. 2013; Walther, Hesselbart and Wendland 2014; Mertens et al. 2015). The aromatic profiles were shown to vary significantly depending on the fermentative conditions. Variables such as temperature, oxygen dissolved in the media, carbon source and concentration of sugar and FAN have been shown to impact the concentration of higher alcohols and esters in the beer (Dzialo et al. 2017). As part of a study to generate new interspecific hybrids, the flavour profiles of seven Group I strains and 10 Group II strains were analysed (Mertens et al. 2015). A meta-analysis of this data reveals that Group II strains produce a better aromatic profile than Group I strains: esters such as ethyl acetate, isoamyl acetate and phenyl acetate were produced at higher levels by Group II strains (Table 2).

While it is difficult to ascertain the contribution of each sub-genome of *S. pastorianus* to the final aromatic profile in lager yeasts, lager-style beer fermentations have been carried out using different strains of the parent *S. eubayanus* (Mardones et al. 2020). These strains were shown to produce different aromatic profiles to *S. pastorianus*, suggesting that the complex hybrid genome may produce a more complex aroma and flavour profile.

## Beer flavour analysis

The synergy of raw materials, process and yeasts culminates in the complex matrix end product – beer, containing a large variety of volatile and non-volatile compounds (Briggs et al.

2004). Among the large number of volatile molecules, only a few dozen play relevant roles in beer sensory properties (Table 3). Higher alcohols and esters are the most important compounds imparting characteristic aromas and flavours to lager, whereas aldehydes and ketones, particularly diketones, are considered off-flavours. For instance, diacetyl and 2, 3-pentadione – by-products from valine and isoleucine biosynthesis in yeasts (Krogerus and Gibson 2013a), are described as butterscotch and undesirable in lager beer (Pires et al. 2015). In addition, sulphur compounds such as hydrogen sulphide (H<sub>2</sub>S) and dimethyl sulphide (DMS) are off-odours. Change of concentrations of these compounds occurs in maturation. Diacetyl can be reduced by yeasts to acetoin and 2,3-butanediol, that possess higher detection threshold than that of diacetyl; however, the removal of diacetyl is strain and condition dependent (Briggs et al. 2004; Krogerus and Gibson 2013b). Hydrogen sulphide is decreased during cold storage and carbon dioxide purging (Briggs et al. 2004). Hops are key ingredients in beer, and hop essential oils are responsible for hoppy smells – particularly terpene hydrocarbons (Hughes 2009). Part of the oxygenated compounds from these terpenes are volatiles adding aromas to beer. Linalool, from  $\beta$ -myrcene and the most abundant terpene alcohol, is an indicator of hop aroma (Almaguer et al. 2014). Other terpene alcohols, such as citronellol and geraniol are present at lower amounts (Dietz et al. 2020; Table 3).

### Measuring flavour compounds

From a flavour detection perspective, higher alcohols and esters are considered ‘easy-to-analyse’ (Martins et al. 2020). They are chemically stable during extraction and their concentrations are above ppb ( $\mu\text{g/L}$ ) level. These compounds can be readily analysed by headspace extraction (static headspace, dynamic headspace and solid phase microextraction) followed by gas chromatography (GC) with flame ionisation detector (FID) or mass spectrometry detector (MSD; Kobayashi, Shimizu and Shioya 2008). Compared to other extraction techniques, solid phase microextraction (SPME) has become very popular due to its ease of use, high sensitivity, reproducibility and low cost. It requires neither solvents nor previous sample preparation and is highly automated (Zhu and Chai 2005). Therefore, for this review, we focus on the measurement of confirmed flavour compounds using the SPME-GC method.

Headspace-SPME-GC has been successfully applied to the determination of flavour compounds in beer (da Silva, Augusto and Poppi 2008; Kobayashi, Shimizu and Shioya 2008; Saison et al. 2008; Cajka et al. 2010; Charry-Parra, DeJesus-Echevarria and Perez 2011; Riu-Aumatell et al. 2014). There are some limitations to the SPME extraction: in a complex system such as beer, analytes may compete for absorption. Modification has been made to overcome these difficulties: higher extraction temperature improves mass transfer in the multi-phase system. Cold fibre SPME was developed to retain the fibre absorption while heating the sample (Wang, Wang and Shu 2020).

### Sensory and current aromatic studies

Consumer’s perception of quality depends on the appearance and the sensory characters of beer and many other psychological determinants; among all these, beer flavour, taste and aroma are crucial (Briggs et al. 2004; Garcia-Munoz et al. 2014). According to definitions in the international methods agreed by the European Brewery Convention (EBC) and the American Society of Brewing Chemists (ASBC), flavour refers to ‘the combination

of olfactory and gustatory attributes perceived during tasting, including tactile, thermal, pain and kinaesthetic effects’. Taste means ‘sensory attribute resulting from stimulation of the gustatory receptors in the oral cavity by certain soluble substances’ and aroma is ‘an odour with a pleasant connotation’ (Analytica 1997). Overall, more than 800 volatile organic compounds (VOCs) have been found in beer (Sohrabvandi, Mortazavian and Rezaei 2011; Donadini, Fumi and Newby-Clark 2014). These compounds contribute to the citrusy, herbal, spicy, flowery, fruity and sometimes also to off-flavours (Ravasio et al. 2018). Therefore, understanding the nature and content of the volatile substances is important for the selection of raw materials and yeast strains (da Silva, Augusto and Poppi 2008).

There are two types of approaches for aromatic study, sensory-guided ‘targeted’ approach and flavour-omics approach. The targeted approach was firstly proposed by Patton and Josephson in 1957, known as odour activity value (OAV) (Patton and Josephson 1957). It and its modifications have been successfully applied to the understanding of the compounds that contribute to beer flavour (Reineccius and Peterson 2013; Witrick, Pitts and O’Keefe 2020). However, the targeted approach can only study compounds that directly produce a sensory response. It ignores potential ingredient interactions (Campo et al. 2006; de-la-Fuente-Blanco et al. 2020). The flavour-omics approach consists of untargeted chemical profiling and multivariate analysis. It can lead to a better understanding of chemical inputs and their impact on beer flavour. A challenge for untargeted chemical profiling-multivariate analysis is beer contains too many volatiles as the variable inputs. A rule-of-thumb applies that the beer sample size should be 10 times that of the independent variables – flavours (Hair, Ringle and Sarstedt 2013), which means the beer samples size is enormous. It’s impossible to perform a study with a complete VOC profiling.

A possible compromised solution would be to select the important volatiles based on the result of non-targeted profiling with semi-quantitative measurement, and then to construct the sensory prediction model with quantitative result of selected volatiles. In non-targeted profiling, the analytical coverage is crucial to prevent the missing of any substance. The coverage must be improved in two ways: a wider range of VOC extraction and better separation. Higher coverage of extraction can be achieved by combining the results from different VOC extraction methods (Zhang et al. 2020). Bidimensional GC is a promising tool for better separation (Ong and Marriott 2002). A high analytical throughout can be achieved by using short GC separation time and VOC extraction time that match each other. A typical fast GC has a running duration of 3–20 min (Zoccali, Tranchida and Mondello 2019). Some faster extraction techniques have been developed, such as vacuum assisted SPME, thin-film SPME and dispersive solid phase extraction (Reyes-Garcés et al. 2018; Milheiro et al. 2019; Vakinti et al. 2019).

### Improving flavour profiles for today’s consumer

There is a desire for continuous improvement in raw ingredients, process and yeast strains by breweries to sustain competitiveness in the market. Technology advancement in the brewing industry is primarily driven by quality improvement and cost reduction. Cost reduction can be achieved by lowering the grain bill through adjunct addition or raw material optimisation, yet the main concern rests on energy consumption (Bamforth 2000; Taylor, Dlamini and Kruger 2013; Yorke, Cook and Ford 2021). Effort has been made to enhance productivity via high gravity

**Table 3.** Selected key aromas in beer.

Selected alcohols	Concentration (mg/L)	Organoleptic description
Ethanol	20 000–80 000	Alcoholic, strong
1-Propanol	3–16	Alcoholic
2-Propanol	3–6	Alcoholic
2-Methylbutanol	8–30	Alcoholic, vinous, banana
3-Methylbutanol	30–70	Alcoholic, vinous, banana
2-Phenylethanol	8–35	Roses, bitter, perfumed
Selected esters	Concentration (mg/L)	Organoleptic description
Ethyl acetate	10–60	Solvent-like, sweet
3-Methylbutyl acetate (isoamyl acetate)	0.5–5.0	Banana, ester, solvent
Ethyl hexanoate	0.1–0.5	Apple, fruity, sweet
Ethyl octanoate	0.1–1.5	Apple, tropical fruit, sweet
2-Phenylethyl acetate	0.05–2.0	Roses, honey, apple, sweet
Selected aldehydes	Concentration (mg/L)	Organoleptic description
Acetaldehyde	2–20	Green, paint
Propanal	0.01–0.3	Green, fruity
Butanal	0.03–0.02	Melon, varnish
2-Methylpropanal	0.02–0.5	Banana, melon
trans-2-Butenal	0.003–0.02	Apple, almond
2-Methylpropanal	0.02–0.5	Banana, melon
Hexanal	0.003–0.07	Bitter, vinous
trans-2-Nonenal	0.00001–0.002	Cardboard
Furfural	0.01–1.0	Papery, husky
5-Methylfurfural	<0.01	Spicy
5-Hydroxymethylfurfural	0.1–20	Aldehyde, stale
Selected vicinal diketone	Concentration (mg/L)	Organoleptic description
2,3-Butanedione (diacetyl)	0.01–0.4	Butterscotch
3-Hydroxy-2-butanone (acetoin)	1–10	Fruity, mouldy, woody
2,3-Butanediol	50–150	Rubber, sweet, warming
2,3-Pentanedione	0.01–0.15	Butterscotch, fruity
3-Hydroxy-2-pentanedione	0.05–0.07	
Terpens derived from hops	Concentration (ug/L)	Organoleptic description
Linalool	1–470	-
Citronellol	1–90	-
Geraniol	1–90	-
$\alpha$ -Terpineol	1–75	-
$\alpha$ -Eudesmol	1–100	-
Humulenol	1–1150	-
Clovanediol	51–677	-

Adapted from Baxter and Hughes, 2001. hyphen (-) no descriptor

(13–18°C) or very high gravity (>18°C) brewing and heat-tolerant yeast strain development (Puligundla et al. 2020). On the other hand, product development is thriving in the pursuit of diverse and novel flavours. In contrast to traditional beer consumers, current consumers favour more flavoursome choices or flavour diversity as evidenced by the Craft beer movement. Healthy living trends also propels brewers to explore options like low alcohol beer, light beer (low calorie beer) and gluten free beer. Here we describe some approaches that have been taken to improve product diversity and flavour profiles.

### Flavoursome choices

The application of pure starter culture in industrial brewing secures fermentation stability and product quality (Walther, Hesselbart and Wendland 2014), but restricts flavour diversity; thus, many brewers look for alternative ways to enhance the flavour profile. One natural approach, often referred to as bioflavouring is to conduct fermentations with

non-conventional yeasts (NCY; Vanderhaegen et al. 2003). NCYs are also playing an important role in the pursuit of new varieties of beers such as non-alcoholic and low-alcohol beer (NABLAB). This beer type is highly desirable in the current societal climate of responsible drinking and healthy living. NCYs can be used either as a pure starter culture (Steensels and Verstrepen 2014; Steensels et al. 2015; Michel et al. 2016; Bellut et al. 2018) or as a supplement in various mix-fermentation methods (Canonico et al. 2016; Michel et al. 2016; Ravasio et al. 2018; Tan et al. 2021). The selection criteria for a brewing-suitable strain includes wort carbohydrate utilisation, ethanol tolerance, flocculation behaviour and fruity flavour production. Most non-conventional yeasts produce isoamyl acetate (Steensels and Verstrepen 2014), a banana note and a standard indicator of fruity flavour. A total of two NCYs currently in commercial use are *Saccharomyces ludwigii* and *Pichia kluyveri* (Saerens et al. 2010; Craface et al. 2013). Other yeast species being explored for beer production include but are not limited to *Torulasporea delvruueckii*, *Brettanomyces anomalus*, *Brettanomyces bruxellensis*, *Candida shehatae*,



*Candida tropicalis* and *Zygosaccharomyces rouxii* (Michel et al. 2016). Additionally, NCYs can generate novel fruity flavours; some of these are not present in traditional beer (Gamero et al. 2016; Michel et al. 2016; Bellut et al. 2018,2019; Bellut and Arendt 2019; Bellut, Krogerus and Arendt 2020). Health concerns propel the development of light (low calorie) beer and gluten free beer. These products derive from enzymatic manipulation of amylolysis and proteolysis of raw materials in the brewing process and are extensively reviewed elsewhere (Cela et al. 2020; Gumienna and Gorna 2020).

### Yeast strain improvement

Improvement of desirable traits in the yeast strains complement and enhance the alterations to the brewing process for today's consumer. Flavour profiles can be expanded or improved by altering amino acid metabolism in the lager yeast. One approach is to increase flux through the Ehrlich pathway to achieve higher concentration of higher alcohols and acetate esters. The biosynthesis of amino acids in yeast cells is regulated by feedback inhibition by the final product at the first irreversible reaction step in the biosynthetic pathway, the committed step. For the branched-chain amino acid, the committed steps are the first steps in leucine and isoleucine biosynthesis catalysed by the Leu4p and Leu9p for the former and Ilv1p for the latter (Holmberg and Petersen 1988; Kohlhaw 2003). For the aromatic amino acids, the committed step is the production of 3-deoxy-D-arabino-heptulosonate-7-phosphate by the enzymes Aro3p and Aro4p (Fig. 2; Braus 1991). Mutants that disrupt the negative feedback regulation can be obtained by growing yeasts in the presence of analogues of the terminal amino acid. Strejc et al used the amino acid analogue 5,5-trifluoro-DL-leucine to increase flux through the leucine biosynthetic pathway. The resultant mutant strains produced three times more isoamyl acetate (Strejc et al. 2013).

Classical mutagenesis, involving UV radiation and chemical mutagens such as ethyl methane sulfonate (EMS), methyl methane sulphonate (MMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), have been used to isolate mutants with altered flavour profiles. This approach needs a robust selection method due to the high number of mutants produced. Growth on the amino acid analogue, thiaisoleucine, was used as a selection following chemical mutagenesis to obtain yeast strains overproducing the flavour compound 2-methyl-butanol (Kielland-Brandt, Petersen and Mikkelsen 1979).

Mutants with reduced off flavours can also be produced. In one study, cells were treated with EMS and exposed to chlorosulfuron, which inhibits acetohydroxy acid synthase, the enzyme responsible for  $\alpha$ -acetolactate production. The selected strains had reduced levels of diacetyl, an off flavour with a smell of butter or butterscotch (Gibson et al. 2018). UV mutagenesis followed by selection in the presence of disulfiram and ethanol was used to reduce the production of acetaldehyde, a flavour that imparts notes of green apples, fresh cut grass and walnuts (Shen et al. 2014).

Hybridisation is a common technique that can be used to obtain new yeast strains with improved flavour profiles. *Saccharomyces pastorianus* is a sterile hybrid and does not produce many viable spores, making it difficult to produce new strains by classic mating and sporulation. Several new approaches such as spore-to-spore mating, rare mating and mass mating have been developed to create new inter- and intraspecies hybrids (Steensels et al. 2014). Several new hybrids of *S. cerevisiae* and *S. eubayanus* were generated through spore-to-spore mating. The resultant hybrids not only showed a wide aromatic compound

production but also a higher tolerance to higher temperatures. Some of the hybrids produced a better aromatic profile compared to both parental strains (Mertens et al. 2015).

Rare mating and mass mating between natural auxotrophs have also been used to create hybrids (Zaret and Sherman 1985; Boeke et al. 1987; Toyn et al. 2000; Krogerus et al. 2015, 2016). Rare mating involves the generation of a hybrid from strains of different ploidy, obtaining a final hybrid with a high ploidy that must be submitted to a stabilisation process. In mass mating, spores from both parental strains are mixed, obtaining strains with a ploidy roughly of  $2n$ . These techniques have been extensively reviewed by (Krogerus et al. 2017).

Another interesting approach is directed evolution, whereby cells are grown for several generations (100–200 generations) under a specific selection pressure. Growth in the presence of chlorosulfuron, produced strains with reduced levels of the off-flavour diacetyl following selection (Gibson et al. 2018). Directed evolution has also been used to generate strains with improved thermotolerance, maltotriose utilisation and ethanol tolerance (Gibson et al. 2020). In one such study, an adapted strain produced a 5-fold uptake of maltotriose resulting in higher alcohol production, improved fermentation attenuation and had no impact in aroma formation (Brickwedde et al. 2017). Directed evolution following UV mutagenesis produced an *S. eubayanus* strain capable of utilising maltotriose. The new strain contained a novel chimeric MALT gene resulting from the recombination of MALT genes. This novel gene is similar to MTY1 gene and enables the utilisation of this sugar (Brouwers et al. 2019).

High-gravity wort and very-high gravity wort are defined as fermentations with a higher content of fermentable sugars and increased FAN. Such fermentations have the potential to produce higher concentrations of ethanol, higher alcohols and esters. However, the high concentration of sugars increases osmotic pressure producing negative effects on the cells, such as increased cell volume, decreased cell viability and ethanol toxicity (Casey, Magnus and Ingledew 1984; Cahill et al. 2000). This can lead to stuck and sluggish fermentations and problems in repitching (Sigler et al. 2009). To overcome these problems, some strains have been generated by different techniques. James et al. evolved strains using EMS and growth in high-gravity wort at high temperatures (James et al. 2008). The selected strains produced higher concentrations of ethanol but additionally showed chromosome rearrangements, demonstrating that the plasticity of this hybrid genome may play a key role in the adaptation to stresses like heat or high gravity wort. Similar approaches have generated strains with a better tolerance of ethanol and increase fermentation performance in VHGF fermentation (Blieck et al. 2007; Huuskonen et al. 2010).

### Future perspectives

*Saccharomyces pastorianus* strains are unique hybrids found in breweries but not in natural environments. As newly evolved strains, dating back to just the Middle Ages, their genomes are still in flux and can undergo rearrangements in response to stress. The strains show a wide range of diversity in chromosome and gene content and such genome plasticity appears to provide the classic 'hybrid vigour' that has allowed adaptation to the unique environs of the lager fermentation vat. The presence of two sub-genomes has not only generated a unique set of hybrid genes but also allows for the potential of trans-regulation and the formation of chimeric protein complexes between the products of the two sub-genomes. A re-examination of the proteome and transcriptomes using current technology and the

quantitative integration of this data with more fully annotated and assembled genomes will greatly expand our understanding of the role of yeast metabolism in flavour compound production.

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