



Recent developments in high gravity beer-brewing

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ABSTRACT

Beer-brewing under high gravity (HG, specific gravity of 13–18 °P) and very high gravity (VHG, specific gravity of > 18 °P) wort conditions has a wide range of benefits, from improved process economics to reduced environmental impact. This review highlights the research developments in HG and VHG brewing over the last 10 years. New research emphasizes the importance of multifaceted strategies to achieve comprehensive development in HG brewing. As the brewing yeast is exposed to a variety of stresses (e.g., osmotic stress, ethanol stress) during HG conditions, stress-alleviating strategies have been the prime focus of researchers. Studies have focused on mitigating nitrogen limitation, promoting inexpensive brewing adjuncts, and realizing the potential of exogenous enzymes in HG brewing. In addition, recent studies have addressed the importance of hybrid and mutant yeasts as well as yeast flocculation and immobilization under high specific gravity conditions. Furthermore, studies to unravel yeast physiology under HG brewing and process modelling have received much attention.

1. Introduction

With growing competition in the brewing industry, high gravity (HG) and very high gravity (VHG) brewing techniques have become increasingly popular for productivity enhancement. High gravity refers to brewing of beer using an increased original gravity wort (wort specific gravity of 13–18 °P [degrees Plato] in HG and > 18 °P in the case of VHG), and subsequent dilution of fermented beer with carbonated water to a prescribed gravity or alcohol concentration (Puligundla, Smogrovicova, Obulam, & Ko, 2011; Stewart, 2010). The term ‘original gravity’ refers to specific gravity of the finished wort before fermentation starts (Thesseling, Bircham, Mertens, Voordeckers, & Verstrepen, 2019). The ‘Plato’ unit is defined as the extract in percent by weight of the wort, beer, or other solution (Weissler, 1995). HG brewing technology has become popular due to increased production capacities (productivity) in the brew house without making costly investments. Furthermore, it has ecological aspects as well, because its advantages include a reduction in water, energy, labor, cleaning, and waste costs (Gibson, 2011). Consequently, the sustainability of the process is enhanced. Other advantages of this technology include improved flavor stability of beer and reduced risk of contamination. The use of HG wort (16–18 °P) has become common practice in many breweries, while pilot-scale plants have demonstrated fermentation of VHG worts (20–25 °P) without major detriment to beer flavor characteristics.

Under higher wort gravity conditions, yeast metabolic activity and ethanol production are elevated. However, when dissolved solids concentration in the medium exceed a certain level, the effect of osmotic pressure, carbon dioxide concentration, nutrient limitation (especially free amino nitrogen [FAN] and dissolved oxygen), and toxicity of the produced ethanol cause yeast growth inhibition, decrease in yeast viability and the rate and extent of fermentation, as well as changes in the yeast metabolism (Smogrovicova, 2013). Partial substitution of barley malt with cheaper adjuncts has the potential to reduce costs as well. However, a combination of HG brewing with high proportions of adjuncts, especially unmalted grains, may lead to problems such as insufficient yeast nutrition, unsatisfactory fermentation results and product quality, in addition to wort separation problems and fluctuation of lautering time. Potential disadvantages of altering wort composition and yeast metabolism include longer fermentation time, decreased beer foam stability, and problems with beer flavor matching compared with sales-gravity brewed beers (Casey, Magnus, & Ingledew, 1984; Dragone, Silva, de Almeida e Silva, & de Almeida Lima, 2003; Puligundla et al., 2011; Stewart, 2010; Verstrepen et al., 2003).

Many authors intended to provide comprehensive information on the improvement of yeast performance under stressful conditions during HG beer fermentation by wort enrichment and supplementation. Various yeast nutrient supplements used or recommended include lipid and lipid components such as sterols and fatty acids, specific metal ions

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and free amino nitrogen, usually supplied in the form of a complex yeast food as described in a review article by Gibson (2011). The addition of yeast extracts provides a multitude of essential nutrients for the fermenting yeast, such as free α -amino nitrogen, minerals and vitamins, or generates an added benefit for a product as a result of their antioxidative properties, as has been summarized in a review by Jacob, Michel, Zarnkow, Hutzler, and Methner (2019). The combination of adaptive laboratory evolution and metabolic engineering of yeast cells offers possibilities to obtain improved brewing strains with enhanced resistance to stress and increased fermentation performance for VHG wort fermentations. Targeted genetic modification of yeast strains is one way to increase beer quality and to improve the economics of beer production. A review by Stewart, Hill, and Russell (2013) has focused on a brief description of genomics, proteomics, and metabolomics as applied to brewer's yeast strains, the uptake and metabolism of wort sugars and amino acids, yeast flocculation, the question of process intensification, with particular attention to HG brewing. Saini, Beniwal, Kokkilgadda, and Vij (2018) summarized the developments in understanding the response of yeast towards various environmental stresses during fermentation, such as heat shock, ethanol stress and osmotic stress, and different strategies for improvement of industrial strains' survival under these stresses. Current knowledge on traditional approaches and recent achievements in rational metabolic yeast engineering for improving brewing strains were summarized in a review by Karabin, Jelinek, Kotrba, Cejnar, and Dostalek (2018). Certain disadvantages associated with HG brewing and suggested interventions are included in Table 1.

The present review focuses on possible solutions and recommendations published in the last 10 years, which can be applied to enhance the efficiency and quality of HG processes, with the aim of producing beer with improved quality, stable foam, and suitable flavor profile at lower cost. Several studies evaluated the influence of barley adjuncts in HG brewing, sources and importance of nitrogenous supplements, possible protease assistance during mashing process for better nutrient utilization, and response of yeast to different fermentable sugars. In addition, researchers have focused on understanding the effect of wort gravity on physiology and accumulation of stress protectants in brewer's yeast, construction of brewer's yeast strains with increased resistance to various stresses as well as increased HG and VHG fermentation performance, and aspects related to yeast immobilization under HG conditions.

2. Yeast nitrogen nutrition

2.1. Nitrogenous supplements

Nitrogenous compounds that are readily assimilable by brewer's yeast are known as free amino nitrogen (FAN), which represents the sum of individual wort amino acids, small peptides (di- and tripeptides), and ammonium ions (Lei, Zhao, & Zhao, 2013). For prediction of viability, proper yeast growth, and fermentation efficiency, FAN has been regarded as a superior index (Inoue & Kashiwara, 1995). Appropriate FAN levels in wort assure efficient yeast multiplication and hence desirable fermentation performance. FAN composition of wort has considerable impact not only on fermentation performance, but also on beer flavor because amino acids are involved in most of yeast metabolic pathways. Beer sensory quality depends on the formation of esters, diacetyl (vicinal diketones), and sulfur compounds, which are produced as metabolic by-products of amino acid synthesis. Amino acids are also precursors of higher alcohols and aldehydes, two important classes of compounds responsible for the development of off-flavors in beer. Therefore, all influences of nitrogen supplementation should be in consideration.

During VHG brewing, amino nitrogen supplementation (e.g., amino acids, yeast extract, urea, ammonium salts) has been shown to improve yeast growth and fermentation (Puligundla et al., 2011). In VHG (20 °P) brewing, the supplementation of wort with 1.0% wheat gluten hydrolysate (a mixture of amino acids and peptides) has shown significant improvement in terms of yeast growth and fermentation, especially yeast biomass accumulation, ethanol production, and utilization rate of free amino nitrogen were improved by 4.6%, 15.2%, and 41.2%, respectively (Zhao, Lu, Zhou, Yang, & Meng, 2017). The addition of wheat gluten peptides of different molecular weights (MW) has been shown to improve the propagation and fermentation performance of brewer's yeast under HG brewing (Zhao, Mo, Zhao, & Liu, 2014). In that study, the supplementation of the peptides with varying molecular weights (5–10 kDa, 3–5 kDa, and < 3 kDa) during fermentation resulted in an enhancement of FAN utilization rate, biomass, and fermentation ethanol yield by 11%, 30%, and 5%, respectively. Furthermore, the smallest peptides (MW < 3 kDa) exerted an increased synthesis of total alcohols by 68% and a decreased production of total esters by 17%, which brought more harmonious taste (a balanced taste of sweet, sour, acidity, bitter and other tastes). In a recent study, wheat gluten hydrolysates (WGH) and their fractions (through ultra-filtration membranes) were shown to exhibit improved yeast viability, biomass accumulation, FAN and sugar consumptions, and ethanol yield under HG fermentation (Zhou et al., 2018). In that study, the fractions with MW < 3 kDa peptides and the supernatant of WGH treated with

Table 1
Certain disadvantages associated with HG brewing and suggested interventions.

Drawback	Intervention	Reference
1) Stuck and sluggish fermentations, and poor yeast viability	- A combination of nutrient supplementation and increased pitching rates has resulted in rapid fermentation of HG wort	Casey & Ingledew, 1983; Casey, Magnus, & Ingledew, 1983
2) Multiple stresses and low fermentation performance	- Correction of wort nutritional deficiencies - Yeast immobilization – significant increase of fermentation rate of VHG wort using brewer's yeasts entrapped in gel matrixes	Gibson, 2011; Smogrovicova, 2013
3) Reduced foam stability in the final diluted beer due to greater losses of hydrophobic polypeptides during the HG brewing process	1. To partially overcome this drawback, the use of hop extracts, wheat malt and increased sparging of the mash with either a lauter tun or a mash filter has been recommended 2. Deletion of the gene (<i>PEP4</i>) that encodes for proteinase A (PrA), a proteolytic enzyme	Stewart, 2010
4) The production of disproportionately high levels of esters	- The use of very high maltose syrup as an adjunct has been recommended	Stewart, 2010
5) The inability of yeast to completely utilize the major fermentable wort sugar – maltotriose.	- Evolutionary engineering for strain improvement	Brickwedde et al., 2017
6) Diminished oxygen availability	- Partially overcame using preoxygenated yeast	Debourg, 2010
7) Poor hop utilization	- Post-fermentation hopping, using isomerized hop extracts (e.g. iso- α -acids produced from CO ₂ hop extract)	Barth-Haas Group, 2018

ethanol precipitation exerted significantly better growth- and fermentation-promoting activity for brewer's yeast. The presence of peptides having high levels of leucine, lysine, histidine and arginine, and relatively low molecular weight has been proposed as a possible reason for bioactivity of these fractions. In another study, the supplementation of wheat gluten hydrolysate fractions separated by macroporous resin was shown to improve the ethanol and osmotic stress tolerance of brewer's yeast in HG brewing (Yang, Zong, Cui, Mu, & Zhao, 2018a). In that study, a significant improvement in ethanol tolerance and the viability of brewer's yeast was noted by the supplementation of 40% ethanol fraction separated by XAD-16 resin. In addition, 0% ethanol fraction separated by the resin increased the osmotic stress tolerance and the viability of brewer's yeast.

Amino acid supplementations have been shown to improve the fermentation performance and stress resistance of the lager yeast (*Saccharomyces pastorianus*) during HG fermentation (Lei, Feng, Peng, & Xu, 2019). In that study, ethanol (10%, v/v) toxicity was shown to be the predominant cause of decreased cell viability, yeast growth, and wort fermentability in comparison with high wort gravity (24 °P). Significant changes in the assimilation of free amino acids (FAA) were induced by high ethanol toxicity, positive correlations between the assimilation amounts of FAA (aspartic acid, serine, glycine, arginine, tyrosine, valine, methionine, lysine, isoleucine, and leucine) and fermentation performance (cell viability, fermentability, and ethanol production) under different ethanol toxicity conditions were noted. The expression level of *HSP12* (encodes one of the two major small heat-shock proteins) was upregulated and enhanced intracellular accumulation of trehalose in yeast was noted following supplementation of these FAA, indicating stimulation of stronger protective mechanism.

Lysine and histidine are the key amino acids for lager yeast under normal gravity as well as HG conditions. The roles of lysine and histidine in nitrogen regulation under HG conditions were further elucidated by Lei, Li, et al. (2013). They showed that the supplementation of lysine, histidine and their mixture enhanced yeast growth, wort fermentation, flavor volatiles formation, and ethanol yield. Substantial upregulation of *Ssy1p-Ptr3p-Ssy5p* (SPS)-sensor-regulated genes was observed upon lysine supplementation when compared with nitrogen catabolite repression (NCR)-sensitive genes. Transport of amino acids into yeast cells occur by general and specific transport systems. Based on the availability of the corresponding amino acids present in medium, the SPS sensor regulates the expression of some genes encoding specific permeases (membrane transport protein). On the other hand, slight activation of SPS-sensor-regulated genes in exponential phase and a significant repression of NCR-sensitive genes throughout the fermentation were observed upon histidine supplementation. On supplementation of lysine and histidine, the consumption of glutamic acid and phenylalanine was increased, and the consumption of serine, tryptophan, and arginine was decreased. In addition, lysine and histidine supplementations exerted identical effects on the fermentation performance and were more efficacious than their mixture addition when the same dose was used.

In another study, the superior effect of supplementation of peptide Lys-Leu over a FAA mixture of lysine and leucine on the improvement of fermentation performance, physiological activity, and nitrogen metabolism of brewer's yeast during VHG (24 °P) wort fermentation has been shown (Yang, Zong, Cui, Mu, & Zhao, 2018b). In that study, the supplementation of peptide Lys-Leu was shown to increase the assimilation of aspartic acid, but decreased the assimilation of glycine, alanine, valine, cystine, isoleucine, leucine, tyrosine, phenylalanine, lysine, arginine, and proline. However, the FAA mixture supplementation only promoted the assimilation of lysine and leucine, while reduced the absorption of total amino acids to a greater extent.

The potential of walnut meal protein hydrolysates, which were prepared using commercial proteolytic enzymes Neutrase®, Protamex®, and Flavorzyme® with/without ultrasonic treatment, for improving fermentation performance of lager yeast in HG (24 °P) brewing has been

shown (Li et al., 2019). In that study, compared with conventional enzymolysis, ultrasonic-assisted enzymolysis and ultrasonic pretreatment followed by enzymolysis were found to improve the degree of hydrolysis significantly. Relatively high degree of hydrolysis was noted following treatment with Protamex than Neutrase or Flavorzyme. The fermentation capacity of yeast was substantially enhanced by wort supplementation with walnut meal protein hydrolysates prepared by ultrasonic-assisted enzymolysis using any of these enzymes, especially those prepared using Protamex with the highest major fractions of MW < 1 kDa exhibited enhanced wort fermentability by 15% and ethanol production by 17%. The stability of beer foam was improved by ultrasonic-assisted enzymolysis using Flavorzyme with the highest major fractions of MW > 3 kDa. In addition, walnut meal protein hydrolysates supplementations significantly increased cell viability and yeast growth (consequently, improved rate of wort fermentation and ethanol yield), promoted trehalose (stress protectant) and glycogen accumulation, upregulated *HSP12* and *SSA3* (stress-related genes) expression in yeast cells, enhanced the formation of higher alcohols and esters, and increased higher alcohols-to-esters ratio, indicating a better balanced taste of final beers.

2.2. Nitrogen levels

Under high wort gravity conditions, the rate of sugar consumption (glucose, maltose, and maltotriose), expression levels of *ATF1* (encodes an alcohol acetyl transferase which catalyzes the synthesis of acetate esters from acetyl CoA and several kinds of alcohols), *BAP2* (encodes a branched-chain amino acid permease), *BAT1* (encodes a branched-chain amino acid transaminase), *HSP12*, and *TDH* (encodes a glyceraldehyde-3-phosphate dehydrogenase) genes, and net cell growth have been found to be relatively low (Lei, Zhao, Yu, & Zhao, 2012). In that study, with increasing level of nitrogen in the wort, all these processes were shown to be increased. In addition, the levels of higher alcohols and esters were found to be high at low nitrogen level under high wort gravity. These results demonstrated that during beer-brewing, wort gravity and nitrogen level can significantly affect yeast physiology and flavor balance.

2.3. Protease assistance

In HG beer-brewing, nitrogen limitation results in stuck or sluggish fermentations due to reduced yeast viability. On the other hand, during fermentation, brewer's yeast cannot assimilate extracellular proteins and long-chain peptides since most yeasts does not secrete acid protease activity into the surrounding medium. In a study, the possibility for utilizing unavailable nitrogen from HG (20 °P and 24 °P) worts by adding commercial proteases (Neutrase, Flavorzyme, and Protamex) at the beginning of fermentations was investigated (Lei, Zhao, & Zhao, 2013). Results showed that the supplementation of the proteases significantly increased the levels of wort FAN and thus the number of suspension yeast cells in the later stages of fermentations. Flavorzyme supplementation exhibited the best fermentation performance among the tested proteases in terms of significantly improved wort fermentability, higher ethanol yield, and flavor volatiles formation. In addition, the proteases did not alter foam stability of final beers compared to control at each of the corresponding gravities.

In another study, changes in FAA composition following the supplementation of these three proteases at the start of wort mashing, and the supplementation effects on FAA assimilation pattern and fermentation performance of lager yeast during normal and HG fermentations were monitored (Lei, Zheng, Wang, Zhao, & Zhao, 2013). Significant improvements in the extract yield and FAA level of mashed worts were observed by proteases supplementation. HG worts treated with Neutrase and Protamex exhibited increased fermentability, flavor volatiles concentration, and ethanol production compared with controls. On the other hand, these beneficial effects were observed in normal gravity

worts treated with Neutrase and Flavorzyme. Changes in FAA assimilation pattern with alteration in wort gravity were proposed to be a reason for these results. Enhanced assimilation ratios of leucine, arginine, phenylalanine, histidine, aspartic acid, and valine were observed under increased wort gravity. In HG fermentations, strong correlations between the assimilation amounts of lysine and histidine and fermentability were observed. These results concluded that optimizing FAA composition (through proteases supplementation) during wort mashing was advantageous to beer-brewing in terms of yeast fermentation performance and flavor volatiles formation. The impact of wort amino acids composition on beer flavor has been elaborately discussed in a review by Ferreira and Guido (2018), and flavor-related aspects of beer fermentation are discussed below (see Section 6), as well.

3. Brewing adjuncts and wort sugars

Brewing adjuncts are materials other than malted barley that bring additional sources of carbohydrates and protein into wort (Goode & Arendt, 2006). Different cereals, including wheat, barley, maize sorghum, rice, and oats, are commonly used (in different forms: whole cereal, grits, flaked, extrusion cooked, terrified/micronized, flour/starch, syrup, and malted cereals) as brewing adjuncts. The application of liquid adjunct materials has been suggested for a significant reduction of production costs and to increase production output in HG brewing worts. In addition, they contribute to product character (Goode & Arendt, 2006).

Low-cost and abundant starch-rich raw materials such as rice and corn starch are widely used as adjuncts (up to 40–50%) in HG beer-brewing. Such a high proportion of adjunct necessitates increasing the ratio of material to water (up to 1:3.0–3.5) during adjunct liquefaction, which, in turn, leads to problems like instable process of wort separation and fluctuation of lautering time (Zhu, Ma, Li, & Li, 2015). In a previous study, a designed observing filter device was used to observe the dynamic formation of spent grain layer (SGL) during lautering (a process in which wort filters through SGL) of mash. The permeability of SGL significantly influences the lautering time in high-adjunct-ratio brewing process apart from wort viscosity. A dense, viscous, and layered SGL was induced by a high proportion of corn starch, which reduced the permeability of SGL. On the other hand, a loose, polyporous, and uniform SGL (increased permeability of SGL) was resulted by rice flour. Particle properties of adjunct were shown to be a key blocking factor. A complete degradation of residual fine particles was suggested to improve the lautering process under HG beer-brewing (Zhu et al., 2015). Fine particles of corn starch could be degraded using exogenous α -amylase application. Corn starch has been a preferred adjunct in HG beer-brewing because of its high fermentability (Kang, Wu, Li, & Zheng, 2009). However, when corn starch proportion increases up to 30–50%, issues like extension of wort lautering (due to residual fine corn starch particles) may occur, which in turn decreases brewery productivity. The use of mesophilic α -amylase was shown to be an effective, simple, and practical solution for improving the lautering performance of high-corn-starch-ratio wort preparation (Zhu, Ma, Mei, & Li, 2017). Routinely used viscosity-reducing enzymes failed to improve the lautering rate. Factors responsible for the inhibition of wort filtration was found to be low water content in liquefaction, but not the resistant starch content and amylopectin proportion.

Partial substitution of barley malt with adjuncts has the potential to reduce costs. Effect of substituting malted barley with 10–40% unmalted oats on the quality of HG mash/worts was investigated (Schnitzenbaumer & Arendt, 2014). In that study, increased color/pH values, mash consistencies, wort viscosities 12%, β -glucan concentrations, and filtration times were observed when mashing with up to 40% oats. However, FAN and extract contents were decreased. On the other hand, the application of Ondea® Pro (an enzyme cocktail consisting of pullulanase, α -amylase, endoprotease, β -glucanase, xylanase, and lipase) significantly increased the processability and quality of 30 or 40%

oat-containing mash/worts. Nevertheless, mashing with up to 20% unmalted oats did not require the addition of exogenous enzymes.

With increased proportions of starch adjuncts, time needed for complete fermentation may be extended. A novel approach has been suggested to improve starch digestibility in a previous study; starches treated by a nonthermal plasma exhibited enhanced *in vitro* starch digestibility (Gao et al., 2019). In that study, DBD plasma-modified starches exhibited an increased accessibility to enzymes and the plasma treatment reduced the viscosity of starch. They concluded that the plasma can potentially be used as a pretreatment agent to accelerate starch hydrolysis during processes such as HG brewing. However, further studies are required to investigate whether plasma-processed starches can be readily used in brewing with no concerns regarding potential toxicity and allergenicity.

The response of lager yeast, *S. pastorianus* CGMCC No. 4466, to glucose, sucrose and maltose, the three main fermentable sugars in brewing, under high gravity (18 °P) and very high gravity (24 °P) fermentations was investigated by Lei et al. (2016). Throughout 18 °P wort fermentation, glucose or sucrose supplementation had improved the fermentation performance of the lager yeast. However, compared with sucrose, in the later stage of 24 °P wort fermentation, the yeast fermentation performance was greatly improved by maltose supplementation, which increased wort fermentability and ethanol production by 14% and 10%, respectively. By maltose supplementation with increase of the wort gravity from 18 °P to 24 °P, higher HSP12 expression level and more intracellular trehalose accumulation in yeast cells were observed, indicating higher stress response of yeast cells. Maltose supplementation stimulated the excretion of glycine and alanine, and the absorption of proline in the later stage of fermentation. It has been shown that, during HG fermentation, proline could increase the stability of membranes of yeast cells or proteins (Lei et al., 2016; Takagi, 2008). Additionally, with increase of the wort gravity from 18 °P to 24 °P, esters formation was increased significantly with glucose supplementation, while higher alcohols level was decreased with maltose supplementation. In another study, the production of maltose (the primary product of starch degradation during mashing), as could be expected in HG mash/worts, was shown to significantly increase the activity and thermostability of barley malt α -amylase (Duke & Henson, 2016).

4. Microbiological aspects

4.1. Hybrid and mutant strains

Hybridization is a potential tool for the generation of novel non-genetically modified brewer's yeast strains with unique properties (Krogerus, Magalhães, Vidgren, & Gibson, 2017). Fermentation traits of newly created interspecific lager yeast hybrids, whose genomes are often unstable, can be further improved by adaptive evolution. To generate variant strains with improved fermentation performance in HG wort, adaptive evolution in high ethanol media was successfully applied. In a study, this approach was used to generate stable and superior variant strains from four different brewing strains, three of which were *de novo* interspecific lager yeast hybrids with different ploidy levels (Krogerus, Holmström, & Gibson, 2018). In that study, during wort fermentation, these adapted variants outperformed the strains from which they were derived. In addition, the majority variant strains possessed several desirable brewing-relevant traits, such as increased ethanol tolerance and ester formation, and decreased diacetyl formation.

Evolutionary engineering or whole-cell directed evolution is a simple and effective conventional genetic engineering method to improve industrial strains. The potential of evolutionary engineering for strain improvement of brewing yeast (hybrid, allopolyploid) has been shown (Brickwedde et al., 2017). In that study, for improving maltotriose (the second most abundant sugar of brewer's wort after maltose and is not fermented by several industrial yeasts) fermentation kinetics,

a new evolutionary engineering strategy was tested. Spontaneous mutants with enhanced affinity for maltotriose were selected by chemostat cultivation (under anaerobic and prolonged carbon-limited conditions) of the reference strain *S. pastorianus* CBS1483 on a maltotriose-enriched sugar mixture. Compared with the parental strain, the evolved strains exhibited a higher ethanol concentration, improved attenuation, and a substantially lower (~5-fold) residual maltotriose concentration (4.75-fold higher transport capacity for maltotriose). These improvements were also demonstrated in pilot-scale (1000 L) HG wort fermentations with no negative effects on flavor compound profiles.

Compared with *S. cerevisiae* strains (ale yeasts), lager yeasts are more sensitive to high-salt stress and high-temperature stress. On the other hand, because of allotetraploid nature of lager yeasts, selection of novel strains for improved fermentation performance or traits is laborious. Sanchez, Solodovnikova, and Wendland (2012) generated novel lager $\times$ ale hybrid strains with increased stress resistance (resistance against high ethanol concentrations or high osmolality) and growth at elevated temperature via improvements in classical yeast breeding. At first, they developed F1 hybrids by classical genetics, using spore clones of lager and ale yeast and complementation of auxotrophies of the single strains upon mating. New hybrids (resembling zygotes) were isolated by micromanipulation and derived colonies were analysed by mating type PCR. Certain F1 hybrids exhibited increased attenuation of sugars and all hybrids showed increased survival of cells at the end of fermentation. Some of the hybrids exhibited improved fermentation rates using 18 °P wort at 18 °C and 25 °C.

In lager hybrid yeast, the contribution of parental subgenomes to phenotypic traits such as aroma production, fermentation performance, and stress tolerance was elucidated by Krogerus et al. (2016). They have generated three *de novo* lager yeast hybrids, using hybridization techniques, without genetic modification. These hybrids exhibited different ploidy levels (allodiploid, allotriploid, and allotetraploid). During fermentation, hybrids with higher DNA content performed better and produced higher concentrations of flavor-active esters in both HG wort (15 °P) and VHGW (25 °P). The hybrid strains also outperformed the parent strains (*S. cerevisiae* and *S. eubayanus*) in terms of fermentation rate and higher concentrations of certain flavor-active esters in produced beer. In higher ploidy hybrid strains, the presence of several genes related to the formation of flavor-active esters (*ATF1*, *ATF2*, *EHT1*, *EEB1*, and *BAT1*) in higher copy numbers was noted. The results could facilitate targeted strain development through interspecific hybridization.

It is well known that, under HG brewing, yeast cells are exposed to multiple stresses, such as elevated ethanol concentration, higher osmotic pressure, and nutritional imbalance. These stresses, in turn, enhance yeast autolysis, which adversely affects beer flavor and quality. In order to improve anti-autolytic ability, strain with overexpression of the *FKS1* gene (which encodes a subunit of 1,3- β -D-glucan synthase) was constructed by 18S rDNA (Li, Wang, & Li, 2015). The overexpression strain exhibited relatively high concentration (62% more) of β -1, 3-glucan than that of wild-type strain. In addition, the *FKS1* overexpression strain shown increased stress-coping ability under salt stress (0.4 mol/L NaCl), ethanol stress (8% ethanol), and starvation stress. When subjected to simulated autolysis, strain overexpressing *FKS1* showed superior anti-autolytic ability.

Since it is known that robust yeast strains that are tolerant to different environmental stresses could overcome the disadvantages of HG brewing, stress tolerance and fermentation performance of mutants of *S. pastorianus* FBY0095 were tested for VHGW brewing using 15% (w/v) maltose and 15% (w/v) ethanol (Yu et al., 2012a). In that study, one of two useful variants, namely L6, exhibited better growth, wort consumption, and ethanol production rates of 0.036, 1.13, and 0.49 g/L/h, respectively, when compared with wild type (0.028, 0.98, and 0.4 g/L/h, respectively). In addition, improvements in the vitality of the variant as well as flavor of resulting beer were noted when compared with wild-type.

Conventional UV mutagenesis strategy has been used generate two stress-resistant lager yeast mutants, MR0-8 and MR1-2 (Li, Wang, Phornsanthia, Yin, & Li, 2014). These mutants exhibited thickened cell walls. Compared with the parent strain (brewing yeast Y-1), the mutant yeast cells (MR0-8 and MR1-2) possessed 26% and 33% higher mannan contents, 64% and 52% higher glucan contents, and 34% and 31% higher chitin contents, respectively. These three major components together form approx. 90% of the entire cell wall, which is the first line of defense against external stresses. During HG brewing, these mutants exhibited enhanced cell viabilities, increased fermentation rates as well as intracellular trehalose levels when compared with the parent strain. In addition, relatively low diacetyl levels and balanced flavor profiles were observed in beers fermented by the mutants.

In order to decrease the production of higher alcohols and increase yeast flocculation under HG beer fermentation and at high temperature, a mutant yeast strain S6-BF2 was constructed by replacing *BAT2* gene (encodes a branched-chain amino acid transaminase) by the *FLO5* expression cassette [encodes lectin-like cell wall protein (floculin) involved in flocculation] in wild-type strain S6 (Sun et al., 2019). The flocculation ability of the mutant enhanced by 63% compared with the wild-type strain. Under high temperature and high gravity beer fermentation by the mutant S6-BF2, higher alcohols concentration was reduced from 175.58 to 159.58 mg/L, the repression of activity of mitochondrial branched-chain amino acid transaminase was observed. In addition, the ratio of higher alcohols/esters in beer was reasonable.

De novo yeast hybrids have been shown to exhibit a number of desirable characteristics relevant to HG brewing, including enhanced aroma formation, swift fermentation rates, and higher stress tolerance. Such interspecific yeast hybrids may possess better sugar (e.g., maltotriose) utilization capacity and low temperature tolerance (suitable for lager brewing) than parents (Gibson et al., 2017).

5. Physiological aspects

5.1. Physiology of brewer's yeast

Yeasts can swiftly adapt their physiology to surrounding environment by reorganizing their genomic expression (Gasch & Werner-Washburne, 2002). The transcript analysis with aid of affinity capture (TRAC) method was successfully used to monitor the expression of a focused set of genes at frequent intervals throughout VHGW (25 °P) fermentations (Rautio, Huuskonen, Vuokko, Vidgren, & Londesborough, 2007). Their results showed strong upregulation of genes involved in glycolysis, ergosterol synthesis, and maltose metabolism in 2–6 h following pitching. In addition, genes of oxygen-requiring pathways were shown to be upregulated during the final fermentation stages. The results indicated that yeast performance in a complex process environment can be monitored and optimized through this approach.

The effect of initial wort gravity [normal gravity (NG), high gravity (HG), and very high gravity (VHG)] on physiological activity of brewer's yeast has been studied (Yu et al., 2012b). In that study, specific fermentation rate and viability of lager yeast FBY009505 (*S. pastorianus*) and ale yeast FBY0099 (*S. cerevisiae*) were shown to be inversely related to wort gravity. In addition, reductions in α -glucosides transport rate and glycolytic enzyme activities were observed with increased wort gravity. On the other hand, adenylate energy charge in yeast cells as well as the levels of intracellular trehalose and glycerol were found to be directly proportional to wort gravity.

In another study, the combined effect of high gravity and high-cell-density fermentations on yeast physiology and flavor compound production was evaluated (Dekoninck, Verbelen, Delvaux, Van Mulders, & Delvaux, 2012). They showed that yeast viability decreased, trehalose levels increased, and expression levels of stress-related genes increased with increasing wort density. Additionally, stimulating effect of worts with high sucrose levels on yeast growth, glycogen formation, the uptake of amino acids, ethyl ester synthesis, and the turnover rate of total

diacetyl was shown. However, sucrose-based wort exerted relatively high osmotic stress on yeast and remarkably decreased acetate esters. Hence, a combination of sucrose and maltose syrups was recommended to increase the wort density.

The role of S-adenosylmethionine (SAM), the principal methyl donor for numerous methylation reactions, in HG brewing has been determined (Oomuro et al., 2018). In that study, the addition of exogenous SAM exhibited a positive effect (increased fermentation rate) on alcoholic fermentation in *S. pastorianus* cells in both high-maltose synthetic medium and high gravity wort. In high-glucose synthetic medium, *S. cerevisiae* cells with deleted adenosine kinase gene *ADO1* (which leads to SAM accumulation) exhibited elevated fermentation rate at 15 °C but not at higher temperatures. These results concluded that SAM acts as a positive regulator in HG brewing at low temperatures. However, the fermentation rate was not affected by the addition of methionine or adenosine (the components of SAM).

Factors affecting extracellular osmolality during brewing fermentations were quantitatively measured and their effects on yeast at the physiological and molecular level were determined (Zhuang, Smart, & Powell, 2017). Osmolality was shown to increase by ethanol production. High osmolality exerted a negative impact on yeast viability, physiology, and vitality. The fluidity of cell membrane was found to be altered and genome integrity was unaffected. These results provide an explanation for reduced yeast quality typically observed under HG conditions.

5.2. Role of vacuolar H^+ -ATPase (V-ATPase)

Ethanol-induced growth inhibition and fermentation in yeast is mediated by different mechanisms, including intracellular acidification (Rosa & Sá-Correia, 1996). Yeast V-ATPase transports intracellular H^+ to the vacuole and this may contribute to yeast's recovery from intracellular acidification. V-ATPase has been shown to be associated with the tolerance of yeast cells to various types of stresses (osmotic, ethanol, air-drying, freeze-thaw, and acid stresses) (Hasegawa et al., 2012). Under HG conditions, bottom-fermenting yeast strains (in which the V-ATPase-related genes *DBF2*, *VMA41/CYS4/NHS5*, and *RAV2* were overexpressed) exhibited relatively high ethanol tolerance and increased fermentation rates compared with parent strain. These results indicate that the ethanol tolerance of bottom-fermenting yeast is directly proportional to V-ATPase activity (Hasegawa et al., 2012).

5.3. Proton efflux rate

To assess the quality of beer fermentation, the proton efflux rate (PER) test is largely employed. Increased initial wort gravity has been shown to decrease PER in lager yeast (*S. pastorianus*) (Yu, Zhao, Wan, Sun, & Zhao, 2011). They showed that, from normal to VHG brewing, the difference in PER values at the beginning of the fermentation was lower than at the end of fermentation. Under HG and VH conditions, PER in *S. pastorianus* was inhibited in the later stages of brewing. Wort ethanol concentration at > 4% (w/v) was significantly negatively correlated with PER value. Therefore, the performance of yeast in HG and VH brewing can be conveniently monitored and explained by determining changes in PER.

6. Flavor-related aspects

Flavor constituents of beer can be divided into four main groups, namely primary (main), secondary, tertiary, and background flavor components. Ethanol, carbon dioxide, and bitter hop substances (isohumulones) belong to primary group, and their removal will lead to a drastic change in a product's flavor (Kosiv, Kharandiuk, Polyuzhyn, Palianytsia, & Berezovska, 2017). The concentrations of primary flavor compounds usually exceed twice the sensitivity threshold. Crucial beer flavor compounds such as ethyl caprylate, isoamyl acetate, ethyl

acetate, and amyl alcohols belong to secondary group; and their concentrations are 1.2-times higher than the sensitivity threshold. Flavoring components with concentrations 0.1–0.5 below the sensitivity threshold belong to tertiary group (e.g., phenylethyl acetate and acetoin). Substances in concentrations of > 10 times below the sensitivity threshold belong to background components (hundreds of different components include in this group), and they form background taste of beer. All flavor components are responsible for product sensory quality and they act together.

6.1. Flavor profile

Flavor profiles of young beers obtained by fermentation of HG worts with dry matter concentrations of 14, 16, and 18 wt%, and at temperatures of 8.85, 13.85, and 18.85 °C, were determined (Kosiv et al., 2017). They showed that the concentrations of flavor components (by-products of fermentation) of young beer, including *n*-propanol, *n*-butanol, acetaldehyde, vicinal diketones, *i*-butanol, 2-methyl-butanol, 3-methyl butanol, ethyl acetate, and isoamyl acetate, were varied depending on wort gravity and fermentation temperature; and beer with good flavor and aroma profile could be obtained from HG fermentation of 16% wort at 13.8 °C, followed by dilution using prepared water.

The flavor of a beer depends not only on the content of its compounds, but also on their ratio (Kunze, 1996; Polednikova, Voborsky, Chladek, & Sruma, 1993). The optimum higher alcohols-to-esters ratio in lager beers according to Polednikova et al. (1993) as well as Brányik, Vicente, Cruz, and Teixeira (2004) is considered to be between 4:1 and 4.7:1. Lager beers with balanced flavor have been shown to possess the higher alcohols-to-esters ratios close to 2.5–3:1 (Dragone, Mussatto, & Almeida e Silva, 2007).

6.2. Ester synthesis

During brewery fermentation, volatile esters are one of the important flavor-active compounds derived from yeast. Dissolved oxygen concentration influences ester synthesis. The effects of O_2 exposure time at vessel filling on the synthesis of esters during fermentation was evaluated by Silverstone, White, and Boulton (2015). In that study, all the yeast was pitched into 10 L of HG wort (16.5 to 17.0 °P) in control fermentations. In trial fermentations, all the yeast was pitched at the start of collection and wort was added as three 3.33-L batches or five 2-L batches over 12 or 24 h, respectively. Wort oxygenation was maintained at a level of 15 ppm for each experiment, with uniform temperature profiles and pitching rates. Compared with the controls, increasing the number of batch fills to three and five reduced the final concentration of isoamyl acetate by 15.7 and 34%, respectively, and ethyl acetate by 25 and 39%, respectively. Further decrease of acetate esters was observed upon the application of hydrostatic pressure. Data indicated that vessel size was inversely proportional to brew length and ester production.

7. Aspects of cell immobilization

7.1. Yeast flocculation

Yeast flocculation has been employed in brewing industry for a long history, which enables convenient and low-cost cell separation. In general, nutrient starvation and/or stress conditions induce flocculation in brewer's yeast. The presence of residual sugars, such as glucose, maltose, and sucrose in fermenting medium, which is generally seen in HG wort, negatively influences flocculation. However, higher nitrogen levels in HG brewing have been shown to improve the flocculation ability (Lei et al., 2012). The flocculation intensity of engineered flocculating yeasts (constructed by substituting silenced native promoters of the *FLO* genes with inducible promoters, such as *HSP30*, *ADH2*, and *JEN1*, for induced flocculation or constitutive promoters, such as *ADHI*

and *TDH3*, for constitutive flocculation) has been shown to be inhibited under circumstances. For instance, when the ethanol level reaches 6% (v/v) during fermentation, yeast flocculation (induced by *HSP30* promoter-controlled expression of *FLO* genes) was found to be inhibited (Govender, Domingo, Bester, Pretorius, & Bauer, 2008). Therefore, for developing yeasts for brewing or ethanol fermentations under HG or VH conditions, this strategy proved to be unsuitable. Alternatively, a cell-cell flocculation induced by trehalose-6-phosphate synthase 1 (*TPS1*) promoter was proposed in a previous study (Li, Zhao, Chang, Zhang, & Bai, 2012). In that study, the non-flocculating industrial yeast *S. cerevisiae* 4126 was transformed (with the cassette *HO-TPS1-FLO1_{SPSC01}-KanMX4-HO*) into a new flocculating yeast strain, ZLH01, by chromosome integration. Flocculation of the ZLH01 was found to be triggered by 3% (v/v) ethanol and a complete flocculation was observed when ethanol concentration further enhanced to 8% (v/v).

Premature yeast flocculation (PYF), which is characterized by the settling of yeast out of fermenting wort more heavily or earlier than usual and prior to complete attenuation, is occasionally observed in brewery fermentations. PYF negatively impacts beer flavor and alcohol yield (lower than expected). Malt-originated factors such as antimicrobial peptides and high molecular weight polysaccharides are believed to induce this accelerated flocculation. The effects of PYF factors, under HG (15 °P) conditions, on yeast physiological characteristics and metabolite profile were determined (Panteloglou, Smart, & Cook, 2013). In that study, minor differences were noted in terms of yeast growth and metabolic aspects (gravity drop, CO₂ evolution, and ethanol yield) between PYF+ and PYF- fermentations. Tested PYF+ malt was known to have caused PYF in brewery fermentations. The 'bridging' polysaccharide mechanism was very likely responsible for inducing PYF. Rapid yeast growth and fermentation were prominent in stirred fermentations (mechanical agitation) than in unstirred fermentations.

The feasibility of continuous HG brewing in an airlift bioreactor over a long period (54 days), using flocculated biomass (*S. pastorianus* strain no. 96), was studied (Pires et al., 2014). The yeast cells (up to 53 g dry wt/L) were remained in the immobilized state regardless of constant liquid agitation and washout of biomass. Two types of beer were produced based on two types of wort, namely a Pilsener (15.6 ± 0.3 °P) and a dark lager wort (14.4 ± 0.03 °P), without process interruption, indicating flexibility of the system. Even during the inlet of HG wort, desired attenuation was achieved with no need for either a supplementary second stage bioreactor or recirculation. The characteristics of green beers produced from these worts were comparable to the standards of regular finished primary beer fermentation.

Flocculation and cell surface hydrophobicity (CSH) of two lager and two ale brewing yeast strains (in YPD medium and in 12 and 18 °P all-malt worts) were determined in anaerobic, static, and aerobic shaken stationary (72- and 96-h growth) cultures (Kopecká et al., 2015). In that study, data indicated that *FLO* genes (the major factor affecting both flocculation and CSH appears to be the supply of oxygen during cultivation) were observed equally in lager and ale yeast strains. In anaerobically grown cultures, both flocculation and CSH were high and very similar irrespective of culture medium and yeast strain. On O₂ supply, certain strain-specific and medium-dependent variations in both parameters appeared. Under low O₂ supply (in static cultures), the variations in flocculation became moderately medium- and strain-dependent, whereas under high O₂ supply (in aerobic cultures), the impact of culture medium (YPD versus worts of different gravity) on flocculation became very strong, with lager strains being much more flocculent than ale strains. In tested strains, the CSH in lager strains (except under anaerobic conditions) was found to be (about 20%) higher than in ale cultures, and no differences in CSH were observed between cells grown aerobically in 12 and 18 °P worts. CSH was reduced in wort-grown aerobic cultures and enhanced in wort-grown static cultures relative to YPD-grown cultures.

7.2. Yeast encapsulation

The immobilization of yeast in gel matrix protects them against osmotic stress under HG conditions. Smogrovicova (2013) has reported a significant increase of fermentation rate of VH wort (24 °P) for brewer's yeasts entrapped in gel matrixes in contrast to free yeast cells. In beers produced through VH fermentation (24 °P) in continuous 'gas-lift' reactor, the concentrations of esters and higher alcohols were higher, compare to normal gravity (12 °P) beers, in both cases – using free and entrapped yeasts in calcium pectate. Beer produced from VH wort by yeasts entrapped in calcium pectate had suitable higher alcohols-to-esters ratio, contrary to beer produced by free control yeasts in VH conditions. However, purchasing encapsulation matrices adds an additional cost to the total.

In another study, at different original wort gravities (9 to 17 °P; batch fermentations at 15 °C), immobilized yeasts (encapsulated in alginate/chitosan matrix) exhibited a high real degree of fermentation and produced slightly higher levels of total fusel alcohols and esters compared with free cells. On the contrary, slightly high levels of aldehydes were produced by free cells. Relatively high specific growth rate and high specific ethanol production rate were observed for the immobilized cells. It was concluded that yeast immobilization did not affect the fermentation process and the formation of by-products (Naydenova, Vassilev, Kaneva, & Kostov, 2013).

8. Miscellaneous aspects

8.1. Process modelling

Full factorial designs are used to study the combined influence of different experimental variables on a response. A full factorial design established using JMP 5.01 software (SAS Institute) was applied to evaluate the effect of environmental factors (wort concentration, dissolved oxygen, fermentation temperature, and yeast pitching rate) on fermentation parameters (fermentation time, final ethanol concentration, final apparent extract, real degree of fermentation, and residual fermenting sugar concentration) during VH brewing (22 °P) fermentations (Lima, Brandão, Lima, & Teixeira, 2011). This study results demonstrated that worts at 22 °P can be fermented in the same period of time as a 15 °P wort, by increasing the oxygen level to ~22 ppm, the temperature to 18 °C, and the pitching rate to 22 × 10⁶ cell/mL. Upon diluting to obtain an 11.5 °P beer extract, the volumetric brewing capacity increased 91% for the 22 °P wort fermentation.

A mathematical model was developed to calculate the amount of higher alcohols and esters, under HG brewing conditions, depending on wort initial concentration and fermentation temperature (Kosiv, Kharandiuk, Polyuzhyn, Palianytsia, & Berezovska, 2016). In that study, the regression equation ($Y_i = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2$) was used; where *Y* – response function (parameter of the process efficiency) depends on process conditions; *X* – independent coded variables (process conditions). The optimal conditions of the main fermentation were found to be fermentation temperature of 284–285 K and dry matter concentration in wort of 14–15%.

8.2. Vitamins in brewing

The effects of various fermentation conditions and variations in yeast strains on vitamin (thiamine and riboflavin) content in final beer were studied by Hucker, Wakeling, and Vriesekoop (2016). They showed that the thiamine content of wort was found to be quickly utilized during the initial stages of a standard fermentation and its uptake was unaffected by increases in wort gravity. Intracellular accumulation of both free thiamine and thiamine diphosphate during wort fermentation was observed; however, no significant changes were noted in extracellular phosphorylated vitamins of thiamine. On the other hand, inefficient utilization of extracellular riboflavin vitamins

was observed during beer fermentations; however, rapid intracellular accumulation of flavin mononucleotide was noted. The thiamine or riboflavin utilization was unaffected when yeast was exposed to an all-malt HG wort. However, in adjunct-driven HG worts, thiamine utilization was reduced. Regardless of the lowered thiamine uptake under HG conditions, minor improvements in yeast viability and fermentation performance were noted. Under both normal and HG conditions, thiamine addition to an all-malt wort did appear to enhance yeast viability.

8.3. Bacterial spoilage

Enterobacter (including *E. agglomerans*), *Hafnia* (*Flavobacterium* or *Obesumbacterium*), *Citrobacter*, and *Klebsiella* species are common bacterial contaminants through most stages of conventional gravity brewing. VHGBrewing has been shown to decrease the risk of bacterial spoilage problems (Magnus, Ingledew, & Casey, 1986). Their results show that, though coliforms failed to survive in final VHGBrews, lactic acid bacteria, acetic acid bacteria, and *Zymomonas* can persist and could still cause spoilage problems. The majority of Gram-positive beer-spoiling bacteria are known to be inactivated by nisin, a bacteriocin. In order to eradicate beer spoilage bacteria, the applicability of nisin-producing *Lactococcus lactis* subsp. *lactis* in brewing process was evaluated (Müller-Auffermann, Grijalva, Jacob, & Hutzler, 2015). Results indicated that among strains capable of producing nisin (NCIMB 8780, 8586, 701402, and 701403), the strains NCIMB 8780 and 8586 produced relatively higher amounts of nisin (up to 268 IU) and were more tolerant to ethanol, hop bitterness, low extract conditions, and high gravity (15–18 °P).

8.4. Xanthohumol-enriched beer

Positive health effects of a hop polyphenol, xanthohumol, are well known. In a study, special malts, namely roasted malt and melanoidin malt, were used to prepare HG sweet worts having excessive xanthohumol content (Karabin et al., 2013). Relatively high solubility of xanthohumol was noted in sweet worts prepared from roasted malts than in worts prepared from Pilsen or melanoidin malts. The most suitable temperature for the addition of xanthohumol-rich hop product to wort was 60 °C (where xanthohumol loss by isomerization reactions was minimal). Xanthohumol-enriched beer was prepared by mixing pale-hopped wort with dark Congress wort rich in xanthohumol (at 9:1 ratio). Finished dark beer contained 1.91 mg xanthohumol/L. Diatomaceous earth (filtration material) and the polyamide sorbents (PVPP, BEERPAP and Polyamide 6) (stabilization materials) slightly decreased xanthohumol in enriched beer. In contrast, tannic acid (stabilization material) exerted a significant reduction in xanthohumol content in the beer.

9. Conclusion

Stresses experienced by yeasts under HG or VHGBrewing conditions can be substantially mitigated by the supplementation of nitrogenous nutrients in the form of protein hydrolysates. Hence, the suitability of unconventional and underutilized food proteins for the supplementation, after taking into consideration the impact on quality and stability of beer, need to be explored. The addition of exogenous proteases and amylases has proved useful for improving yeast assimilable nitrogen and lautering in high-adjunct (starches) HG worts, respectively. Yeast strains with improved fermentation performance under HG conditions have successfully been developed using strategies, such as adaptive evolution, evolutionary engineering, hybridization, gene over-expression, gene replacement, and UV mutagenesis. Our understanding of the effect of wort gravity on different physiological activities of yeast, such as α -glucosides transport rate, glycolytic enzyme activity, intracellular trehalose and glycerol levels, uptake of amino acids, and ester synthesis, has substantially improved in recent years.

Immobilized yeast may significantly benefit continuous HG beer-brewing. Stress-tolerant strains of self-flocculating yeast, having good fermentation abilities, may have great importance in HG brewing. High-throughput techniques could be used for the discovery of new strains suitable (e.g., stress tolerance, ethanol tolerance, flavor balance) for HG brewing purposes in a fast and cost-effective manner. In addition, microfluidics platforms (e.g., droplet microfluidics) could be used to identify superior yeasts for HG brewing. Factorial modelling could help improve the economics of the process. Prior to malting process, the application of non-thermal grain decontaminating agents (e.g., ion beams, plasma discharges) could potentially eliminate spores of toxic fungi (thereby reduced over-foaming or gushing of beer) and detoxify mycotoxins. Flow cytometry and confocal imaging could be used for simultaneous measurement and analyses of multiple characteristics of yeast cells, such as cell viability, intracellular pH, mannan, damaged cells, proteinase A, and intercellular glycogen and trehalose; and therefore these tools may be helpful in effective monitoring of HG brewing process.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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