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Aroma-active ester profile of ale beer produced under different fermentation and nutritional conditions

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A broad range of aroma-active esters produced during fermentation are vital for the complex flavour of beer. This study assessed the influence of fermentation temperature, pH, and wort nutritional supplements on the production of yeast-derived ester compounds and the overall fermentation performance. The best fermentation performance was achieved when wort was supplemented with 0.75 g/l L-leucine resulting in highest reducing sugar and FAN (free amino nitrogen) utilization and ethanol production. At optimum fermentation pH of 5, 38.27% reducing sugars and 35.28% FAN was utilized resulting in 4.07% (v/v) ethanol. Wort supplemented with zinc sulphate (0.12 g/l) resulted in 5.01% ethanol (v/v) production and 54.32% reducing sugar utilization. Increase in fermentation temperature from 18°C to room temperature (±22.5°C) resulted in 17.03% increased ethanol production and 14.42% and 62.82% increase in total acetate ester concentration and total ethyl ester concentration, respectively. Supplementation of worth with 0.12 g/l $ZnSO_4$ resulted in 2.46-fold increase in both isoamyl acetate and ethyl decanoate concentration, while a 7.05-fold and 1.96-fold increase in the concentration of isoamyl acetate and ethyl decanoate, respectively was obtained upon 0.75 g/l L-leucine supplementation. Wort supplemented with L-leucine (0.75 g/l) yielded the highest beer foam head stability with a rating of 2.67, while highest yeast viability was achieved when wort was supplemented with 0.12 g/l zinc sulphate. Results from this study suggest that supplementing wort with essential nutrients required for yeast growth and optimizing the fermentation conditions could be an effective way of improving fermentation performance and controlling aroma-active esters in beer.

[**Key words:** Aroma-active esters; Ethanol; Fermentation pH; Free amino nitrogen; L-Leucine]

The preferable flavours of beer depend on the balance of volatile constituents such as acids, alcohols, aldehydes, ketones, and esters (1). These flavour-active compounds are produced by yeast during fermentation (2). Although, there are many strains of brewing yeast (*Saccharomyces cerevisiae*) for beer production (3), the choice of suitable yeasts to produce desirable tastes and flavours in beer is very important and significant. Two types of brewing yeast were originally classified based on their flocculation behaviour: top fermenting (ale yeast) and bottom fermenting (lager yeast) (4). The two main classes of beer types (ales and lagers) are based on the two yeast types because of their distinct behaviour during fermentation. Ale yeast (top yeast) exhibit flotation and have the ability to trap CO_2 bubbles to form a yeast 'head' at the top of fermentation vessels whereas with lager strains (bottom yeasts), the cells clump together, resulting in flocs that sediment from the medium to settle at the bottom of the fermentation vessel. This phenomenon, flocculation, is strain-dependent (2,5) and evidence exists that a number of *FLO* (flocculation) genes play an important role in flocculation (6). Ale yeast is genetically more diverse and ferment at higher temperatures (18–24°C) whereas lager yeasts are more conserved and ferment at lower temperatures (8–14°C) (7). Although, both yeast types need a little oxygen to initiate their metabolism; the alcoholic fermentation is anaerobic (3).

One of the most important factors affecting ester production during fermentation is the yeast strain type and the relative proportions of each individual ester produced. Furthermore, the influence of fermentation parameters, such as oxygen and temperature, is highly dependent on the strain background (8,9). Previous studies suggested that the differences in ester production between yeast strains are due to differences in alcohol acetyltransferase activity (10). Many studies have shown that the AATase activity of *S. cerevisiae* is greatly reduced by aeration or addition of unsaturated fatty acids (11,12). Researches showed that more esters are produced when fermentation is started at relatively high temperatures, and subsequently decrease after the maximal CO_2 production rate occurs (13). The reason for the temperature dependence of ester synthesis is as yet unknown, although it has been suggested that temperature affects acetyl transferases (AATase) activity and/or formation (14).

Wort composition and fermentation conditions are known to affect fermentation performance and flavour profiles (15). Beer brewing yeast strains respond differently to the various nutritional and fermentation conditions (9) which creates unique flavour

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profiles in the final product. Generally, ale yeast strains produce a higher concentration of flavour-active esters in beer (7). Therefore, this study aims at investigating the effects of various nutritional and fermentation conditions on fermentation performance and ester profiles in beer produced by an ale yeast strain. This could help brewers to identify the nutritional and fermentation conditions that could produce the desired amounts of esters in accordance with specific consumer preferences.

MATERIALS AND METHODS

Yeast strain and cultivation conditions All experiments were carried out using an ale *S. cerevisiae* strain (Safale S-04, Belgium). Yeast was grown in malt extract broth for 24 h at 30°C with shaking at 120 rpm. Two millilitre pre-culture was inoculated into 200 ml malt extract broth for 6 h at 30°C with shaking at 120 rpm until it reached an OD_{600} of 0.451. Samples were centrifuged at 4000 rpm for 15 min at 4°C and the pellet was resuspended in 200 ml wort. Twenty-millilitre inoculum was used to pitch 2 l wort at a pitching rate of 6×10^6 cfu/ml.

Wort preparation Malt wort was prepared by adding 3.080 kg of crushed pale malt to 9.2 l of water. The malt was kindly donated by the South African Breweries (SAB), Prospecton, South Africa and it originated from SAB Maltings, Caledon, South Africa. Malting was carried out at the following temperatures: 63.5°C for 60 min to allow for β-amylase activity, 71°C for 30 min to allow for α-amylase activity and 74°C for 10 min to inactivate all enzymes. Mash was then centrifuged to separate spent grain and wort. The residual mash was washed with approximately 4 l of water. Mash was then transferred to beakers and washed with 9 l of warm water to remove residual sugars. The wort was then brought to a boil; 5 g of Southern hops was added and allowed to boil for 1 h, followed by the addition of 2.5 g Saaz hops and allowed to boil for a further 10 min. Both the Southern hop and Saaz hop used in this study were kindly donated by the SAB and they originated from Southern Cape, South Africa and Czech Republic, respectively.

Wort fermentation Fermentations were set up to determine the effect of temperature, pH, addition of zinc sulphate ($ZnSO_4$) (Sarchem, South Africa) and L-leucine (Merck, Germany), on fermentation performance and ester production using mini-fermenters (3.5 l) to facilitate the fermentation process on a small scale. All fermentations were carried out in triplicate in fermentation vessels containing 2 l of wort which were fermented under various supplementations and fermentation parameters as follows: $ZnSO_4$ (0.03, 0.06, and 0.12 g/l); L-leucine (0.25, 0.50 and 0.75 g/l), temperature (14, 18 and room temperature [±22.5°C]); and pH (3, 5 and 7), which was adjusted using HCl or NaOH. Control set up was performed at 18°C and at pH 5. Fermentations were monitored by an air lock mechanism to ensure the fermentations are not stuck. During fermentation, samples were withdrawn at regular intervals and analyzed as described below. Once fermentation was complete fermenter vessels were incubated at 4°C for 5 days to allow for yeast settlement.

Fermentation analysis Samples were removed daily in order to determine reducing sugar content, free amino nitrogen (FAN) content, ethanol concentration and total yeast cell density. These parameters were analyzed using the Dinitrosalicylic acid method (16), Ninhydrin Assay (16), Gas Chromatography (17) and standard spread plate technique (17), respectively.

Bottling and conditioning Settled yeast was removed from each fermenter and beer was transferred into sterile 750 ml sample bottles. The bottles containing the beer were allowed to stand for 30 min before capping. Eight millilitres of a brown sugar (1 g/ml) was added to each bottle to allow for carbonation. The bottles were capped and incubated at 14°C for 5 days for conditioning, and thereafter stored at 4°C until required for further analysis.

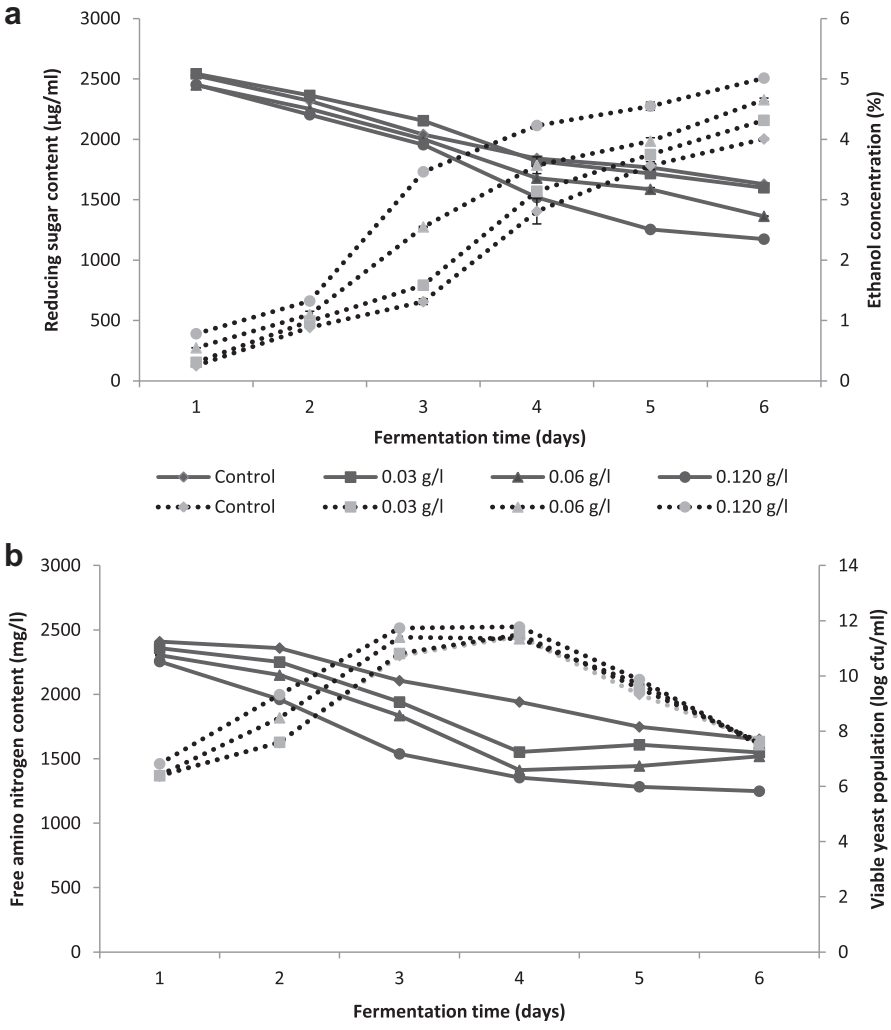

FIG. 1. Profiles of (a) reducing sugar and ethanol content and (b) free amino nitrogen concentration and yeast cell density in the wort during fermentation at varying concentrations of zinc sulphate. Solid lines represent reducing sugar and free amino nitrogen content and dotted lines represent ethanol concentration and yeast cell density. Values are averages of triplicate results ± standard deviation.

Measurement of foam head stability Foam head stability was assessed according to the modified mini foam shake tests (18). Twenty millilitres of beer was dispensed into 50 ml glass measuring cylinders, in triplicate and all of the cylinders were sealed with parafilm. Each set of cylinders were shaken at the same time, vigorously up and down 10 times, after which the cylinders were set down on the counter and the parafilm pieced, and a timer set for 15 min. After 15 min the foam was evaluated visually and the cylinders were rated from best to worst. Ratings of 1 through 5 were given, where 5 is the greatest stability and 1 is the worst. A commercial beer served as the positive control.

Analysis of beer colour Beer colour was measured spectrophotometrically at a wavelength of 430 nm (19) using distilled water as a blank. Experiments were performed in triplicate and a commercial beer was included in the analysis as a positive control.

Measurement of spent yeast density Spent yeast density was measured by the method described by Solay et al. (20). Ten millilitre sample was centrifuged (6000 rpm for 10 min at 4°C). The pellet was then resuspended in a NaCl solution (0.9%, w/v), filtered through a previously dried and weighted Whatman grade GF/A (Ø 47 mm) glass microfiber filter, and dried at 105°C to a constant weight. Thereafter, weight of the filter was subtracted from the weight of the filter containing the dried cellular material to obtain the mass of spent yeast produced.

Analysis of beer volatile esters The composition and concentrations of acetate and ethyl esters in the samples were measured by headspace analysis of the samples in a gas chromatograph (GC) (Agilent 6820) coupled with flame ionization detector (FID). The beer samples (100 ml) were collected in 250 ml serum bottles (Wheaton) and were immediately closed. Samples were heated for 25 min at 70°C in a water bath before injecting 1 ml of the headspace into the GC with a capillary column (DB1) using a gas tight syringe (Hamilton). The oven temperature was held at 50°C for 5 min, then increased to 200°C at a rate of 5°C per min and finally held at 200°C for 3 min. The FID temperature was kept constant at 250°C and nitrogen was used as the carrier gas. Standards were run using varying concentrations of each ester compound to generate standard curves from which the concentration of esters present in the sample was estimated.

RESULTS

Fermentation performance under different nutritional and fermentation conditions The profile of sugar utilization and ethanol production during the fermentation period in the absence and presence of varying concentrations of $ZnSO_4$ is shown in Fig. 1a, while the FAN content and yeast density at the same period is shown in Fig. 1b. Fermentation performance improved with the addition of $ZnSO_4$ into wort. Wort supplemented with 0.120 g/l of $ZnSO_4$ resulted in the highest utilization of reducing sugars and FAN (54.32% and 49.82%, respectively), after the 6-day fermentation period, values which are 48.42% and 48.14% higher than that utilized in the unsupplemented wort. Furthermore, addition of $ZnSO_4$ (0.12 g/l) resulted in the highest production of 5.01% (v/v) ethanol which was 24.94% higher than the control. Wort supplemented with 0.06 and 0.12 g/l $ZnSO_4$ increased the yeast cell density up to day 3 of fermentation achieving a maximum cell population of 2.54×10^{11} and 5.42×10^{11} cfu/ml, respectively, thereafter yeast cell density gradually decreased (Fig. 1b).

The sugar utilization and ethanol production profiles during the fermentation period in the absence and presence of varying

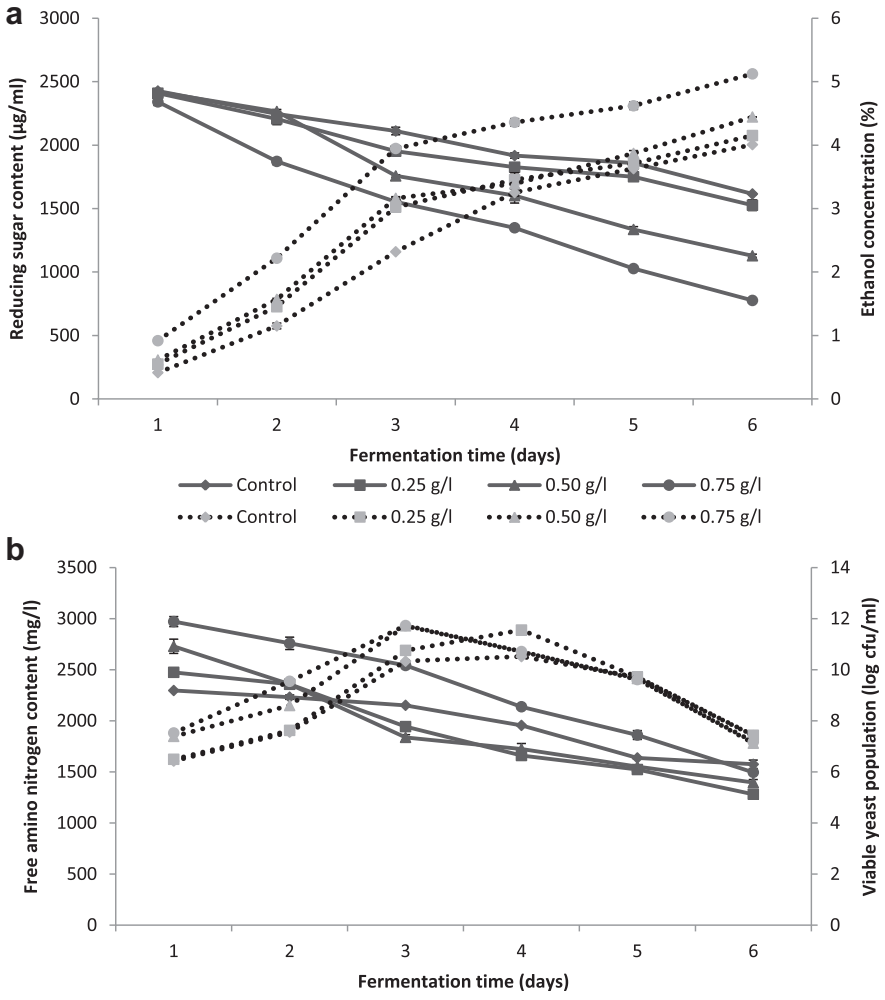

FIG. 2. Profiles of (a) reducing sugar and ethanol content and (b) free amino nitrogen concentration and yeast cell density in the wort during fermentation at varying concentrations of L-leucine. Solid lines represent reducing sugar and free amino nitrogen content and dotted lines represent ethanol concentration and yeast cell density. Values are averages of triplicate results ± standard deviation.

concentrations of L-leucine is shown in Fig. 2a, while the FAN content and yeast density during the same period is shown in Fig. 2b. In the control experiment, 35.73% of the available reducing sugars was utilized. Increasing concentration of L-leucine in wort resulted in an increase in reducing sugar utilization. Wort supplemented with 0.75 g/l L-leucine resulting in 69.11% reducing sugar utilization which was 93.42% higher than the unsupplemented control. Addition of L-leucine to wort resulted in an increased amount of FAN concentration in the medium. The control experiment resulted in 32.10% of FAN utilization by yeast, with increasing concentration of L-leucine in the medium resulting in more FAN being consumed by yeast. In the control experiment, 4.01% (v/v) ethanol was produced whereas wort containing 0.75 g/l L-leucine produced 5.12% (v/v) ethanol which was 27.68% higher than that in the unsupplemented control.

Sugar utilization and ethanol production profiles during wort fermentation at varying temperatures is shown in Fig. 3a, while the FAN content and yeast density at the same period is shown in Fig. 3b. Increased fermentation temperature resulted in greater utilization of both the reducing sugar and FAN. Fermentation at 14°C resulted in 33.58% reducing sugar utilization; while 37.10% and 51.77% reducing sugar was utilized at 18°C and room temperature, respectively. Fermentation at 18°C and room temperature (22.5°C) resulted in 33.85% and 52.40% FAN utilization, respectively (Fig. 3b). Furthermore, the highest fermentation temperature resulted in the highest ethanol production (4.74% v/v) which was 17.03% more than fermentation at 18°C. Fermentation at 14°C gradually increased the yeast cell density up to day 4 reaching a peak of 2.55×10^{11} cfu/ml and thereafter decreased. Fermentation at 18°C and room temperature resulted in an increase in yeast cell density until day 3 attaining a maximum of 2.64×10^{11} and 3.95×10^{11} cfu/ml respectively, thereafter, a decrease in yeast cell density was observed.

The profile of sugar utilization and ethanol production during fermentation at different pHs is shown in Fig. 4a, while the FAN content and yeast cell density at the same period is shown in Fig. 4b. pH 5 was found to be optimum as the highest utilization of nutrients was observed. Fermentation at pHs 3, 5 and 7 resulted in 21.09%, 38.27% and 26.31% reducing sugar utilization, respectively. At pH 5, 35.28% of FAN was utilized whereas fermentation at pH 3 and 7 resulted in 22.19% and 30.74% FAN utilization, respectively. Furthermore, at pH 5 the highest amount of ethanol (4.07% [v/v]) was produced which was 77.73% and 81.70%, respectively, higher than alcohol produced than at pH 3 and 7. Yeast cell density increased during fermentation at pH 5 and 7 up to day 4 reaching a maximum of 3.48×10^{11} cfu/ml and 2.54×10^{10} cfu/ml, respectively, thereafter decreased. At pH 3, yeast cell density increased until day 3 of fermentation attaining a maximum of 2.22×10^{9} cfu/ml and gradually decreased thereafter.

Spent yeast density Spent yeast density determined under the various nutritional and fermentation conditions ranged from 1.985 to 2.848 mg/ml. Supplementation of wort with $ZnSO_4$ and L-leucine, resulted in an increase in spent yeast density (Fig. 5).

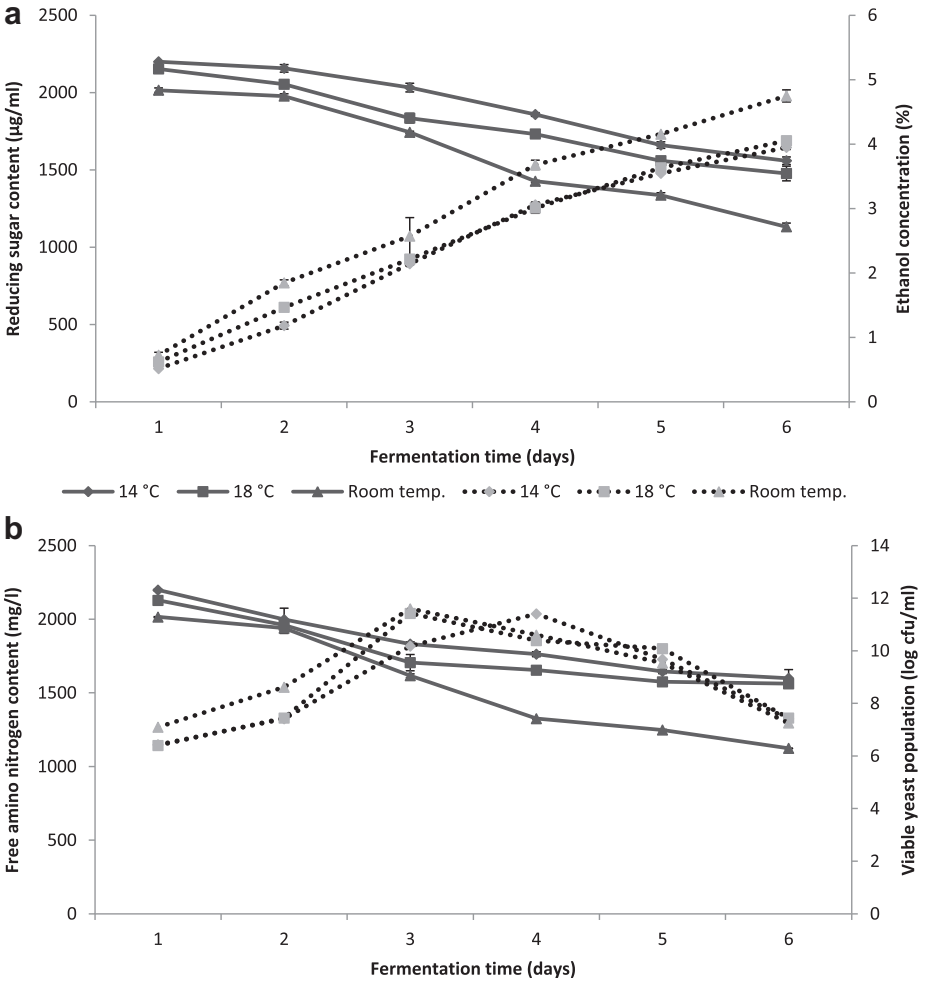

FIG. 3. Profiles of (a) reducing sugar and ethanol content and (b) free amino nitrogen concentration and yeast cell density in the wort during fermentation at varying temperatures. Solid lines represent reducing sugar and free amino nitrogen content and dotted lines represent ethanol concentration and yeast cell density. Values are averages of triplicate results ± standard deviation.

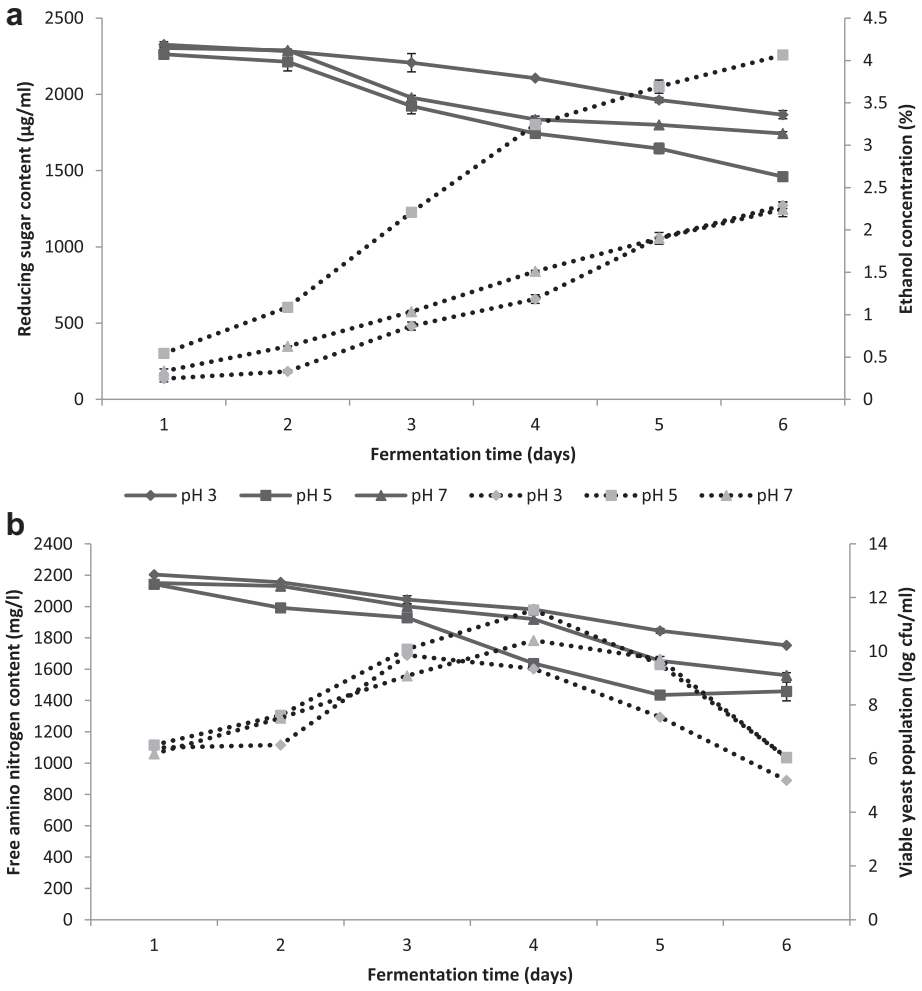

FIG. 4. Profiles of (a) reducing sugar and ethanol content and (b) free amino nitrogen concentration and yeast cell density in the wort during fermentation at varying pHs. Solid lines represent reducing sugar and free amino nitrogen content and dotted lines represent ethanol concentration and yeast cell density. Values are averages of triplicate results ± standard deviation.

Fermentation in the presence of 0.12 g/l $ZnSO_4$ produced the highest spent yeast density of 2.848 mg/ml which was 32.52% more compared to that obtained during the fermentation without any $ZnSO_4$. Addition of 0.75 g/l L-leucine into wort resulted in 17.01% increase in spent yeast density compared to the unsupplemented sample. Similarly, increase in fermentation temperature from 18°C to room temperature resulted in 10.31% increase in spent yeast density. However, increase in wort acidity and alkalinity resulted in a decrease in spent yeast density. The highest spent yeast density was observed at pH 5, with 8.82% and 7.73% reduction in spent yeast density observed at pH 3 and 7, respectively, compared to that at pH 5.

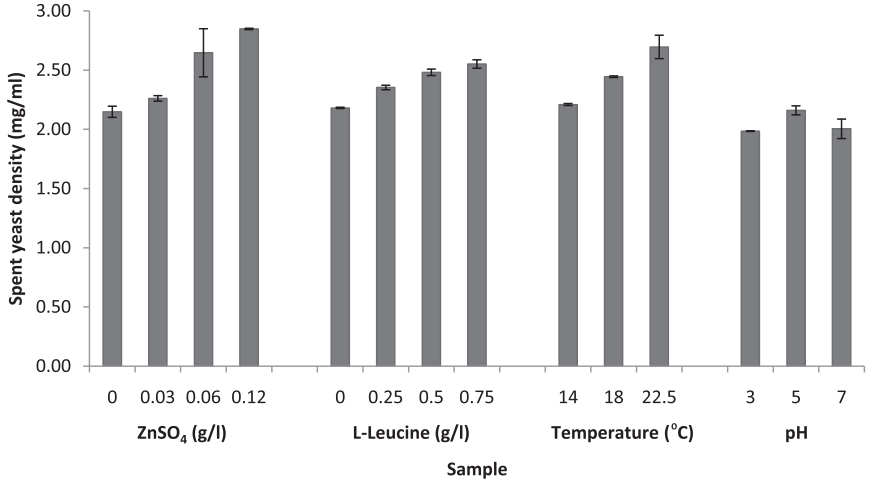

FIG. 5. Spent yeast density produced under various nutritional and fermentation conditions. Values are averages of triplicate results ± standard deviation.

Beer colour and foam head stability The colour intensity of the beers produced under the different conditions was similar. A commercial beer which served as a control had the deepest colour intensity with an absorbance value of 0.862 (Table 1). The beer produced from wort supplemented with 0.75 g/l L-leucine resulted in the deepest colour intensity compared to other experimental beers compared with an absorbance of 0.683. Similarly, the commercial beer had the best foam head stability to all the experimental beers. The best foam head stability was produced in the experimental beers with 0.75 g/l L-leucine, retaining as high as 53.4% foam head stability compared to the commercial beer, while those prepared at pH 7 and at room temperature had the least foam head stability rating (Table 1). An increase in $ZnSO_4$ in wort from 0.03 g/l to 0.12 g/l resulted in a decrease in foam head stability while increasing L-leucine concentration resulted in an increase in foam head stability. Wort supplemented with 0.75 g/l L-leucine had 14.59% better foam head stability than the unsupplemented control. Alteration of fermentation temperature resulted in a decrease in foam head stability at room temperature, decreasing by 28.33%. However, increasing the pH of the fermentation medium resulted in a decrease in foam head stability.

Beer volatile ester compounds composition and concentration The volatile ester compounds produced during the fermentations as quantified by Gas Chromatographic analysis of the headspace samples are shown in Table 2. Addition of 0.12 g/l $ZnSO_4$ into the fermentation medium resulted in a 27.70% increase in total acetate esters and 123.02% increase in total ethyl esters compared to the unsupplemented sample (Table 2). Ethyl acetate constituted roughly 72.5% of the total esters, while ethyl octanoate was present in very small amounts. Supplementation of wort with 0.120 g/l $ZnSO_4$ resulted in an increase in all ester concentrations with the highest increase of 145.85% in ethyl decanoate concentration obtained. This was followed by isoamyl acetate, phenyl ethyl acetate, ethyl acetate, ethyl hexanoate and ethyl octanoate increasing by 145.78%, 43.06%, 18.08%, 15.26% and 14.76%, respectively, compared to the unsupplemented control. There is a good correlation between total acetate ester concentration and $ZnSO_4$ concentration ($R^2 = 0.953$) (Fig. 6a). Of these acetate esters, isoamyl acetate showed a good correlation with $ZnSO_4$ concentration ($R^2 = 0.980$). There was also a good correlation with $ZnSO_4$ concentration and ethyl ester concentration ($R^2 = 0.988$) specifically ethyl decanoate ($R^2 = 0.985$) and ethyl octanoate ($R^2 = 0.980$).

TABLE 1. Colour profiles and foam head stability of ale beer produced under different nutritional and fermentation conditions.

Sample	Beer colour (OD_{430nm})	Foam head stability rating
Zinc sulphate (g/l)		
0.00	0.655 ± 0.00	2.33 ± 0.58
0.03	0.653 ± 0.00	2.33 ± 0.58
0.06	0.655 ± 0.010	2.00 ± 0.00
0.12	0.647 ± 0.00	2.00 ± 0.00
L-Leucine (g/l)		
0.00	0.654 ± 0.010	2.33 ± 0.58
0.25	0.665 ± 0.00	2.33 ± 0.58
0.50	0.674 ± 0.00	2.33 ± 0.58
0.75	0.683 ± 0.00	2.67 ± 0.58
Temperature (°C)		
14	0.661 ± 0.00	2.33 ± 0.58
18	0.666 ± 0.010	2.33 ± 0.58
Room temperature (22.5)	0.678 ± 0.170	1.67 ± 0.58
pH		
3	0.653 ± 0.010	1.33 ± 0.58
5	0.648 ± 0.00	2.33 ± 0.58
7	0.638 ± 0.010	1.67 ± 0.58
Commercial beer	0.862 ± 0.00	5.00 ± 0.00

Values are averages of triplicate results ± standard deviation.

Addition of L-leucine (0.750 g/l) into the fermentation medium resulted in a 41.27% increase in total acetate ester concentration and 83.76% increase in total ethyl ester concentration compared to the control which was not supplemented with L-leucine (Table 2). The highest increase was observed for isoamyl acetate which increased by 605.21% compared to the unsupplemented control. This was followed by ethyl decanoate, phenyl ethyl acetate, ethyl acetate, ethyl hexanoate, and ethyl octanoate increasing by 96.46%, 52.86%, 19.02%, 6.96% and 6.57%, respectively. There was a good correlation between increasing L-leucine concentration and phenyl ethyl acetate ($R^2 = 0.961$) (Fig. 6b).

Increasing the fermentation temperature from 18°C to room temperature (22.5°C) resulted in an increase in total acetate ester concentration by 14.42%, and total ethyl ester concentration by 62.82% (Table 2). The highest increase of 89.25% was observed for ethyl decanoate, followed by isoamyl acetate and ethyl acetate increasing by 22.80%, and 17.46%, respectively, compared to the control. However, there was a decrease in phenyl ethyl acetate, ethyl octanoate and ethyl hexanoate. There was a good correlation between total acetate esters ($R^2 = 0.999$), specifically isoamyl acetate ($R^2 = 0.965$) and the fermentation temperature (Fig. 6c). Of the ethyl esters, a good correlation between fermentation temperature and ethyl decanoate ($R^2 = 0.982$) was observed.

Fermentation pH that produced the highest concentration of esters was 7 resulting in a 13.08% increase in total acetate ester and 6.76% total ethyl ester production when compared to the control (pH 5) (Table 2). There was a 60.17% increase in isoamyl ester production at pH 7, followed by phenyl ethyl acetate, ethyl octanoate, ethyl acetate, ethyl decanoate, and ethyl hexanoate increasing by 14.56%, 9.60%, 9.21%, 6.27% and 1.13%, respectively. There was an 18.40% decrease in total ester concentration at pH 3, with isoamyl acetate decreasing by 43.64%. There was a good correlation between fermentation pH and total acetate ester concentration (R^2= 0.986) (Fig. 6d). All acetate esters and ethyl decanoate exceeded their threshold level under all fermentation pH.

DISCUSSION

In this study, the influence of fermentation temperature and pH and wort composition on fermentation performance and the production of important aroma active esters were assessed. It was not surprising that wort supplemented with $ZnSO_4$ or L-leucine lead to an increase in utilization of nutrients and a higher ethanol production, as metal ions such as zinc and amino acids such as L-leucine are essential for yeast growth and metabolism (7). In general, metal ions can impact on the metabolic processes during fermentation by influencing several important parameters including yeast growth, viability, enzyme activities, alcohol fermentation and stress tolerance (21). Metal ions act as co-factors for important fermentation enzymes and also as modulators of environmental stress. Amino acids play a crucial role in yeast nutrition as it is utilized for protein formation (structural and enzymic) required for growth (22). Of the different fermentation temperatures and pHs investigated in this study, optimum yeast performance was achieved at RT and at pH 5, respectively. Increasing fermentation temperature from 18°C to RT (±22.5°C) resulted in a faster fermentation and a higher utilization of nutrients. Fermentation temperature and pH affect not only the fermentation kinetics (rate of fermentation) but also the yeast metabolism which determines the chemical composition of the beer. Generally, increased fermentation temperatures in the range of 10–25°C leads to increased ester production in ale beer (9). It has been suggested that temperature affects alcohol acetyl transferases (AATase) activity (9,14) and may also cause changes in the availability of fusel alcohols that are necessary for ester formation (23). Increased temperature also affects the thermodynamic distribution coefficient favouring ester accumulation in the aqueous medium

TABLE 2. Ester concentrations in ale beer produced under different nutritional and fermentation conditions.

Sample	Acetate ester concentration (mg/l)			Ethyl ester concentration (mg/l)		
	Ethyl acetate	Isoamyl acetate	Phenyl ethyl acetate	Ethyl decanoate	Ethyl hexanoate	Ethyl octanoate
Zinc concentration (g/l)						
0.00	34.45 ± 1.78	2.56 ± 0.04	2.09 ± 0.02	1.96 ± 0.04	0.255 ± 0.01	0.159 ± 0.00
0.03	36.34 ± 1.64	3.93 ± 0.02	2.52 ± 0.08	2.48 ± 0.10	0.287 ± 0.00	0.168 ± 0.01
0.06	39.54 ± 0.10	4.75 ± 0.02	2.39 ± 0.07	3.59 ± 0.10	0.266 ± 0.03	0.172 ± 0.01
0.12	40.68 ± 0.12	6.29 ± 0.10	2.99 ± 0.09	4.82 ± 0.14	0.293 ± 0.01	0.183 ± 0.00
L-Leucine concentration (g/l)						
0.00	31.46 ± 0.11	1.15 ± 0.01	4.44 ± 0.06	2.53 ± 0.03	0.259 ± 0.00	0.158 ± 0.00
0.25	32.55 ± 0.03	6.02 ± 0.01	5.15 ± 0.06	2.31 ± 0.02	0.262 ± 0.00	0.156 ± 0.00
0.50	32.64 ± 0.03	6.66 ± 0.14	5.59 ± 0.02	4.21 ± 0.04	0.254 ± 0.00	0.166 ± 0.00
0.75	32.36 ± 1.06	8.11 ± 0.05	6.79 ± 0.04	4.97 ± 0.20	0.276 ± 0.00	0.169 ± 0.00
Fermentation temperature (°C)						
14	28.64 ± 0.08	3.24 ± 0.02	3.85 ± 0.05	1.17 ± 0.00	0.259 ± 0.00	0.566 ± 0.01
18	30.59 ± 0.02	5.35 ± 0.02	5.87 ± 0.04	2.28 ± 0.05	0.253 ± 0.01	0.569 ± 0.00
Room temp (±22.5)	35.93 ± 0.66	6.57 ± 0.07	5.34 ± 0.03	4.32 ± 0.04	0.231 ± 0.01	0.504 ± 0.00
Fermentation pH						
3	25.52 ± 0.02	1.33 ± 0.03	3.59 ± 0.05	2.54 ± 0.02	0.157 ± 0.00	0.297 ± 0.02
5	30.66 ± 0.05	2.36 ± 0.00	4.93 ± 0.01	2.43 ± 0.01	0.155 ± 0.00	0.439 ± 0.00
7	33.47 ± 0.04	3.73 ± 0.11	5.65 ± 0.04	2.58 ± 0.02	0.170 ± 0.00	0.444 ± 0.00
Threshold	30	1.2	3.8	1.5	0.21	0.9

Values are averages of triplicate results ± standard deviation.

(15). High cell densities were achieved under all experimental fermentations and the control experiment. This may have occurred due to oxygen been introduced into the fermentation vessels when samples were taken daily. This will create an aerobic environment that will result in more yeast growth.

The appearance of beer is an important quality in the final product therefore, colour and foam head stability were analyzed in the ale beer produced in this study. Colour arises in raw materials primarily as a result of the Maillard reaction also called, descriptively, nonenzymic or nonoxidative browning (24). Since all wort was prepared with the same raw ingredients, the colour profiles were similar. Beer foam head stability was increased with the addition of L-leucine into the fermentation medium, whereas the addition of $ZnSO_4$ and increase in the fermentation temperature resulted in a decrease in foam head stability. Since amino acids are building blocks for proteins, increase in L-leucine concentration allows for more proteins to interact with Protein Z, the protein responsible for the stability of beer foam head, therefore resulting in an increase in foam head stability (24). Brewers' spent yeast is usually sold for use as food and animal feed due to its high vitamin content (25), production of a high spent yeast density is therefore beneficial. Addition of $ZnSO_4$ or L-leucine into the fermentation

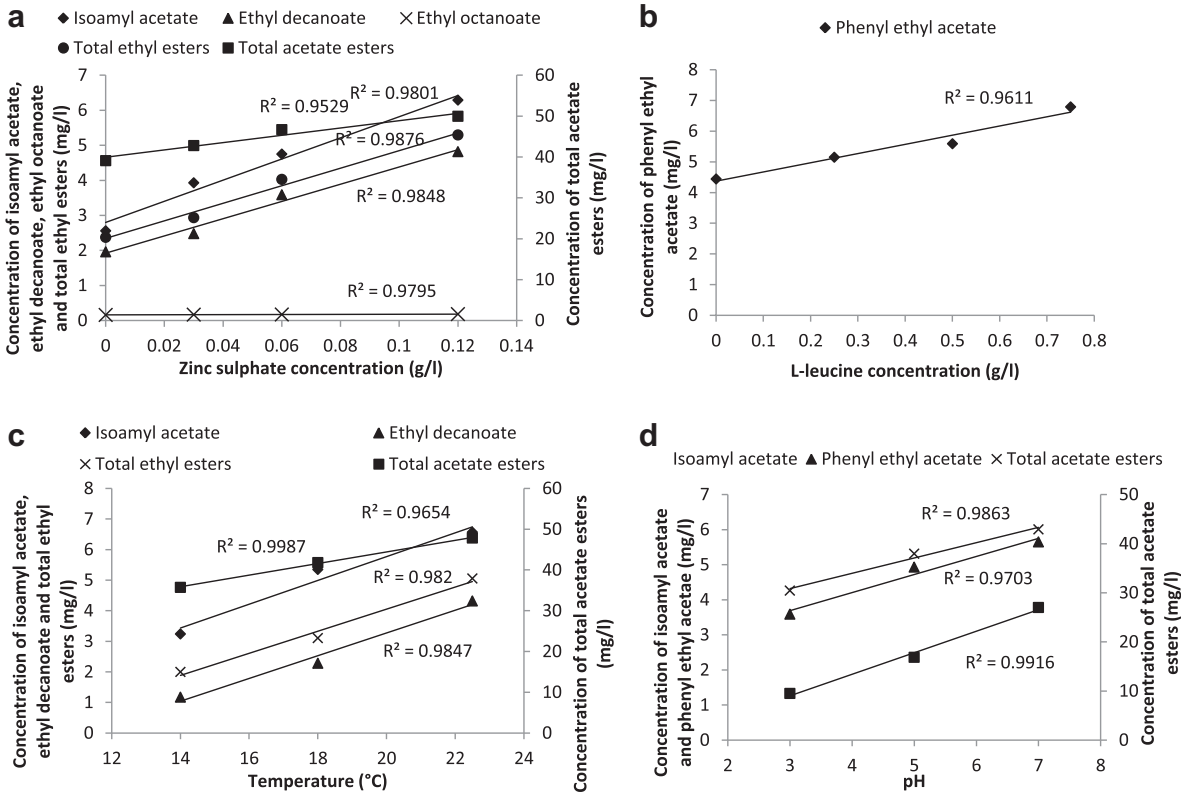

FIG. 6. Profiles of esters in ale beer showing strong correlations with (a) concentrations of zinc sulphate, (b) concentrations of L-leucine (c) fermentation temperature and (d) fermentation pH. Values are averages of triplicate results ± standard deviation.

medium resulted in an increase in spent yeast density possibly due to the fact that these supplements stimulated yeast growth, thus resulting in a higher yield spent yeast density yield.

Addition of $ZnSO_4$ (0.120 g/l) into the fermentation medium resulted in an increase in acetate and ethyl esters. This could be due to the stimulation of higher alcohol production by zinc, which can subsequently be converted to esters (2). Similarly, addition of L-leucine into the fermentation medium resulted in an increase in acetate and ethyl esters. The concentration and composition of wort FAN can have impact on the production of higher alcohol and esters, due to the role of amino acid metabolism in the formation of these flavour compounds (22,26). Genome-wide gene analysis of yeast expression profiles during flavour formation when cultivated on L-leucine and ammonia revealed a group of 117 genes that were more than two-fold up- or downregulated (27,28). The gene expression groups consisted of genes encoding proteins involved in amino acid metabolism. It was concluded that amino acid metabolism pathways, other than the branched chain amino acids (BCAA) pathway, play significant roles in the formation of volatile compounds. Hence, the observed increase in acetate and ethyl ester concentration with increasing fermentation temperature. Optimum fermentation pH for ester production in this study was pH 7, resulting in higher acetate and ethyl ester production compared to the control (pH 5). It had been observed that the activity of AATase in brewer's yeast increases with increasing pH (29,30), which may be responsible for the increased ester production with increasing temperature.

In conclusion, fermentation conditions and nutritional supplements are important in beer brewing due to its influence on fermentation performance and the final product characteristics. Supplementing wort with essential nutrients and altering important fermentation conditions can be used to alter ester formation in beer. Moreover, since these conditions increase the rate of fermentation, for faster nutrient utilization and ethanol production, the fermentation time can be reduced. This is promising for a reduced operational cost for the production of ale beer with the desired flavour properties. However, the expression level of the ester synthetase genes under the different conditions should be determined to develop best fermentation conditions for optimum ester production.

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References

1. **Patel, S. and Shibamoto, T.:** Effect of 20 different yeast strains on the production of volatile components in symphony wine, J. Food Compos. Anal., **16**, 469–476 (2003).
2. **Verstrepen, K. J., Derdelinckx, G., Dufour, J., Winderrikx, J., Thevelein, J. M., Pretorius, I. S., and Delvaux, F. R.:** Flavour-active esters: adding fruitiness to beer, J. Biosci. Bioeng., **96**, 110–118 (2003).
3. **Bamforth, C. W.:** Beer: an ancient yet modern biotechnology, Chem. Ed., **5**, 102–112 (2000).
4. **Jentsch, M.:** Top-fermented beer specialities in focus, Brauwelt Int., **5**, 332–334 (2007).
5. **Speers, R. A., Tung, M. A., Durance, T. D., and Stewart, G. G.:** Biochemical aspects of yeast flocculation and its measurements: a review, J. Inst. Brew., **98**, 293–300 (1992).
6. **Teunissen, A. W. R. H. and Steensma, H. Y.:** Review: the dominant flocculation genes of *Saccharomyces cerevisiae*, Yeast, **11**, 1001–1013 (1995).
7. **Lodolo, E. J., Kock, J. L. F., Axcell, B. C., and Brooks, M.:** The yeast *Saccharomyces cerevisiae* the main character in beer brewing, FEMS Yeast Res., **8**, 1018–1036 (2008).
8. **Anderson, R. G. and Kirsop, B. H.:** The control of volatile ester synthesis during the fermentation of wort of high specific gravity, J. Inst. Brew., **80**, 48–55 (1974).
9. **Peddie, H. A. B.:** Ester formation in brewery fermentations, J. Inst. Brew., **96**, 327–331 (1990).
10. **Ramos-Jeunehomme, C., Laub, R., and Masschelein, C. A.:** Why is ester formation in brewery fermentations yeast strain dependent?, pp. 257–264, in: Proceedings of the 23rd European Brewery Convention Congress, Lisbon. Oxford University Press, Oxford, UK (1991).
11. **Fujii, T., Kobayashi, O., Yoshimoto, H., Furukawa, S., and Tamai, Y.:** Effect of aeration and unsaturated fatty acids on expression of the *Saccharomyces cerevisiae* alcohol acetyltransferase gene, Appl. Environ. Microbiol., **63**, 910–915 (1997).
12. **Fujii, T., Nagasawa, N., Iwamatsu, A., Bogaki, T., Tamai, Y., and Hamachi, M.:** Molecular cloning, sequence analysis, and expression of the yeast alcohol acetyltransferase gene, Appl. Environ. Microbiol., **60**, 2786–2792 (1994).
13. **Sablayrolles, J. M. and Ball, C. B.:** Fermentation kinetics and the production of volatiles during alcoholic fermentation, J. Am. Soc. Brew. Chem., **53**, 71–78 (1995).
14. **Mason, A. B. and Dufour, J.:** Alcohol acetyltransferases and the significance of ester synthesis in yeast, Yeast, **16**, 1287–1298 (2000).
15. **Saerens, S. M. G., Delvaux, F., Vertrepen, K. J., van Dijck, P., Thevelein, J. M., and Delvaux, F. R.:** Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation, Appl. Environ. Microbiol., **74**, 454–461 (2008).
16. **Sadasivam, S. and Manickum, A.:** Biochemical methods, chapter 1 carbohydrates, 2nd ed. New Age International Publishers, India (1996).
17. **Hiralal, L., Pillay, B., and Olaniran, A. O.:** Stability profile of flavour-active ester compounds in ale and lager beer during storage, Afr. J. Biotechnol., **12**, 491–498 (2013).
18. **Van Nierop, S. N. E., Evans, D. E., Axcell, B. C., Cantrell, I. C., and Rautenbach, M.:** Impact of different wort boiling temperatures on the beer foam stabilizing properties of lipid transfer protein 1, J. Agric. Food Chem., **52**, 3120–3129 (2004).
19. **Seaton, J. C. and Cantrell, I. C.:** The determination of beer colour – collaborative trial, J. Inst. Brew., **99**, 21–23 (1993).
20. **Solay, A., Lecina, M., Gamez, X., Cairo, J. J., Riu, P., Rosell, X., Bragos, R., and Godia, F.:** On-line monitoring of yeast cell growth by impedance spectroscopy, J. Biotechnol., **118**, 398–405 (2005).
21. **Venkateshwar, M., Chaitanya, K., Altaf, M., Mahammad, E. J., Hameeda, B., and Reddy, G.:** Influence of micronutrients on yeast growth and β-D-fructofuranosidase production, Indian J. Microbiol., **50**, 325–331 (2010).
22. **Pierce, J. S.:** The role of nitrogen in brewing, J. Am. Soc. Brew. Chem., **93**, 378–381 (1987).
23. **Calderbank, J. and Hammond, J. R. M.:** Influence of higher alcohol availability on ester formation by yeast, J. Am. Soc. Brew. Chem., **52**, 84–90 (1994).
24. **Lewis, M. J. and Bamforth, C. W.:** Essays in brewing science. Springer Science and Business Media, New York (2006).
25. **Ingledew, W. M., Langille, L. A., Menegazzi, G. S., and Mok, M. H.:** Spent brewer's yeast - analysis, improvement, and heat processing considerations, MBAA, **14**, 231–237 (1977).
26. **O'Connor-Cox, E. S. C. and Ingledew, W. M.:** Wort nitrogenous sources – their use by brewing yeasts: a review, J. Am. Soc. Brew. Chem., **47**, 102–108 (1989).
27. **Schoodermark-Stolk, S. A., Jansen, M., Verkleij, A. J., Verrips, C. T., Euverink, G. J. W., Dijkhuizen, L., and Boonstra, J.:** Genomewide transcription survey on flavour production in *Saccharomyces cerevisiae*, World J. Microbiol. Biotechnol., **22**, 1347–1356 (2006).
28. **Styger, G., Jacobson, D., and Bauer, F. F.:** Identifying genes that impact on aroma profiles produced by *Saccharomyces cerevisiae* and the production of higher alcohols, Appl. Microbiol. Biotechnol., **91**, 713–730 (2011).
29. **Horton, C. E., Huang, K., Bennett, G. N., and Rudolph, F. B.:** Heterologous expression of the *Saccharomyces cerevisiae* alcohol acetyltransferase genes in *Clostridium acetobutylicum* and *Escherichia coli* for the production of isoamyl acetate, J. Ind. Microbiol. Biotechnol., **30**, 427–432 (2003).
30. **Saerens, S. M. G., Delvaux, F. R., Verstrepen, K. J., and Thevelein, J. M.:** Production and biological function of volatile esters in *Saccharomyces cerevisiae*, Microbiol. Biotechnol., **3**, 165–177 (2010).