Research article

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The influence of yeast strain on the oxidative stability of beer

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Flavour stability, or instability, relates to the rate of flavour change through the shelf-life of packaged beer. There are several control points in the production of beer where flavour stability may be altered. These include fermentation and the influence of yeast is key. Greater insight into the yeast traits which contribute to flavour stability may help yeast strain selection in the future. Knowledge of the key phenotypes may also lead to improved yeast handing or monitoring practices. In this study, 11 yeast strains, previously characterised according to their sensitivity to oxidative stresses (induced by menadione and hydrogen peroxide) were screened using miniature (100 mL) fermentations and the oxidative stability of the resultant green beer assessed using Electron Paramagnetic Resonance Spectroscopy. The selection of strains with high resistance to multiple oxidative stresses was shown to be a good indicator that yeast would produce a more oxidatively stable beer, although the mechanisms determining this are unknown. The relevance of selecting yeast based on their oxidative sensitivity, their potential to remove metals and sulphur dioxide production are discussed. © 2021 The Authors. *Journal of the Institute of Brewing* published by John Wiley & Sons Ltd on behalf of The Institute of Brewing & Distilling

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Introduction

Brewers invest a significant amount of time and effort ensuring that their product presents the desired flavour profile. However, during the time it takes for the product to reach the consumer this flavour profile can change, losing positive attributes and developing aging characteristics. These characteristics are typically due to the formation of several aldehydes through oxidative and non-oxidative routes, although many other pathways may also contribute to flavour change, as reviewed by Vanderhaegen *et al.* *(1)*. Oxygen content *(2)* and exposure to elevated temperatures and agitation during transport can accelerate the aging process *(3)*. The assessment of flavour deterioration is ideally conducted sensorially on packaged beer after storage for appropriate periods, since this indicates the changes likely to be perceived by the consumer. The ability to optimise the brewing process in a way which limits flavour deterioration is hampered by the time required for sensorial assessment. Therefore, the need exists to accurately predict these flavour changes before they happen. Many researchers and brewers have adopted assays utilising Electron Paramagnetic Resonance (EPR) spectroscopy, also known as Electron Spin Resonance (ESR), to achieve this aim *(4, 5)*. Beer is heated to increase the formation of short lived radicals, which can then be captured by added spin traps. The spin trap radical adducts are more stable and can be detected and measured using EPR *(6)*. The changing profiles of the curves generated from an EPR oxidative stability assay from sweet (unhopped) wort through to final beer have been described previously *(7)*. Un-boiled sweet wort produces relatively few radicals compared to hopped boiled wort, with oxygenated pitching wort having a still higher potential for radical formation *(7)*. During fermentation a lag time is typically generated, characterised by an absence of, or reduced, adduct formation observed at the beginning of the assay. This is an indication of the antioxidant capacity of the beer *(7)*. Amongst early adopters of this technique, the lag time was shown to predict the sensorial flavour stability (in days to develop stale character when stored at 30°C *(8)*) and this measure is the one recommended by the ASBC *(9)* for assessing the oxidative stability of light (pale) beers. Another measure commonly derived from the forcing test is the EPR intensity after a given time (TXXX), which is most commonly 150 min (T150). This metric is recommended for dark or amber coloured beers which may lack a lag time. This provides a measure of free radical formation after a standardised time interval during the forcing assay. In some instances, using the two measures together can provide useful information, with the lag time (antioxidant capacity) indicating when a beer may begin to stale and the subsequent T150 value suggesting how severely a beer will stale. Thus, brewers target longer lag times and/or smaller TXXX for improved flavour stability. Marquez and co-workers identified the total area under the EPR curve as a further metric indicative

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of the total free radical generation across the EPR assay *(10)*. When applied across three brewing sites, a reduction in the area under the EPR curve was suggested to correlate with the improved sensory evaluation of beers *(10)*.

Generating beers with enhanced oxidative stability and thus a potentially longer shelf life would increase customer satisfaction and realise potential savings in the supply chain. Fermentation is one step amongst many in the process which may contribute to the final flavour stability of a beer. There is an ever increasing range of yeast strains available to brewers but knowing which phenotype will result in increased flavour stability is not clear. Yeast interaction with hop compounds, metal ions *(11)*, production of sulphur dioxide (SO_2) *(12)*, extracellular proteins and reducing impact on the environment may all contribute to the overall flavour stability *(13)*.

Selection of yeast based on their resistance to oxidative stress has been proposed as a criterion which may produce more oxidatively stable beers *(11, 14)*. Oxidative stress is a broad term covering stress originating from reactive oxygen species (ROS) of which brewing yeast may encounter many types and from many sources. Yeast which can manage these stresses more effectively may also change their environment (beer) in a positive manner with regard to oxidative stability. Berner and Arneborg *(11)* screened yeast for their resistance to paraquat and diamide, before selecting high and low resistant strains for fermentation trials. Diamide induces the formation of disulphide bonds, thus reducing the antioxidant capacity of yeast. The action of paraquat is through the generation of the superoxide ROS (O_2^-), potentially at the expense of NADPH or NADH *(15)*. Berner and Arneborg *(11)* selected three strains of differing resistance and found that the least oxidative stress resistant strain produced the more oxidatively stable beer. Hoff *et al*. *(14)* also selected high and low oxidative stress tolerant yeast, although did not specify the particular oxidative stress used. In this case, the strain with lower stress resistance led to fresh beer with an increased rate of radical formation, suggesting lower oxidative stability, although the significance of the difference at this point was not discussed.

In addition to the observations on oxidative stress of yeast, Berner and Arneborg *(11)* also noted that increased metal ion removal by yeast coincided with improved oxidative stability, suggesting that this was the more important trait. Pro-oxidant metal ions are known to contribute to beer flavour instability *(16–18)*, so increased removal during fermentation should directly improve oxidative stability. Metal ions may also result in oxidative stress in yeast through the generation of ROS *(19)* or direct interaction with ROS defence systems such as the binding of copper to glutathione *(20)*, although the concentrations typically found in wort are unlikely to elicit this response. In contrast, many of the functions of yeast are dependent on metal ions, which are key components of cellular enzymes, including those involved in the response to oxidative stress. Catalase, a key defence against hydrogen peroxide, contains iron as a functional component *(21)*, whilst the Cu-Zn superoxide dismutase plays a key role in detoxification of the superoxide ROS *(22)*.

The present study was designed to evaluate the impact of yeast strain on the EPR oxidative stability of beers. The same wort sample was fermented in triplicate at laboratory scale using 11 commercial yeast strains under parallel fermentation conditions. The yeast strains were further categorised into four sub-groups, according to their resistance to two oxidative stresses – hydrogen peroxide and menadione. The impact of each yeast strain and of their oxidative-stress resistance group on the EPR oxidative stability of beer was determined. Furthermore we report, in each case, the levels and uptake of iron, manganese and copper across fermentation, as well as the levels of SO_2 developed. These data provide new information regarding the role of yeast strain, and its phenotype, on factors which determine the oxidative stability of beer.

Materials and methods

Yeast strains

The yeast strains were all *Saccharomyces pastorianus* and are proprietary brewing strains.

Yeast propagation

Yeast strains were retrieved from long term storage at -80°C and propagated in wort at 25°C and shaken at 120 rpm. The cultures were transferred from 10, 100 and 1000 mL sequentially every 48 h. Cells were harvested by centrifugation (4°C and 3000 *g*), washed with sterile deionised water, resuspended to a standard viable yeast concentration and 500 μl pitched into miniature FV. This resulted in 1.5 x 10^7 viable cells per mL, assessed by methylene blue staining *(23)*.

Miniature fermentations

Hopped wort was produced in a 10 hl batch, using a pale lager malt. The mash had the following standpoints 50°C (15 min), 63°C (20 min), 72°C (15 min), and 76°C (5 min). The 60 minute boil was hopped with Magnum T90 pellets resulting in wort of 1.0664 original gravity, 20 IBU and pH 5.5. Miniature 100 mL fermentation vessels (FVs) were based on the method of Quain et al. *(24)*, with wort aerated at 15°C by agitation with a headspace of air for 24 h. FVs were continuously stirred using a multi position (15 places) magnetic stirring plate (VWR International Ltd, UK) and magnetic stirring bar. A Bunsen valve allowed the exit of gas generated during fermentation. Yeast strains were assessed in triplicate and fermentations were stopped when the weight of the vessels was constant, when the yeast was removed by centrifugation (4°C and 3000 *g*). Green beer was analysed with an Alcolyzer-Plus: beer system, which incorporated a DMA 4500 density meter (Anton Paar, Graz, Austria).

Beer oxidative stability assay

α-(4-Pyridyl N-oxide)-N-tert-butylnitrone (POBN) (Sigma-Aldrich, USA) was prepared as outlined in Kunz et al. *(25)*. 168 mg POBN was dissolved in 1 mL of water and 50 μL of this stock solution was added to 12 mL of beer. The Electron Paramagnetic Resonance (EPR) was measured using an E-Scan Beer Analyser system (Bruker, Billerica MA, USA). The spectrum parameters were set up to capture the central peak of the triplet peaks generated, with a centre field of 3475 G and a sweep width of 14 G. The microwave bridge had a power of 2.31 mW and frequency of 9.78 GHz. Receiver gain was 2000, modulation frequency 86 kHz, modulation amplitude 1.49 G, modulation phase 0.85 deg, time constant 20.48 mS based on the settings of Kunz et al. *(25)*. Samples were inserted into a heating block (60°C) at 163 second intervals. Assays were controlled by EPR Liquid and Beverage Analyser software (ELBA 2.0.2, Bruker, Billerica MA, USA) which took 17 samples over 450 minutes. 18 scans were aggregated and the peak to peak

height of the first derivative of the EPR spectra was recorded as the intensity value at a given time point.

EPR curve analysis

A sigmoidal equation was fitted to the data using Prism 6 (GraphPad Software, La Joila, USA). From the resulting fitted curve, lag times were determined as the point at which the modelled curved reached an intensity equal to 12% of the difference between the upper and lower limits of the curve. The T450 was the value of the curve at 450 minutes. The rate at the point of inflection was determined as the maximum rate (EPR Intensity/min) of the curve. In cases where the signal intensity reduced late in the assay, curves were fitted to the sample points prior to this reduction.

Total sulphite

Total sulphite was quantified enzymatically using a commercial kit (K-ETSULPH 02/15, Megazyme, Bray, Ireland). Sulphite in the sample is oxidised to sulphate with the production of hydrogen peroxide. The hydrogen peroxide is reduced in the presence of the NADH peroxidase and NADH, resulting in a decrease in absorbance at 340 nm. The assay was performed in 96 microwell plates at room temperature (20°C) with 0, 1, 5 and 10 mg/L sulphite standards. A final absorbance (340 nm) was read after 20 min and used to determine the amount of sulphite in the samples.

Metal ion analysis

Wort samples were diluted 1:10 with nitric acid to a final concentration of 2% (v/v) prior to analysis. Nitric acid (trace metal grade, Fisher Scientific, Loughborough, UK) was added to beer samples to a final concentration of 2% (v/v). Iron, copper and manganese was quantified using Inductively coupled plasma mass spectrometry (ICP-MS) (Thermo-Fisher iCAP-Q) with a Flatopole collision cell (charged with Helium gas) upstream of the analytical quadrupole. Internal standards were introduced to the sample stream via a T-piece with Sc (50 μg/L), Ge (20 μg/L) Rh (10 μg/L) and Ir (5 μg/L) included in the matrix of 2% (v/v) nitric acid. External calibration standards for iron, copper and manganese were run at 0, 20, 40 and 100 μg/L. Samples were introduced via an autosampler (Cetac ASX-520) through a nebuliser. Sample processing was undertaken using Qtegra software (Thermo-Fisher Scientific).

Statistical analysis

Trial data were analysed using a one-way ANOVA with yeast strain as factor using Minitab statistical software v17.2.1. To investigate potential impacts of the oxidative stress grouping of yeast strains on the measured variables, further one-way ANOVA was performed with yeast oxidative stress group as the factor. In each case the criterion $p<0.05$ was used to assess statistical significance.

Results and discussion

Yeast strains

The strains of *S. pastorianus* were classified *(26)* according to their sensitivity to two oxidative stresses (0.5 mM menadione and 4 mM hydrogen peroxide) incorporated into wort as a growth medium. A Biolog Omnilog phenotypic-microarray (Biolog, Hayward CA, USA) was used to measure cellular respiration with a signal intensity less than 75% of that of a control (no oxidative stress addition) considered to be the threshold for sensitivity to that stress. Hydrogen peroxide is a common stress formed within the yeast cell as a by-product of metabolism. It has been shown to be produced by *S. cerevisiae* under ethanol stress which may subsequently result in autophagy *(27)*. When yeast are exposed to an external source of hydrogen peroxide, sensitivity to this stress has been linked to the amount of hydrogen peroxide which crosses the cell membrane. Overexpression of aquaporins have facilitated hydrogen peroxide influx and resulted in increased sensitivity of those yeast strains *(28)*. Menadione leads to the formation of the superoxide radical, which may subsequently lead to hydrogen peroxide formation in certain systems. Although the two oxidative stresses are closely related, the response of yeast to each stress has been shown to differ *(29)*. In the present research, Group One strains had been classified as sensitive to both hydrogen peroxide and menadione. Group Two were sensitive to hydrogen peroxide, but resistant to menadione, whilst Group Three yeast were resistant to hydrogen peroxide but sensitive to menadione. Group Four were resistant to both menadione and hydrogen peroxide *(26)*. All yeast were strains of *S. pastorianus* which displayed varying fermentation characteristics (Table 1), but their genetic relatedness was not explored.

Fermentation and oxidative stability assay

Of the 12 strains selected, strain D failed to ferment the wort adequately and was removed from the trial. Given that this yeast has previously been used in beer production this failure to attenuate was unexpected; the cause remains unknown. The remaining 11 yeasts reached attenuation after five to eight days of fermentation (Table 1). The ethanol concentration in the beers ranged between 7.39-8.14% ABV and the pH was similar for all strains apart from strain E, which resulted in a significantly higher pH (4.24) than all other strains ($p<0.05$; Table 1).

Rate of attenuation and ethanol yield are important considerations when brewers are selecting yeast. When attenuation was reached, the green beer was removed, and the oxidative stability assayed. The T450, maximum rate of adduct formation and lag time (if present) were derived for all trial beers (Table 2). Assessment of the oxidative stability of the resulting green beer (with yeast removed) was made at the end of fermentation without any additional maturation or processing.

In this study there was significant variation in the oxidative stability of beer produced with different yeast strains (Table 2). For example, T450 values varied from 2.17-14.5 x 10^5, with Strain E (Group 1) resulting in a significantly higher T450 than any other yeast strain ($p<0.05$). Strain C gave rise to the lowest T450 value, which was statistically similar to that for strains F, J, K and L but significantly lower than for all other strains in the study ($p<0.05$; Table 2). Differences in the oxidative stability of the beers were solely attributable to the action of the yeast strains, since the wort composition and fermentation practices were otherwise consistent across all trials. Such differences may have arisen due to the extent of removal of components of wort such as metals or reducing sugars, or differences in the production of antioxidant compounds such as SO_2. Interestingly, only five strains produced the characteristic sigmoidal curve which enables a lag time to be calculated (Figure 1). The other strains produced beers which exhibited immediate spin adduct formation during the assay, characteristic of an absence of antioxidant activity or excessive radical production which negates any antioxidant effect. This

Table 1. Fermentation performance of 11 yeast strains classified according to oxidative stress sensitivity. Data are the mean ± SD of three biological replicates.

Strain ID	Oxidative stress sensitivity Group*	Attenuation (days)	Final density (g/mL)	Ethanol (% v/v)	Viability (%)	Beer pH
A	One	5	$1.0059^{b} \pm 0.0004$	$7.73^{abcd} \pm 0.14$	$94^{c} \pm 1$	$3.87^{b} \pm 0.03$
E	One	7	$1.0073^{ab} \pm 0.0020$	$7.81^{abcd} \pm 0.09$	$88^{d} \pm 2$	$4.24^{a} \pm 0.07$
B	Two	5	$1.0062^{b} \pm 0.0002$	$7.65^{bcd} \pm 0.07$	$95^{abc} \pm 1$	$3.77^{b} \pm 0.05$
F	Two	6	$1.0085^{a} \pm 0.0008$	$7.39^{d} \pm 0.35$	$97^{abc} \pm 1$	$3.84^{b} \pm 0.1$
G	Two	8	$1.0070^{ab} \pm 0.0003$	$7.88^{abc} \pm 0.10$	$97^{a} \pm 1$	$3.86^{b} \pm 0.02$
H	Two	5	$1.0055^{b} \pm 0.0007$	$7.98^{ab} \pm 0.27$	$96^{abc} \pm 2$	$3.82^{b} \pm 0.01$
I	Three	5	$1.0056^{b} \pm 0.0001$	$8.14^{a} \pm 0.02$	$98^{ab} \pm 1$	$3.87^{b} \pm 0.02$
C	Four	7	$1.0077^{ab} \pm 0.0007$	$7.47^{cd} \pm 0.14$	$95^{bc} \pm 1$	$3.82^{b} \pm 0.01$
J	Four	8	$1.0072^{ab} \pm 0.0007$	$7.52^{bcd} \pm 0.09$	$96^{abc} \pm 1$	$3.76^{b} \pm 0.08$
K	Four	6	$1.0067^{ab} \pm 0.0001$	$7.52^{bcd} \pm 0.08$	$96^{abc} \pm 1$	$3.88^{b} \pm 0.01$
L	Four	5	$1.0065^{ab} \pm 0.0001$	$7.53^{bcd} \pm 0.09$	$94^{abc} \pm 2$	$3.87^{b} \pm 0.01$

* Group One strains were sensitive to both hydrogen peroxide and menadione stress. Group Two were sensitive to hydrogen peroxide, but resistant to menadione whilst Group Three yeast were resistant to hydrogen peroxide but sensitive to menadione. Group Four were resistant to both menadione and hydrogen peroxide stresses.

[a] Superscripts denote one-way ANOVA group codes. Reading down each column, mean values that do not share a letter are significantly different ($p<0.05$).

absence of lag time has been noted in previous studies *(14)* and other measures from the assay curve such as rate (Max EPR Intensity/min) and intensity at specific times (TXXX) have been used to describe the oxidative stability of these beers. Group Four yeasts in the study are resistant to the oxidative stresses menadione and hydrogen peroxide and yeast strains C, K and L within this group produced beers with clear lag times (236, 158 and 137 min respectively). Strain J, also classified as a Group Four yeast, produced no lag time but resulted in in a beer exhibiting one of the lowest T450 and rate of adduct formation values. Strains A and E were the only other yeast to produce beers with lag times (78.5 and 82.4 min respectively), the shorter length indicating a lower oxidative stability relative to strains C, K and L. Although the curves generated during the oxidative stability assay were sigmoidal there was significant spin adduct formation during the early part of the assay similar to those where a lag time could not be assigned (Figure 1). Indeed Strain E produced a beer with a high capacity to generate radicals (highest T450 value; Table 2), indicating a low oxidative stability. Strain E had the lowest viability post-fermentation (88%, compared with 94-97% for all other strains (Table 1)), which may have contributed to higher radical formation, although the reasons remain to be determined. Overall the Group Four strains had the lowest mean maximum rate and T450 values and were significantly lower for the maximum rate parameter relative to both Group One and Group Three yeast strains ($p<0.05$; Table 3). This supports the hypothesis that beers produced from the most oxidative stress resistant strains would potentially be the most flavour stable. It also suggests, according to the current classification, that strain resistance to menadione stress might be a better indicator of EPR oxidative stability. One Group Two strain (F) also performed well based on T450 and rate of adduct formation. The properties that provide these yeast strains with greater resistance to ROS or which result in more oxidatively stable beer are yet to be fully understood. There was no apparent link with rate of fermentation, and it may be that yeasts which are better able to resist the stresses of fermentation remain more vital. Healthy yeast with high vitality (acidification power) have been shown to produce more flavour stable beer *(30)*. Alternatively, the specific responses which allow the yeast to survive multiple ROS stresses (menadione and hydrogen peroxide), which are currently unknown, may be beneficial for the oxidative stability of beer.

Yeast influence on the metal ion content of beer

In addition to being crucial for many metabolic pathways in yeast, the metal ion content has a direct impact on the oxidative stability of beer *(8)*. The wort used in this study contained 135 μg/L manganese, 85 μg/L iron and 59 μg/L copper (Table 2). The concentrations reported in this study were lower than is typical, based on the limited publications for wort. However, variation in metal ion concentrations is to be expected as they are influenced by the materials and processes used in wort production. Mochaba et al. *(31)* demonstrated batch to batch variation for iron in one brew house, making the same brand, between 210-800 μg/L. Another industrial scale (600 hL) study measured 250 μg/L iron and 90 μg/L copper *(32)*, whilst manganese has been shown to vary between 60-140 μg/L in a pilot scale brew *(33)*.

After fermentation the residual metal ion concentrations in the green beers were measured. Of the initial content in wort, between 74% (yeast strain E) and 86% (I) of manganese, 15% (F) and 53% (A) of iron and 17% (F) and 61% (E) of copper remained in the green beer (Table 2). Small additions of metal ions (10 μg/L) have been shown to negatively impact the oxidative stability of beers, reducing lag time and increasing T450 measures as the metal ion concentration is increased *(34)*. Residual iron levels in the present trial beers ranged from 13-45 μg/L, relative to the initial wort concentration of 85 μg/L (Table 2). There was a low degree of correlation between iron content and the measured T450 values. Plotting the maximum rate of adduct formation against the iron concentration post-fermentation suggested that there was a positive correlation, albeit in two potential groups of strains (Figure 2). Strains A, C, K and L formed one group, and were also four of the five strains to produce beers with a lag time. Beers produced using these strains had some of the highest residual iron contents in

Table 2. Total sulphite, manganese, iron and copper concentrations at the end of fermentation and the oxidative stability parameters derived from the curves generated during the EPR assay. Data are the mean ± SD of three biological replicates. Initial metal ion concentrations in the wort are included.

Strain ID	Group	lag time (min)	T450 x 10^{-5}	Rate (Max EPR Intensity/min)	Total sulphite (mg/L)	Mn (μg/L)	Fe (μg/L)	Cu (μg/L)
Wort Content		N/A	N/A	N/A	N/A	135 ± 3	85 ± 6	59 ± 1
A	One	78.5^{c} ± 14	6.25^{bc} ± 0.31	2980^{bcd} ± 199	6.3^{de} ± 0.6	103^{abc} ± 6	45.4^{a} ± 2.5	22.4^{b} ± 3.4
E	One	82.4^{c} ± 7	14.49^{a} ± 2.50	6250^{a} ± 179	7.9^{bc} ± 0.2	100^{c} ± 3	31.4^{c} ± 3.3	36.4^{a} ± 2.5
B	Two	N/A^{d}	5.19^{bcd} ± 1.02	3300^{bcd} ± 905	5.9^{e} ± 0.5	105^{abc} ± 6	26.9^{c} ± 3.3	17.2^{bcd} ± 4.3
F	Two	N/A^{d}	2.44^{de} ± 0.42	1070^{d} ± 351	8.2^{bc} ± 0.2	102^{bc} ± 3	12.7^{d} ± 1.6	10.3^{d} ± 2.2
G	Two	N/A^{d}	5.12^{bcd} ± 0.66	2370^{cd} ± 50	8.8^{ab} ± 0.3	113^{ab} ± 4	16.6^{d} ± 0.9	10.8^{d} ± 1.4
H	Two	N/A^{d}	6.58^{bc} ± 0.08	3960^{bc} ± 520	9.1^{ab} ± 0.3	103^{abc} ± 5	26.6^{c} ± 2.7	14.0^{cd} ± 2.4
I	Three	N/A^{d}	6.83^{b} ± 1.09	4830^{ab} ± 753	8.7^{ab} ± 0.1	116^{a} ± 2	30.9^{c} ± 4.5	16.0^{bcd} ± 0.4
C	Four	236^{a} ± 3	2.17^{e} ± 0.31	1160^{d} ± 263	7.3^{cd} ± 0.1	110^{abc} ± 3	31.5^{c} ± 3.3	17.3^{bcd} ± 0.9
J	Four	N/A^{d}	3.87^{cde} ± 1.24	2320^{cd} ± 1083	8.8^{ab} ± 0.5	112^{abc} ± 2	17.3^{d} ± 2.8	11.5^{cd} ± 2.2
K	Four	158^{b} ± 17	4.82^{bcde} ± 0.11	2360^{cd} ± 90	9.4^{a} ± 0.5	106^{abc} ± 2	40.2^{ab} ± 3.0	20.5^{bc} ± 0.7
L	Four	137^{b} ± 12	4.18^{bcde} ± 0.52	1950^{cd} ± 424	8.9^{ab} ± 0.7	112^{abc} ± 8	34.7^{bc} ± 3.1	22.1^{b} ± 3.3

[a] Superscripts denote one-way ANOVA group codes. Reading down each column, mean values that do not share a letter are significantly different ($p<0.05$).

the study, but relatively low rates of spin adduct formation. The removal of copper, another pro-oxidant metal ion found in beer, was closely correlated with that of iron, with the exception of Strain E, which demonstrated a significantly higher residual level of copper than all other strains (36 μg/L; $p<0.05$; Table 2). In a previous study *(14)*, a high oxidative stress resistant yeast strain produced beer with the lowest levels of iron and copper *(14)*. Here, the initial wort contained 314 μg/L iron and 101 μg/L copper, whilst the subsequent beers contained 52 and 33 μg/L iron and 30 and 14 μg/L copper *(14)*. In a separate study, wort containing iron between 600-815 μg/L, resulted in beers ranging from 57-396 μg/L with the less oxidative-stress tolerant strain removing the most iron and producing the most oxidatively stable beer *(11)*. The lower initial iron content of the wort used in this investigation may explain why there was less impact from the removal of iron in this current study than was previously reported.

Here, none of the strains entirely removed the metal ions monitored in this study from fermenting wort. This may be attributed to a proportion of such ions not being biologically available due to the formation of complexes with other wort components *(35)*. The average uptake of manganese was 30 μg/L, 22% of the total, whilst on average greater proportions of iron and copper (54 and 41% respectively) were removed, despite being present in lower overall concentrations in the wort. Metal ion concentrations were measured when fermentation stopped, and changes across fermentation were not assessed. The end point values may be influenced by cellular leakage or efflux earlier in the fermentation, particularly from less vital yeast, as previously demonstrated *(31)*. Differences in the final metal ion contents of the beers are attributable to the action of yeast, however it was not determined if this was due to extracellular binding or intracellular uptake. If metals are attached extracellularly there may be potential for carry-over into subsequent fermentations on yeast repitching. Intracellular iron concentration has previously been shown to increase with successive fermentations *(31)*, and this may reduce the tendency for yeast to actively uptake additional iron.

Sulphur dioxide (SO_2)

Sulphur dioxide refers to molecular SO_2, bisulphite, and sulphite, with the ratio of these compounds determined by the pH of the solution. The role of SO_2 in flavour stability has been reviewed previously *(36)*. Its potency as an antioxidant is clear, however, SO_2 production during fermentation is viewed as less useful than the addition of SO_2 to the final product *(36)*. This is due to the ability of SO_2 to bind carbonyl compounds. When bound by SO_2, yeast may be unable to reduce the carbonyl compounds to their respective alcohols, resulting in the persistence of such adducts into finished beer and potential release of carbonyls from the adduct during storage *(37)*. SO_2 has been shown to increase the EPR lag time measured by oxidative stability assays *(6, 7, 25)*. The addition of sulphite to beer has previously been shown to adjust a POBN derived lag time by approximately 30 minutes for every 1 mg/L added *(25)*, although the impact will vary depending on the beer.

The concentration of SO_2 at the end of fermentation ranged from 5.9-9.4 mg/L (Table 2). The antioxidant effect of sulphite is often considered to be the primary driver of EPR lag times but did not appear to explain the presence of lag times in these samples. Two strains (L and K) which produced beers exhibiting a lag time also produced SO_2 at relatively high concentrations. However, Strain C, responsible for the most oxidatively stable beer, produced 7.3 mg/L sulphite, which was one of the lowest in the trial.

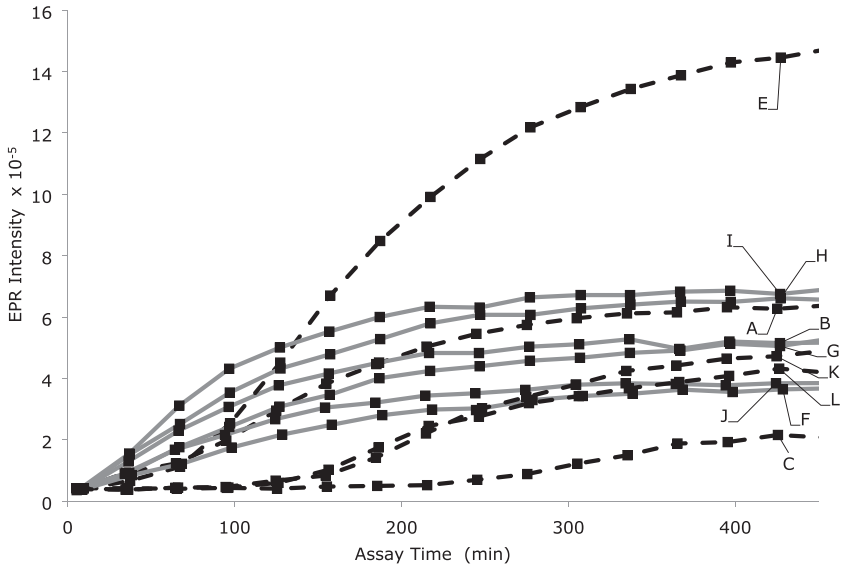

Figure 1. Examples of the curves generated during the oxidative stability assays of the beers at the end of fermentation. The EPR intensity is recorded over 450 minutes. Sigmoidal curves are fitted to the data from which lag time and T450 measures are taken. Dashed lines indicate curves from which a lag time could be derived. Table 2 reports the metrics derived from these assay curves. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 3. One way ANOVA analysis of fermented beer data by yeast oxidative stress group.

Strain ID	Group*	n	pH	Yeast viability (%)	lag time (min)	T450 (x 10^{-5})	Max Rate (EPR Intensity/min)	Total sulphite (mg/L)	Mn (μg/L)	Fe (μg/L)	Cu (μg/L)
A, E	One	6	4.06^{a}	90.8^{b}	80.4^{ab}	10.4^{a}	4620^{a}	7.1^{b}	101^{c}	38.4^{a}	29.4^{a}
B, F, G, H	Two	12	3.83^{b}	96.4^{a}	N/A^{c}	4.83^{b}	2680^{bc}	8.0^{ab}	106^{bc}	20.7^{b}	13.1^{b}
I	Three	3	3.87^{ab}	97.3^{a}	N/A^{bc}	6.83^{ab}	4830^{ab}	8.7^{ab}	116^{a}	30.9^{ab}	16.0^{b}
C, J, K, L	Four	12	3.83^{b}	94.9^{a}	133^{a}	3.76^{b}	1950^{c}	8.6^{a}	110^{ab}	30.9^{a}	17.9^{b}

* Group One strains were sensitive to both hydrogen peroxide and menadione stress. Group Two were sensitive to hydrogen peroxide, but resistant to menadione whilst Group Three yeast were resistant to hydrogen peroxide but sensitive to menadione. Group Four were resistant to both menadione and hydrogen peroxide stresses.

[a] Superscripts denote one-way ANOVA group codes. Reading down each column, mean values that do not share a letter are significantly different ($p<0.05$).

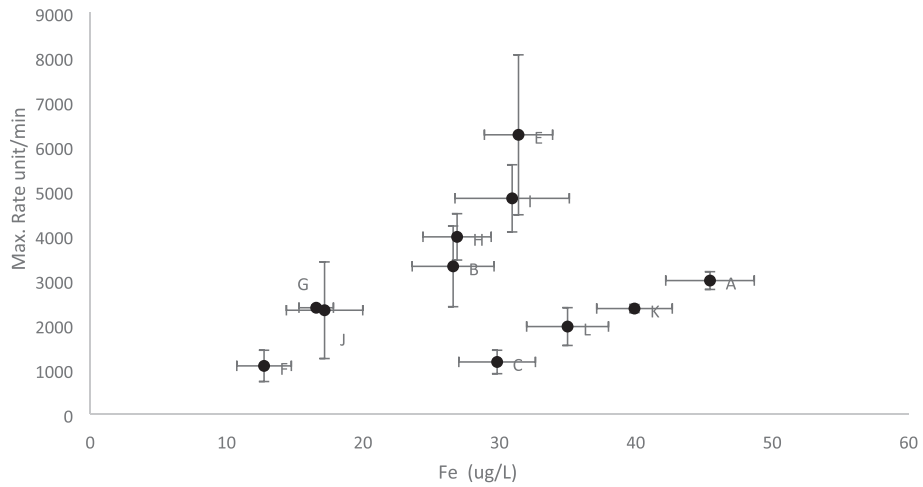

Figure 2. The relationship between residual iron post-fermentation and the maximum rate of spin adduct formation during the EPR oxidative stability assay. Data are the mean and standard deviation of three biological replicate measurements. [Colour figure can be viewed at wileyonlinelibrary.com]

Interestingly, Group Four strains produced significantly higher concentrations of SO_2 than the oxidative stress sensitive Group One strains ($p<0.05$; Table 3). It is thought SO_2 exhibits its antioxidant effect in beer through reaction with hydrogen peroxide *(38)*, but it is unclear if its production may be beneficial to yeast under exogenous hydrogen peroxide stress. Total SO_2 was measured here and does not account for the proportion present in the bound form, whose role in oxidative stability is less clear. However, Andersen et al. suggested that bound SO_2 can still produce an antioxidant effect *(6)*. In addition, only the final SO_2 concentration was determined and any influence of kinetics of its formation were not investigated in the present study.

Fermentation and the influence of yeast strain have been shown to play significant roles in determining the oxidative stability of green beer as measured by EPR. Selection of strains with high resistance to multiple oxidative stresses was shown to be a good indicator that yeast would produce more oxidatively stable beer, although the mechanisms determining this observation are unknown and require further study. The potential to sequester iron ions may contribute positively to oxidative stability, but in the present study this appeared less significant, probably due to the relatively low levels of metal ions in the mother wort. Brewers should consider their wort composition when searching for yeast to improve beer flavour stability. High metal sequestering strains have previously been shown to be beneficial for flavour stability *(11)*, but other factors appeared to be more significant in the present study. There are several other potential mechanisms which may be responsible for the strain differences, including the reducing activity of yeast *(39)*, interaction with hop and polyphenol compounds, alteration of barley proteins *(40)* and production of yeast proteins such as thioredoxin *(41)* - none of which were measured here. The miniature fermentation system used in this study, and elsewhere *(24, 42, 43)*, is an effective screening tool when multiple strains are being assessed. Potential strains of interest can be identified, but further work is required to determine if results are replicated in larger scale systems and when yeast is re-pitched. Further analyses, in particular sensory assessment of the resulting beers would be required to confirm the inferred influence of oxidative stability changes on flavour stability. Ultimately the yeast traits to select for enhanced oxidative stability are likely to be dictated by the wort and beer that is produced.

Author Contributions

David Jenkins - performed the investigation, writing the original draft, conceptualisation and methodology.

Sue James – writing, review and editing, methodology and conceptualisation.

Frieda Dehrmann – writing, review and editing, methodology and conceptualisation.

Katherine Smart – writing, review and editing, methodology and conceptualisation.

David Cook – supervision, writing, review and editing, methodology and conceptualisation.

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