Research article

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Heat resistance of yeast ascospores and their utilisation for the validation of pasteurisation processes for beers

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Beers and other low pH beverages are often stabilised by pasteurisation. There is a lack of guidance as to how many pasteurisation units are required for effective treatment of novel products so as to avoid over-pasteurisation. Yeast are common spoilers of such beverages and some species can produce heat resistant ascospores. As ascospores are more heat tolerant than vegetative cells they are ideal marker organisms for validating the effectiveness of beverage pasteurisation processes. In this study, 63 yeast strains were screened for their ability to produce spores with 30 strains showing different spore configurations. The rate of ascospore development during incubation on sporulation medium was also determined. It was found that the heat resistance of the ascospores of different species/strains varied widely with *Saccharomyces* species producing some of the most heat tolerant spores. Ascospores of *Saccharomyces cerevisiae* BRYC 501 were the most heat resistant with significantly more (over 6–16 times) heat resistance than heat tolerant lactic acid bacteria. The D- and z-values of *Saccharomyces cerevisiae* BRYC 501 ascospores were determined in alcoholic and non-alcoholic versions of two lager beers (American and German). The spores were over 14–18 times more heat tolerant in the non-alcoholic beers and, accordingly, higher PUs need to be applied. Interestingly, at the same/similar alcohol concentration and pH the yeast ascospores were significantly more heat resistant (1.9–2.5 times) in the American than the German beer which may suggest that bitterness contributed to their heat resistance.

Keywords: pasteurisation; yeast ascospores; capillary tubes; heat inactivation; beer

Introduction

Although yeast ascospores have been extensively studied over the past few decades *(1–4)*, there is no data on the utilisation of yeast ascospores as an indicator for the validation of pasteurisation processes of beer and other low pH beverages. Indicators or surrogates are commonly used in the food and drink industry to measure the effectiveness of various processes including pasteurisation. They are frequently used as they are safe, non-pathogenic (BSL 1 - Bio Safety Level 1 or HG 1 – Hazard Group 1) but also because they are more resistant than the target microorganisms *(5)*. Good examples include *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus),* which is commonly used for sterilisation validation as it produces one of the most heat resistant spores *(6)*; *Clostridium sporogenes,* used as a surrogate for the pathogen *Cl. botulinum (7)*; *Listeria innocua,* which can be used instead of *Listeria monocytogenes (8,9)*; *E. coli* O157 VTEC -ve, which is a surrogate for *E. coli* O157 VTEC +ve *(10)* and *Enterococcus faecium* which is frequently used as a surrogate instead of spoilage and pathogenic bacteria *(11,12)*. Although all these species could potentially be used for the process validation of low pH products, they are either too resistant (*Bacillus*, *Clostridium*) or too sensitive (vegetative bacteria). Additionally, most are not usually associated with the product types under study as they cannot grow at low pH, survive for a prolonged time as inactive spores (and do not pose a significant issue) or die rapidly. Yeast ascospores can be more suitable for the purpose of process validation in this type of food/beverage as they can potentially grow in low pH products resulting in product spoilage. Also, they are more heat resistant than any vegetative spoilage, pathogenic bacteria or vegetative yeast cells, but they are less heat resistant than bacterial spores. Ascospores, therefore, are ideally suited for heat process validation trials.

There is a need for this kind of indicator in the brewing and drinks industry as a high number of new beverages are being launched on the market that require adequate pasteurisation e.g. non-alcoholic beers, beer mixes, non-alcoholic spirits, Kombucha, flavoured low pH water and other acidified alcoholic/non-alcoholic beverages. All of these beverages can be stabilised by mild pasteurisation. According to Pasteurisation Guidance from 2006 *(13)*; products with pH values below 3.5 should be pasteurised at 70°C for 5 minutes (equal to 138 PU) and products with a pH of beween 3.5 and 4.0 should be pasteurised at 85°C for 5 minutes (equal to 20,000 PU). Alternatively, the European Brewery Convention (EBC) Manual of Good Practice recommends pasteurising non-alcoholic beers at 80-120 PU, lemonade at 300-500 PU and fruit juices at 3,000-5,000 PU *(14)*. Many of the new beverages currently being developed, however, do not fit into any of these specific product categories. Therefore, selecting effective pasteurisation parameters without conducting validation is difficult. Often, drinks manufacturers opt for higher PU values to avoid the risk of under-pasteurisation and subsequent microbiological spoilage. However, this approach can result in

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an over-pasteurised product with flavour and nutritional deterioration. Furthermore, over-pasteurisation leads to excessive energy and water usage.

As the number of microorganisms which are able to spoil low pH (<4.5) products is significantly lower than higher pH products and, as spore forming bacteria rarely grow in them, yeast ascospores are potentially the most suitable organism for heat treatment tests on low pH products. Most yeast species are safe to use, they are easy to grow and store, and certain species produce heat resistant ascospores. Spores have significantly greater heat resistance than any vegetative bacterial or yeast cells *(15)*. Therefore, if it is confirmed that a pasteurisation process is effective in inactivating a high number of yeast ascospores this will ensure that all other spoilage microorganisms would be inactivated by this process as well.

The aim of this study was to screen various yeast strains for their ability to produce ascospores, measure the heat resistance of spores produced and select the most heat resistant yeast ascospore. Subsequently, a series of heat resistance experiments was conducted in a selection of beers, the D and z-values determined, and optimal pasteurisation parameters calculated. The D-value is the time required at a specific temperature for a decimal (i.e., 1 log or 90%) reduction in the population of a microorganism; the z-value is defined as the change in temperature required for a 10-fold change in the D-value. To show that the heat resistance of yeast ascospores are significantly greater than the heat resistance of a typical beer spoilage vegetative bacteria, additional heat inactivation trials were performed with the common beer spoilage microorganisms; *Lactobacillus brevis* (which has been found to be one of the most heat resistant beer spoilage bacteria in a similar study) *(16)* and *Lactobacillus lindneri* (an emerging beer spoilage bacteria) *(17)*.

Materials and methods

Beers

Two American-style lagers of the same brand (4.5% ABV, 0.05% ABV) and two German-style lagers of the same brand (4.9% ABV, 0.05% ABV) were purchased from a local supermarket and were used in this study. The German-style beers were brewed according to the German beer purity laws using only four ingredients – water, barley malt, hops and yeast - whilst the American-style beers contained water, barley malt, rice, malt extract, hop, hop extract and natural flavours. The bitterness, pH and ABV of the beers were measured and the concentration of carbohydrates, sugars and protein was obtained from the package labels (Table 1). Bitterness was measured by spectrophotometry following the EBC Analytica method 9.8 and expressed in IBU (International Bitterness Units), the ABV was measured as ethanol by volume by gas chromatography following the EBC Analytica method 9.3.2. The pH was measured with an AR15 pH meter (Accumet Research, USA).

Microorganisms

In total, 63 yeast strains were screened for their ability to produce ascospores (Table 2). All yeast strains were stored in liquid nitrogen (Air Products, UK). Fifteen strains were obtained from the National Collection of Yeast Cultures (NCYC) collection, one from the DSMZ (German Collection of Microorganisms and Cell Cultures) collection and three from the Research Center Weihenstephan for Brewing and Food Quality *(18)*. The remaining strains were either isolated from different beverages (beer, kombucha, wine, soft drinks) or were environmental isolates. Species were identified by 26S rDNA D1/D2 sequencing. All yeast strains retrieved from liquid nitrogen were revived in Yeast and Mould broth (YM broth; Oxoid, UK) and on Yeast and Mould agar (YM agar; Oxoid, UK).

Two bacterial species, *Lactobacillus brevis* BSO 566 (which was found to be the most heat resistant bacterium in our previous study *(16)*) and *Lactobacillus lindneri* BSO 943 – an emerging beer spoilage microorganism *(17)* - were employed. The bacteria were stored in liquid nitrogen. When required they were retrieved from long term storage and revived in de Man, Rogosa and Sharpe broth (MRS broth; Oxoid, UK) and on de Man, Rogosa and Sharpe agar (MRS agar; Oxoid, UK).

Preliminary screening of yeast strains

For preliminary trials the heat resistance of yeast ascospores was determined in McIlvaine buffer (pH 4). For full heat inactivation trials, the spores were suspended in the four beers. The McIlvaine buffer (pH 4) was prepared by mixing 7.71 mL of 0.2 M disodium phosphate (Sigma-Aldrich UK) with 12.29 mL of 0.1 M of citric acid (Sigma-Aldrich, UK) *(19)*. The buffer was kept chilled (2-8°C) and used within one month. The pH of the buffer was confirmed before each trial.

Ascospore production

All yeast strains used in this study were retrieved from long term storage (liquid nitrogen) and grown in YM broth. All cultures were grown for 2 days at 25 ± 1°C and 200 µL were spread onto Ascospore Agar (HiMedia, India). The ascospore agar plates were incubated for 9-10 days at 25 ± 1°C and then flooded with 10 mL of sterile distilled water. The ascospores were harvested with an L-shape spreader and the suspension centrifuged at 3,000 x *g* for

Table 1. Analysis of beers

Beer analysis	Alcohol free beer		Alcoholic beer	
	American-style lager	German-style lager	American-style lager	German-style lager
ABV (%) - label	0.05	0.05	4.5	4.9
ABV (%) - analysed	0.05	0.03	4.5	4.9
pH	4.4	4.2	4.3	4.2
Bitterness (IBU)	5.6	25.9	6.9	24.9
Carbohydrates (g/100mL)	8.0	3.1	3.1	2.2
Sugars (g/100mL)	0.5	0.2	0.1	0.1
Protein (g/100mL)	0.2	0.3	0.3	0.37

Table 2. Yeasts screened for the production of ascospores

Yeast	Code	Source
Cryptococcus adeliensis	KY33	Kombucha
Debaryomyces hansenii	BRYC 922	Cider
Debaryomyces hansenii var. *fabryi*	BRX 411	Cider
Debaryomyces marama	BRX 413	Environment
Hanseniaspora sp.	KY12	Kombucha
Hanseniaspora valbyensis	KY13	Kombucha
Hanseniaspora valbyensis	KY34	Kombucha
Harpella melusinae	KY35	Kombucha
Kluyveromyces marxianus	KY36	Kombucha
Kluyveromyces marxianus	KY4	Kombucha
Kluyveromyces marxianus var. *marxianus*	BRYC 515	Cheese; NCYC 179
Kluyveromyces marxianus var. *marxianus*	BRYC 509	NCYC 100
Kluyveromyces thermotolerans	BRYC 407	Brewery
Meyerozyma guilliermondii	BRYC 931	Environment
Millerozyma farinosa	BRX 379	Beer; NCYC 815
Pichia galeiformis	BRX 412	Environment
Pichia kluyveri	BRX 410	Olives; NCYC 246
Pichia kudriavzevii	BRYC 905	Brewery
Pichia kudriavzevii	KY37	Kombucha
Pichia manshurica	BRYC 920	Wine
Pichia membranifaciens	KY7	Kombucha
Pichia membranifaciens	BRYC 918	Wine
Rhodotorula mucilaginosa	KY38	Kombucha
Rhodotorula mucilaginosa	BRYC 928	Brewery
Rhodotorula mucilaginosa	BRYC 932	Brewery
Saccharomyces bayanus var. *uvarum*	BRYC 934	Brewery
Saccharomyces cerevisiae	BRYC 919	Wine
Saccharomyces cerevisiae	KY8	Kombucha
Saccharomyces cerevisiae	KY1	Kombucha
Saccharomyces cerevisiae	KY11	Kombucha
Saccharomyces cerevisiae	BRYC 501	NCYC 74, ATCC 9080
Saccharomyces cerevisiae	BRYC 937	Beer; DSM 1848
Saccharomyces cerevisiae	BRYC 606	Ale production strain; NCYC 1019
Saccharomyces cerevisiae	BRYC 73	Ale production strain; NCYC 1681
Saccharomyces cerevisiae var. diastaticus	BRX 425	Beer
Saccharomyces cerevisiae var. diastaticus	BRX 414	TUM 3-D-2, BLQ
Saccharomyces cerevisiae var. diastaticus	BRX 415	TUM 71, BLQ
Saccharomyces cerevisiae var. diastaticus	BRX 416	TUM 1-H-7, BLQ
Saccharomyces cerevisiae var. diastaticus	BRX 417	Craft beer
Saccharomyces cerevisiae var. diastaticus	BRX 418	Beer; DSM 70487
Saccharomyces spp.	BRYC 938	Brewery
Saccharomyces spp.	BRYC 939	Brewery
Saccharomyces uvarum	KY5	Kombucha
Saccharomycodes ludwigii	BRX 380	NCYC 731 (Type strain)
Saccharomycodes ludwigii	BRYC 935	Bakery
Saccharomycodes ludwigii	BRX 386	Wine; NCYC 849
Schizosaccharomyces pombe	BRYC 906	Distillery
Schizosaccharomyces pombe var. *pombe*	BRYC 511	Millet Beer; NCYC 132
Schizosaccharomyces pombe var. *pombe*	BRYC 527	Raw cane sugar; NCYC 380
Schwanniomyces vanrijiae	BRYC 549	Oak tree; NCYC 577
Torulaspora delbruekii	BRX 409	Yogurt
Wickerhamomyces anomalus	BRYC 500	NCYC 16
Wickerhamomyces anomalus	BRYC 917	Wine
Wickerhamomyces anomalus	BRYC 923	Cider
Wickerhamomyces silvicola	BRX 389	NCYC 413
Zygoascus hellenicus	BRYC 926	Brewery

(*Continues*)

Table 2. *(Continued)*

Yeast	Code	Source
Zygosaccharomyces bailii	BRX 397	Sorghum brandy mash; NCYC 563
Zygosaccharomyces bailii	BRX 394	Red wine; NCYC 573
Zygosaccharomyces bailii	BRX 408	Wine
Zygosaccharomyces bailii	BRX 426	Alcoholic drink
Zygosaccharomyces bailii	BRX 427	Alcoholic drink
Zygosaccharomyces rouxii	BRYC 916	Alcoholic drink
Zygosaccharomyces rouxii	BRYC 528	Raw cane sugar; NCYC 381

5 min. The pellet was re-suspended in the test liquid, stored at 2-8°C and used within 4 hours of preparation.

Determining the sporulation ability of yeast strains

The spore suspensions were microscopically examined with and without ascospore staining. Heat fixed microscopic slides were spore stained using a method adapted from Schaeffer Fulton *(20)*. Heat fixed slides were flooded with 1% (w/v) brilliant green (Sigma-Aldrich, UK) and were gently heated from underneath with a Bunsen burner. Once the stain started to evaporate, the heat source was removed but reapplied once the evaporation stopped. The heating process was maintained for a total of 10 mins, the remaining stain was washed off with tap water and the slide counter stained with safranin for 40 seconds. Immediately thereafter, excess safranin was washed off with water, the slides were dried and then microscopically examined at 400 x and 1000 x (oil immersion) magnification. In addition, unstained wet mounts were prepared and observed for the presence of spores.

Yeast ascospore heat resistance screening

To select the most heat resistant ascospores, the heat resistance of all ascospores obtained from sporulating yeast strains were determined. The ascospores were prepared as above and the heat resistance of the ascospores suspended in McIlvaine buffer determined. The heat resistance (D_{60} – decimal reduction time at 60°C) was determined at one temperature (60°C) using the capillary tube method. Test solution (50 μL, ascospores suspended in McIlvaine buffer) containing between 10^6 and 10^7 CFU/mL were introduced into nine soda glass capillary tubes G119/0,2 (Fisher Scientific, UK); the tube ends were heat sealed and processed within 15 min. As shown by Bradshaw et al. *(21)* and by Campden BRI's internal checks (data not shown), sealing the capillary tubes did not affect the test solution. The ramp up time (heating time required to reach the target temperature) was measured prior to conducting the heat inactivation trials using an ultra-thin (0.075mm Diameter) K-Type thermocouple (RS PRO, UK) placed inside the capillary tube. The temperature was logged with a Squirrel OQ610 Temperature Data Logger (Grant Instruments, UK) every 0.25 seconds. Similarly, as shown by Jordan et al. *(22)* and Basaran-Akgul *(23)* the ramp up time in glass capillary tubes was short and the target temperature was reached within 6-7 seconds (see temperature profiles in Figure 1). The number of the ascospores in three capillary tubes was enumerated without any heat treatment to determine the initial number (N_0) of ascospores. The remaining six, sealed capillary tubes were submerged in a water bath at the set temperature of 60°C. Three capillary tubes were held in the water bath for 1 min and the other three for 10 min. Following heat treatment, the tubes were removed from the water bath and immediately cooled in ice water for approximately 1 min. The ascospore suspensions were then recovered from the capillary tubes and the viable spores enumerated by spread plating. As 50 μL (volume of suspension tested) was rinsed out of the capillary tubes with 450 μL Ringer's Solutions (Oxoid, UK) and 100 μL was analysed, the limit of detection for this method was 100 CFU/mL. The surviving yeast were recovered on YM agar after 10 days of aerobic incubation at 25 ± 1°C. The heat resistance was expressed as the D_{60}-value which was calculated from the time required to decrease the live spores from N_1 (live yeast after 1 min at 60°C) to N_{10} (live yeast after 10 min at 60°C) or from the time required to reduce the live yeast from

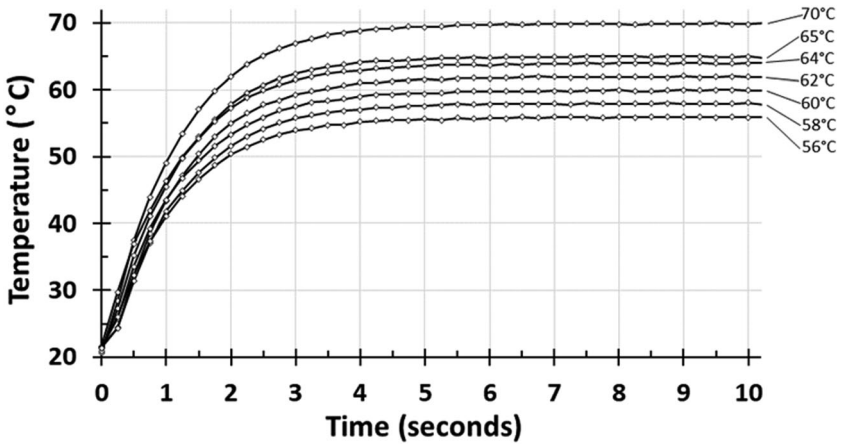

Figure 1. Temperature profile inside the capillary tube during heating to target temperatures of 56-70°C.

N_0 (live cells before trial) to N_{10} if no significant drop between N_0 and N_1 was observed. A significant drop between N_0 and N_1 was expected for very heat sensitive ascospores or in cases where ascospores were present at very low numbers in the initial solution. The D-values and standard errors for all trials were calculated using MiniTab 19. The yeast strain producing the most heat resistant ascospores was selected and used for the further trials.

Determining the yeast sporulation rate

To determine the optimal incubation time to obtain the highest ascospore yield, the sporulation rate and heat resistance of ascospores were measured on consecutive days (day 0 to day 10) of incubation on the sporulation medium. The selected yeast strain (*Saccharomyces cerevisiae* BRYC 501) was incubated on ascospore agar and the live cell/spore concentrations in the suspensions prepared daily were determined. The number of yeast cells without spores and the cells containing ascospores were microscopically counted in three replicates of each suspension. The sporulation rate was expressed as the percentage (%) of ascospore producing cells to total number of cells.

Heat inactivation of yeast ascospores in beer

The selected yeast strain – stored in liquid nitrogen - was resuscitated in YM broth and incubated aerobically at 25°C for 2 days. The exponential phase culture (0.2 mL) was spread onto the ascospore agar and the plates were incubated aerobically. After 9-10 days incubation, the agar plates were flooded with 10 mL of sterile distilled water and the ascospores were harvested with an L-shape spreader. This suspension was centrifuged at 3,000 x *g* for 5 min and the pellet suspended in the test beer. The ascospore solution was stored at 2-8°C and used within 4 hours of preparation. The ascospore heat resistance was measured at four temperatures – at 58, 60, 62 and 64°C for alcoholic beers and at 60, 62, 64 and 65°C for non-alcoholic beers - using the capillary tube method described above. The heat resistance was studied at these temperatures as they are most impactful on the heat resistance and result in a wide range of D values. All live yeast spores were recovered on YM agar and colonies were counted after 10 days of aerobic incubation at 25°C. D- and z-value, 95% confidence interval (CI), standard error (SE) and coefficient of determination (R^2) were calculated using the MiniTab 19 software.

Heat inactivation of *Lactobacillus* species in beer

To demonstrate that beer spoilage bacteria are significantly more heat sensitive than yeast ascospores, a series of trials were performed in the selected beers using two beer spoilage bacteria. *Lactobacillus brevis* BSO 566 and *Lactobacillus lindineri* BSO 943 were recovered from long term storage (liquid nitrogen) and were grown anaerobically in MRS broth at 25°C for 5 days. The broth was then centrifuged, and the resultant pellet was resuspended in the test beer. The bacterial heat resistance was measured at one temperature (60°C) using the capillary tube method described above. Live cells were recovered on MRS agar after anaerobic incubation at 25°C for 10 days. The D_{60} values, 95% confidence interval (CI), standard error (SE) and coefficient of determination (R2) were calculated using the MiniTab 19 software.

Minimum pasteurisation requirements

Based on the determined D- and z-values, the minimum PU required for the elimination of 6 logs of yeast ascospores in the beer were calculated.

Results

Preliminary screening of yeast strains

The preliminary screening showed that 30 of the 63 yeast strains tested were able to produce ascospores (Table 3). Either one, two, three or four ascospores were present within an ascus. Two different ascospore arrangements (chain or cluster) were observed where three or four ascospores were produced within an ascus (Figure 2). Whilst in most cases the presence of ascospores was easily detected by simple wet mount, the staining method was found to be useful when a low number of spores was present. It was observed that yeast strains could produce a mix of asci containing one, two, three or four ascospores and these could be present at different ratios. The sporulation rate determined for *Saccharomyces cerevisiae* BRYC 501 showed that over 50% of cells were able to produce ascospores within the first two days of incubation. A gradual increase in sporulation rate was observed thereafter, and the maximum rate of 94.9% was achieved at day 10 of incubation (Figure 3).

The heat resistance of ascospores of yeast strains able to produce spores was determined at one temperature (60°C). The results showed that the most heat resistant ascospores were produced by *Saccharomyces cerevisiae* BRYC 501. Their heat resistance (D_{60} value) in McIlvaine buffer at pH 4 was 19.7 minutes - nearly twice as high as for the second most heat resistant ascospores (see table 3). Some yeast strains produced heat sensitive ascospores as no survival was recovered after 10 minutes of heat treatment at 60°C and consequently their resistance to heat was below the limit of detection. The results also showed that *Saccharomyces* species produced the most heat resistant ascospores as nine of the most heat resistant ascospores were produced by *Saccharomyces* species. It is noteworthy that this was only a preliminary screen and the D values may not be precise as they were calculated from only two heating time points. Nevertheless, these results clearly show that *Saccharomyces cerevisiae* BRYC 501 produced the most heat resistant ascospores.

Heat resistance of ascospores in beer

The results of the yeast ascospore heat inactivation experiments in beer showed that all inactivation curves were linear (Figure 4 and Figure 5), and first order kinetics were suitable to describe the inactivation patterns. All R^2 values were above 0.95 except in the case of the 4.9% ABV German-style beer where, in three cases, the R^2 values were lower (0.935, 0.940 and 0.939 for the 58, 62 and 64°C trial respectively). The D-values at all four temperatures and the z-values for all four beers were calculated (Table 4) and it was shown that the *Saccharomyces cerevisiae* BRYC 501 ascospores survived the heat treatment considerably better in the American-style lager than in the German-style lager in both the alcoholic and alcohol-free varieties. The D_{60} of *Saccharomyces cerevisiae* BRYC 501 ascospores in the 0% ABV American-style lager was over twice as high as in the 0% ABV German-style lager. This may indicate that composition (bitterness, sugar content etc) or differences in

Table 3. D_{60} of ascospores suspended in McIlvaine buffer

Microorganism	Code	D_{60} (min)	SE (min)	95% CI (lower, upper)
Saccharomyces cerevisiae	BRYC 501	19.73	4.14	(12.5, 47.3)
Saccharomyces cerevisiae	BRYC 939	10.18	0.88	(8.20, 13.40)
Saccharomyces cerevisiae var *diastaticus*	BRX 414	9.06	1.08	(6.81, 13.54)
Saccharomyces cerevisiae var *diastaticus*	BRX 418	8.86	0.37	(7.93, 10.03)
Saccharomyces cerevisiae var *diastaticus*	BRX 415	8.02	0.70	(6.45, 10.59)
Saccharomyces cerevisiae	BRYC 938	7.72	0.88	(5.86, 11.29)
Saccharomyces cerevisiae	BRYC 937	7.59	0.30	(6.85, 8.52)
Saccharomyces cerevisiae var *diastaticus*	BRX 425	7.58	0.58	(6.25, 9.64)
Saccharomycodes ludwigii	BRYC 935	6.60	0.32	(5.82, 7.63)
Saccharomyces cerevisiae	KY 11	4.07	0.25	(3.48, 4.91)
Pichia farinosa	BRX 379	3.85	0.17	(3.42, 4.39)
Kluyveromyces thermotolerans	BRYC 407	<3.00	0.00	(3.00, 3.00)
Zygosaccharomyces bailii	BRYC 408	<2.85	0.14	(2.50, 3.31)
Saccharomyces cerevisiae	BRYC 919	<2.51	0.08	(2.32, 2.75)
Saccharomyces cerevisiae	BRYC 73	<2.27	0.03	(2.18, 2.37)
Saccharomyces cerevisiae	KY 8	<1.98	0.03	(1.91, 2.06)
Saccharomyces cerevisiae var *diastaticus*	BRX 416	<1.97	0.04	(1.87, 2.09)
Kluyveromyces marxianus var *marxianus*	BRYC 509	<1.89	0.02	(1.84, 1.94)
Saccharomycodes ludwigii	BRX 386	<1.82	0.05	(1.70, 1.97)
Wickerhamomyces anomalus	BRYC 923	<1.70	0.01	(1.68, 1.71)
Hansenula silvicola	BRX 389	<1.61	0.01	(1.59, 1.64)
Debaryomyces vanriji	BRYC 549	<1.61	0.03	(1.54, 1.69)
Saccharomyces cerevisiae	KY 1	<1.61	0.01	(1.59, 1.63)
Saccharomyces uvarum	KY 5	<1.54	0.01	(1.51, 1.57)
Pichia manshurica	BRYC 920	<1.53	0.02	(1.48, 1.58)
Saccharomyces bayanus var. *uvarum*	BRYC 934	<1.53	0.10	(1.30, 1.85)
Wickerhamomyces anomalus	BRYC 917	<1.49	0.02	(1.44, 1.54)
Saccharomyces cerevisiae	BRYC 606	<1.44	0.01	(1.41, 1.47)
Pichia subpelliculosa	BRYC 500	<1.43	0.00	(1.42, 1.44)
Saccharomyces cerevisiae var *diastaticus*	BRX 417	<1.43	0.01	(1.40, 1.45)
Zygosaccharomyces bailii	BRX 416	<1.41	0.03	(1.34, 1.49)

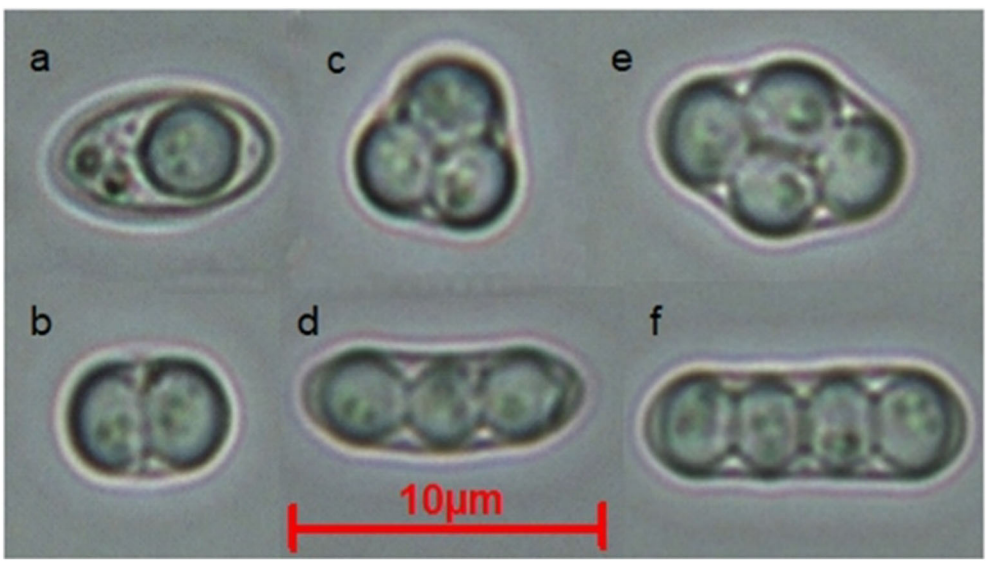

Figure 2. Yeast ascospores and their arrangements within the ascus; a) single ascospore, b) two ascospores, c) three ascospores as a cluster, d) three ascospores as a chain, e) four ascospores as a cluster and f) four ascospores as a chain.

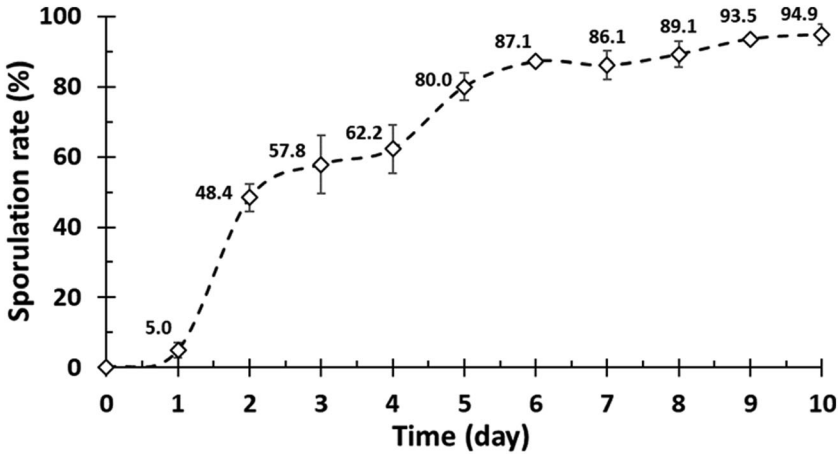

Figure 3. Sporulation rate for *Saccharomyces cerevisiae* BRYC 501.

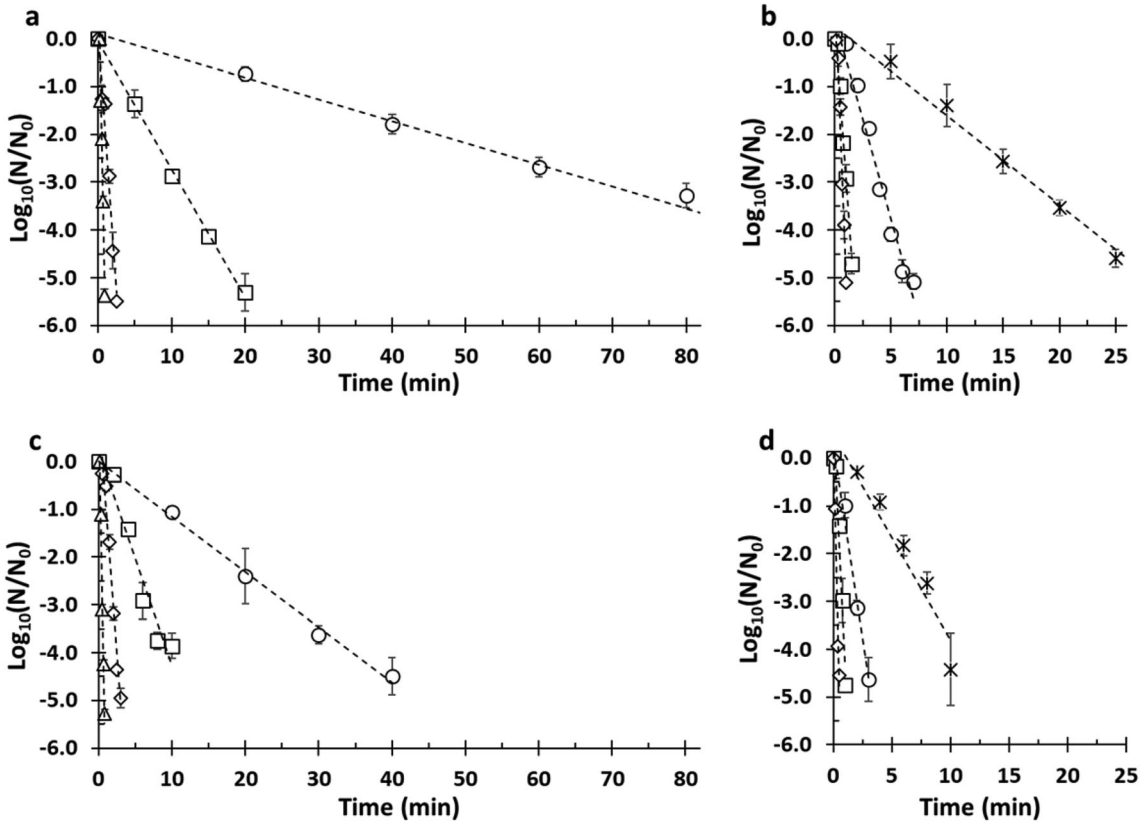

Figure 4. Inactivation curves for *Saccharomyces cerevisiae* BRYC 501 in four beers: a) non-alcoholic American-style lager, b) 4.5% ABV American-style lager, c) non-alcoholic German-style lager and d) 4.9% ABV German-style lager at 58°C (⋇), 60°C (○), 62°C (□), 64°C (◊) and 65°C (△).

manufacturing practices of the 0% ABV beers may have a significant impact on the heat resistance of yeast ascospores.

Bitterness was significantly higher in the German-style lager (24.9-25.9 IBU) than in the American-style lager (5.6-6.9 IBU). Also, the z-values were slightly lower in the case of the American-style beer for both the alcoholic as well as the alcohol-free variant. Furthermore, the z-values for the ascospores in both beer styles were slightly lower in the alcohol-free than in the alcoholic beers. This indicates that this German-style lager can be pasteurised with considerably lower pasteurisation units than the American-style beer studied here. Indeed, it was calculated that the 0% ABV German-style lager requires 52 PU rather than 132 PU required for the American-style beer. Similarly, the alcoholic German-style lager (4.9% ABV) requires only 3.7 PU, which is almost half of the 7.2 PU required for the alcoholic American-style beer (4.5% ABV). These PU values are considerably lower (except for the alcohol-free American-style beer) than the values suggested by the EBC Manual of Good Practice *(14)* according to which lager should be pasteurised at 15-25 PU and alcohol-free beer at 80-120 PU.

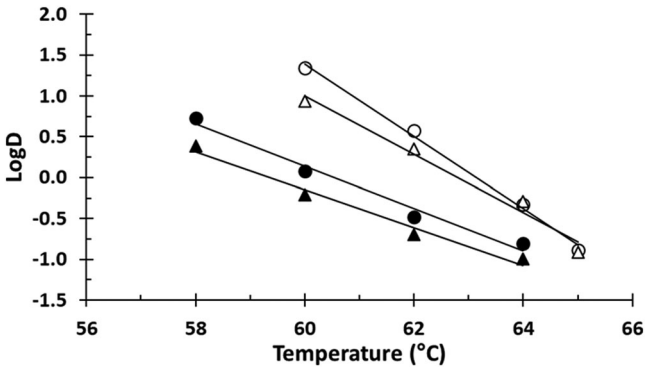

Figure 5. z-values of *Saccharomyces cerevisiae* BRYC 501 in 0% ABV American-style lager (○), 0% ABV German-style lager (△), 4.5% ABV American-style lager (●) and 4.9% ABV German-style lager (▲).

This study also showed that the heat resistance of ascospores is significantly greater than for the tested vegetative bacteria. The D_{60} for the ascospores of yeast *S. cerevisiae* BRYC 501 in the 0% ABV American-style beer was over 16 times higher than the D_{60} for *Lactobacillus brevis* and *Lactobacillus lindneri* (Table 5) in the same beer. Similarly, for the 0% ABV German-style beer the D_{60} is over 11 times higher, for the 4.5% ABV American-style beer seven times and six times higher for the 4.9 % ABV in German-style beer. In fact, if the 0% ABV American-style beer requires 132 PU for an effective pasteurisation, such heat treatment would result in greater than a 100 log reduction of *Lactobacillus brevis* cells. In the 0% ABV German-style beer, effective pasteurisation (52 PU) would reduce the bacterial cells by 70 log, in the 4.5% ABV American-style beer (7.2 PU) by 40 log and in the 4.9% ABV German-style beer by 37 log.

Table 4. D-values and z-values of *Saccharomyces cerevisiae* BRYC 501 in beer together with 95% Confidence Interval (95% CI), standard error (SE), R-squared and the calculated minimum pasteurisation units (min. PU) to achieve a 6-log reduction in ascospores

Beer	Temp (°C)	D (min)	95% CI	SE	R^2	z-value (°C)	SE	R^2	min. PU
0% ABV - American-style lager	60	21.9	(20.7, 23.5)	0.70	0.994	2.26	0.11	0.995	132
	62	3.73	(3.50, 4.00)	0.11	0.998				
	64	0.47	(0.43, 0.53)	0.02	0.967				
	65	0.13	(0.12, 0.14)	0.01	0.976				
0% ABV - German-style lager	60	8.66	(7.83, 9.69)	0.43	0.995	2.80	0.30	0.978	52
	62	2.24	(1.97, 2.60)	0.14	0.949				
	64	0.51	(0.50, 0.71)	0.05	0.965				
	65	0.12	(0.11, 0.13)	0.00	0.987				
4.5% ABV - American-style lager	58	5.37	(5.00, 5.80)	0.19	0.992	3.87	0.39	0.980	7.2
	60	1.20	(1.12, 1.30)	0.04	0.975				
	62	0.33	(0.24, 0.30)	0.01	0.978				
	64	0.16	(0.14, 0.17)	0.01	0.979				
4.9% ABV - German-style lager	58	2.43	(2.07, 2.94)	0.20	0.935	4.32	0.43	0.979	3.7
	60	0.62	(0.81, 1.26)	0.10	0.982				
	62	0.20	(0.17, 0.24)	0.01	0.940				
	64	0.10	(0.08, 0.14)	0.01	0.939				

Table 5. D_{60} of *Lactobacillus brevis* BSO 566 and *Lactobacillus lindneri* BSO 943 in beer

Microorganism	Beer	D (min)	95% CI	SE	R^2	6D (min)
Lactobacillus brevis BSO 566						
	0% ABV American-style	1.30	(1.18, 1.45)	0.06	0.945	7.82
	0% ABV German-style	0.74	(0.68, 0.80)	0.03	0.988	4.42
	4.5% ABV American-style	0.17	(0.15, 0.19)	0.01	0.962	1.01
	4.9% ABV German-style	0.10	(0.09, 0.12)	0.01	0.978	0.61
Lactobacillus lindneri BSO 943						
	0% ABV American-style	0.61	(0.55, 0.68)	0.03	0.958	3.66
	0% ABV German-style	0.45	(0.40, 0.52)	0.03	0.973	2.70
	4.5% ABV American-style	0.15	(0.14, 0.17)	0.01	0.974	0.90
	4.9% ABV German-style	0.08	(0.07, 0.09)	0.01	0.973	0.48

Discussion

This study showed that of the yeast strains tested, *Saccharomyces cerevisiae* BRYC 501 produced the most heat resistant ascospores. This strain is deposited in the ATCC as a *Saccharomyces carlsbergensis* (ATCC 9080), in the NCYC (NCYC 74) and the DSMZ (DSM 70424) as *Saccharomyces cerevisiae*. The yeast has been used in many applications (assay, compounds productions) but data on the heat resistance of this strain is limited to one report *(24)*. Further, although there is only one published study *(25)* which compares the heat resistance of ascospores from many different yeast species, other studies *(4,24,26–28)* show - as was found here - that the ascospores of *Saccharomyces* species are the most heat resistant. The study by Put and De Jong in 1976 *(25)* screened 120 yeast strains, finding that the genus *Saccharomyces* exhibited the highest heat resistance. They reported that *Saccharomyces cerevisiae* and *Saccharomyces chevalieri* were the most heat resistant species amongst all the yeasts investigated; only these two species survived heat treatment of 65°C for 10 minutes. Subsequently, a year later *(3)*, they reported D_{60} = 10 minutes and z = 5°C for ascospore suspensions of both these strains and in 1982 *(29)* they reported D_{60} values between 5.1–19.2 minutes for spore suspensions of a range of *Saccharomyces* species and D_{60} values between 20-40 minutes for spore suspensions of *Kluyveromyces bulgaricus*. Splittstoesser et al. *(2)* reported D_{60} = 6.1 minutes and z = 6.7 for *Saccharomyces cerevisiae* ascospores. Kilgour and Smith *(30)* reported D_{60} of two *Saccharomyces* spp. in non-alcoholic beer (D_{60} = 7.7–23 minutes; z = 3.9-4.1°C) and in 3.7% ABV beer (D_{60} = 1.7–2.9 minutes; z = 5.6-6.9 °C). Milani et al. *(24)* reported the highest D-values for ascospores of *Saccharomyces cerevisiae* strains with D_{60} values between 4.6 and 11.2 minutes and z-values between 11.7 and 14.3 °C. These values are significantly higher than the values reported for other (non-*Saccharomyces*) species. Garg *(26)* reported D_{55} = 15.33 minutes for ascospores of *Zygosaccharomyces bailii*, Raso et al. *(28)* reported D_{50} for *Zygosaccharomyces bailii* ascospores in different juices between 10.4 and 37 minutes.

The results obtained in this study are similar to those published in the literature. However, despite using the same strain as Milani et al. *(24)*, the ascospore heat resistance is significantly different in the two studies. According to Milani et al. for *Saccharomyces cerevisiae* BRYC 501 (ATCC 9080) in degassed and filtered beer, the D_{60} was 4.6 minutes and z = 12.4 °C. However, in this work, the values were much lower with D_{60} values for alcoholic beer between 0.62 and 1.2 minutes and z = 3.9-4.3°C. This may be explained by the different methodologies used for the preparation of ascospores (growth medium, washing), for the recovery of ascospores during the heat inactivation experiments (recovery agar, incubation temperature/time) and/or differences in the heat inactivation trials (pouches, capillary tubes, minimum number of timepoints, level of inactivation, pre-treatment of beer). Such differences may have contributed to changes in yeast physiology and associated heat sensitivity of the ascospores.

The D- and z-values reported in Table 4 suggest that the heat resistance of yeast ascospores in beer is not only significantly affected by the alcohol content, but that bitterness may possibly play a role too. Alternatively, other beer compounds that were not measured in this work could influence the survival of ascospores when heat treated. Whilst the effect of alcohol on the heat resistance of microorganisms is well documented *(2,24,31)*, the possible effect of bitterness has not, to our knowledge, been reported before. However, there is a report of high levels of hop acids resulting in lower yeast viability *(32)*.

The D-values for the American-style beer, which had a significantly lower bitterness than the German beer (over 4.5 times lower for the alcohol free beer and over 3.5 times lower for the alcoholic beer), exhibited more than double the D-values for the German-style beer. Accordingly, at similar alcohol concentration, adequate pasteurisation of the American-style beer would require double the pasteurisation units required by the German style beer.

This study also showed that the heat resistance of yeast ascospores was over 6-16 times greater than the heat resistance of *Lactobacillus brevis* and *Lactobacillus lindneri*. Indeed, of the two bacteria, *L. brevis* was the more heat resistant bacterium tested in this study. This strain was also the most heat resistant vegetative bacterium when tested in ale and stout in our previous study *(16)*. This suggests that when yeast ascospores are effectively inactivated during thermal processing, vegetative bacteria will also be inactivated at significantly greater numbers. A number of studies report that the heat resistance of vegetative bacteria is significantly lower than the heat resistance of yeast ascospores. Tsang et al. *(33)* reported D_{53} = 1.2 and 3.3 minutes and z-values of 15.39 and 11.17°C for *Lactobacillus frigidus* and *Pediococcus acidilactici* respectively in lager beer. L'Anthoën and Ingledew *(31)* showed that the heat resistance of spoilage bacteria was higher than the heat resistance of pathogenic bacteria (*Escherichia coli* and *Salmonella* Typhimurium) in alcohol-free beer but they also demonstrated that the heat resistance of bacteria was significantly lower than that of yeast ascospores. Other publications support this conclusion *(3,24,25,29)*.

The D_{60} values for the *Lactobacillus* strains tested in this study are in line with those published in other studies. Adams et al. *(34)* reported a D_{60} for *Lactobacillus* E93 between 0.31-2.56 minutes and z = 9.17-12.13 °C in alcoholic and non-alcoholic beers respectively. Reveron et al. *(35)* reported a D_{60} of 0.02 minutes and z = 6.49 for *Lactobacillus paracasei* in Pilsner beer and L'Anthoën and Ingledew *(31)* reported a D_{55} for *Lactobacillus delbrueckii* of 7.6 and 2.8 minutes in alcohol-free and 5% ABV beer respectively. However, there are a number of reports that the heat resistance of vegetative bacteria is much higher. Oliver-Daumen *(36)* in his thesis, reported D_{59} = 0.7 minutes, D_{69} = 0.5 minutes and z = 54.8°C for *Lactobacillus lindneri* in alcohol free beer. However, the z-value was calculated from only two temperature data points and the methodology employed for the heat inactivation experiments is a concern. Similarly, L'Anthoën and Ingledew *(31)* report extrapolated D_{60} for *Pediococcus acidilactici* of 7.66 and 1.33 minutes for alcohol free and 5% ABV beer respectively. But here the D_{60} values were extrapolated from the thermal death curve covering a temperature range between 47.0-53.3°C and 49.2-55°C and their reported D- and z-values are therefore debateable.

Conclusions

The ascospores of *Saccharomyces cerevisiae* BRYC 501 were the most thermoresistant spores of the yeast strains studied and were significantly more heat resistant than vegetative beer spoilage bacteria. Therefore, yeast ascospores can be a useful tool for validating pasteurisation processes when determining minimum pasteurisation requirements for different beverages (e.g., zero

alcohol beers) or for verifying whether a current pasteurisation regime achieves sufficient kill. This study has also generated a number of questions which have not been addressed before and can be explored in future investigations. For example, it is unknown if the heat resistance of ascospores is changing during storage, or if (and how) sporulation conditions could affect the heat resistance of ascospores. It is also unknown if detergents, disinfectants or any other chemicals which are commonly used in drink processing plants can impact on ascospores. Additionally, the bitterness of beer may have an impact on the viability or heat resistance of ascospores.

Author contributions

Grzegorz Rachon: Conceptualisation, methodology, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, visualisation, supervision, project administration.
Christopher P. Raleigh: Conceptualisation, validation, investigation.
Karin Pawlowsky: writing – review and editing.

Conflicts of Interest

The authors declare no conflict of interest.

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