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Optimisation of *Saccharomyces cerevisiae* BRYC 501 ascospore formation and recovery for heat inactivation experiments

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Abstract

Why was the work done: Ascospores from *Saccharomyces cerevisiae* BRYC 501 are useful biological indicators for validating the pasteurisation of beer. Ascospores are formed by yeast in response to low nutrient conditions and are characterised by greater resistance to stressors including desiccation, freezing and, during pasteurisation, high temperatures. Accordingly, the sporulation temperature of yeast ascospores, their shelf life, heat resistance and recovery need to be investigated.
How was the work done: The role of temperature in sporulation was investigated at 25, 27 and 30°C by measurement of the rate of sporulation over ten days. Shelf life of ascospores was established in deionised water at 1-5°C over 120 days by the measurement of heat resistance in McIlvaine's buffer. The recovery conditions of ascospores were determined using six agars after heat inactivation experiments in McIlvaine's buffer, alcohol free and lager beer.
What are the main findings: The sporulation rate of *S. cerevisiae* ascospores was highest at 27°C, and lowest at 30°C. Heat resistance of ascospores formed at 30°C was low with a D_{60} value of <3 minutes, whereas at 25 and 27°C the D_{60} was 8.8 and 9.2 minutes. The shelf life of ascospores was 60 days after an initial 14 days of maturation at both 25 and 27°C. The variation of heat resistance for ascospores grown at 25°C was shown to be lower than those grown at 27°C. Use of yeast extract-peptone-glucose agar to recover heat injured cells was > 200% higher than with the control agar.
Why is the work important: Sporulation should be performed at 25°C to produce the maximum number of spores for heat inactivation experiments. Spores can be maintained for 60 days, enabling several experiments to be performed. After heat inactivation experiments, ascospores should be recovered on YEPG agar for 10 days at 27°C

Keywords:
Pasteurisation, heat inactivation, yeast ascospores, beer, lager, alcohol free beer

Introduction

Yeast ascospores are a useful biological indicator for validation of pasteurisation processes of beer (Milani et al. 2015; Rachon et al. 2018, 2021, 2022). Ascospores are a specialised morphology in yeast cells formed in response to low nutrient environments and are a defining feature of the fungal phylum, *Ascomycota*. When vegetative, budding, diploid yeast cells are introduced into an environment with no assimilable nitrogen and a poor carbon source (e.g. acetate), yeast cells exit the mitotic cell cycle and begin meiosis, producing up to four haploid spores within the mother cell (Esposito and Klapholz 1981, Miller 1989). The spores are contained by collapsing the mother cell membrane to create an enveloping membrane or ascus (Coluccio and Neiman 2004; Zhang et al. 2017). The spore wall enables ascospores to be more resistant to factors that cause death in vegetative cells such as desiccation, freezing, starvation, ultraviolet radiation and high temperatures such as those experienced during the pasteurisation of beer (Miller 1989; Smits et al. 2001; Milani et al. 2015; Rachon et al. 2018, 2021, 2022).

Although methods for production of ascospores have been extensively studied (Miller 1989; Neiman 2005; Curran and Bugeja 2006), the practical details of the sporulation medium (Put et al. 1976; Put and de Jong 1982), oxygen requirement (Meier-Dörnberg et al. 2018), and temperature (Lin 1979) are suggested to be strain dependent (Bilinski et al. 1987). The application of different approaches affects the properties of the ascospore structure, germinative ability, and survival under environmental stressors such as high temperature (Suiker et al. 2021).

Ascospore yield is around 50-60% (Truong-Meyer et al.1996; Paulissen and Huang 2016), which restricts their industrial exploitation as bioindicators. When mixed populations of vegetative cells and ascospores are present, selective protocols are required to isolate ascospores. These include treatment with diethyl ether (Dawes and Hardie 1974) where vegetative cells are destroyed, or zymolyase enzymes (Sherman 2002; Bahalul et al. 2010) where the cell wall of vegetative cells and ascus are digested, destroying vegetative cells and freeing spores from their asci. However, both approaches can result in the carry over of vegetative cells (Chatterjee et al. 1982; Sirr et al. 2022).

A better approach is to use a yeast strain that produces a higher number of ascospores, for example *Saccharomyces cerevisiae* BRYC 501 (ATCC 9080, NCYC 74) which yields >94% ascospores (Rachon et al. 2021). This is higher than reported by Milani et al (2015) for this strain where 58% ascospores were used in heat inactivation experiments in beer (4% ABV). Rachon et al (2021) suggested the difference in the number of ascospores could be explained by different methods of preparation. Rachon et al (2021) also reported that ascospores from *S. cerevisiae* BRYC 501 were the most thermoresistant ascospores from the yeast (63 strains) and vegetative bacteria investigated. As such, *S. cerevisiae* BRYC 501 ascospores are an ideal bioindicator to investigate pasteurisation regimes in beer and alcohol-free beer.

The presence of yeast ascospores in the brewhouse has been attributed to sublethal cleaning, disinfection and exposure to heat (Suiker et al. 2023). If a pasteurisation process can kill yeast ascospores in a beer, it is reasonable to assume that vegetative yeast and bacteria are also killed. Accordingly, pasteurisation of beer or alcohol-free beer, validated with *S. cerevisiae* BRYC 501 ascospores, will enable the optimisation of process time and resources (water and energy).

Of particular interest is what temperature should be used to sporulate *S. cerevisiae.* Bacterial spores, like those of *Bacillus* species, are more heat resistant when grown at higher temperatures (Palop et al. 1998) and this characteristic is shared by ascospores from some fungal species when sporulated at higher temperatures (Conner and Beuchat 1987) over long periods (>10 days) (Put and de Jong 1982; Raso et al. 1998). The temperature used to produce yeast ascospores are strain dependent and span the range of 18-30°C (Lin 1979; Bilinski et al. 1987; Milani et al. 2015; Rachon et al. 2021). The most heat resistant yeast ascospores from *S. cerevisiae* BRYC 501 were produced at 25°C (Rachon et al. 2021), but other temperatures have yet to be explored.

Optimal storage for yeast ascospores is also strain

specific, although reports suggest storage in sterile distilled water at 1-5°C does not affect heat resistance for up to one month (Splittstoesser et al. 1986; Rachon et al. 2022). Alternatively, another report suggests the heat resistance of yeast ascospores will reduce when stored for any length of time (Put and de Jong 1982). To draw parallels from bacterial spores, *Bacillus* spores are typically matured for up to four weeks in sterile distilled water before use to harden or mature, increasing their resistance to disinfectants (British Standard 'Evaluation of sporicidal activity' EN 13704:2018). It has not yet been determined how long *S. cerevisiae* ascospores can be stored in sterile distilled water or if the heat resistance alters during storage.

The recovery conditions used for yeast ascospores after heat inactivation experiments also varies. Table 1 shows recovery conditions for yeast ascospores after heat inactivation experiments including recovery media, incubation time, temperature, the liquid matrix, and the D-value (time required at a specific temperature for a decimal reduction in viable microorganisms). Except for Milani et al (2015), these experiments have long incubation times to allow sub-lethally damaged ascospores the potentially to recover. Incubation temperatures are either 25 or 28°C, with EBC Analytica 4.2.2 ('general aerobic count on samples of yeast or fermenting beer'), recommending 27 ± 1°C.

Table 1.

Recovery conditions for yeast ascospores after heat inactivation experiments.

Media	Incubation Duration	Incubation Temp (°C)	Matrix	$D_{(°C)}$ - value		Reference
YPD agar	5 days	25	Alcohol free beer, pH 4.3	D_{52} –	4.0	Suiker et al. 2021
			Physiological saline, pH 6.5	D_{52} –	10.3	
YM agar	10 days	25	McIlvaine buffer, pH 4.0	D_{60} –	19.7	Rachon et al. 2021
			0% lager, pH 4.4	D_{60} –	21.9	
			4.5% ABV lager, pH 4.3	D_{60} –	5.4	
YEPG agar	2 days	28	4% ABV lager	D_{60} –	11.2	Milani et al. 2015
PD agar	5 days	25	Pineapple juice, pH 3.4	D_{50} –	37.0	Raso et al. 1998
MY40 broth	Up to one month	28	0.05M phosphate saline buffer, pH 4.5	D_{55} –	15.2	Garg et al. 1997
PD agar	5-10 days	25	Distilled water	D_{60} –	2.4	Splittstoesser et al. 1986
WLN agar	Unknown	Unknown	0% ABV beer	D_{60} –	7.7[1] 23.0[2]	Kilgour and Smith 1985 (two yeast strains, 1 and 2)
			3.7% ABV beer	D_{60} –	1.7[1] 2.9[2]	
Mycophil agar	10-14 days	25	0.5M phosphate citrate buffer, pH 4.5	D_{60} –	19.2[a] 22.2[b]	Put and de Jong 1982(a), 1982(b)
Plate count agar + 20% sucrose	7 days	25	0.1M PO_4 buffer + 48% sucrose	D_{65} –	2.0	Corry 1976
Mycophil agar	10-14 days	25	0.5M phosphate citrate buffer, pH 4.5	D_{60} –	16.8	Put et al. 1975

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Using *S. cerevisiae* BRYC 501, the aims of this study are (i) to investigate the impact of temperature (25, 27 and 30°C) on the rate of sporulation and heat resistance of ascospores, (ii) to determine the impact of sporulation temperature on storage, shelf life and heat resistance of ascospores and (iii) to investigate the ideal recovery conditions of ascospores after heat inactivation in three matrices - alcohol free beer, beer and McIlvaine's buffer. Optimising the formation and recovery of *S. cerevisiae* BRYC 501 ascospores will enable further research to utilise this biological indicator approach as a standard operating procedure, facilitating heat inactivation experiments.

Materials and methods

Yeast strain

S. cerevisiae BRYC 501 (ATCC 9080, NCYC 74) was stored in liquid nitrogen (Air Products, UK). Strain identity was confirmed by sequencing the D2 region of the large subunit ribosomal DNA (D2 LSU) (Arbefeville et al. 2017). Ten stocks were created by streaking *S. cerevisiae* BRYC 501 on Yeast and Mould agar (YM, CM0920 Oxoid, UK) for three days at 27°C and selecting colonies which were stored on cryobeads (TS/71-YE Technical Service Consultants Ltd) in a -80°C freezer (Hera Freeze 486).

Matrix for heat inactivation experiments

Heat inactivation experiments were performed in McIlvaine buffer at pH 4.0 ± 0.02. This was prepared (Rachon et al. 2021) by combining 7.71 mL of 0.2 M disodium phosphate with 12.29 mL of 0.1 M of citric acid. The buffer was stored at 1-5 ± 1°C for a maximum of one month. Experiments exploring recovery conditions were performed with McIlvaine buffer, alcohol free beer (<0.01% ABV, pH 4.4, bitterness of 14.2 IBU) and lager beer (4.0% ABV, pH 4.3, 10.7 IBU). The physiochemical properties of the two beers are reported in Table 2. Bitterness, as IBU (International Bitterness Units) was analysed according to EBC Analytica method 9.8 while ABV was determined using gas chromatography according to EBC Analytica method 9.3.2. A pH meter (AR15, Accumet Research, USA) was used to measure pH.

Table 2.

Beer composition.

Beer characteristics	Alcohol free beer	Lager beer
ABV (%) (Label)	0.0	4.0
ABV (%) (analysed)	<0.01	4.0
pH	4.4	4.3
Bitterness (IBU)	14.2	10.7
Carbohydrates (g/100mL)	4.3	1.2
Sugars (g/100mL)	2.5	3.2
Protein (g/100mL)	0.0	0.0

Impact of temperature on the sporulation rate of ascospores

Ten replicates of *S. cerevisiae* BRYC 501 were recovered from storage at -80°C and recovered in 5 mL of Yeast and Mould broth (YM broth, Formedium, UK) in 7 mL bijou bottles. The bijou bottles were incubated statically for two days at 25 ± 0.5°C when the yeast was in stationary phase and 200 µL was spread onto Ascospore Agar (HiMedia, India) and incubated aerobically for 10 days at 25, 27 and 30 ± 0.5°C. Plates were incubated lid up, producing a lawn of ascospores. Daily for ten days, ascospores at each temperature were cropped by flooding the agar surface with 10 mL of sterile deionised water, suspended with a L-shaped spreader, and decanted into sterile 50 mL centrifuge tubes. Spore crops were centrifuged at 3,000 x g for 15 minutes before discarding the supernatant and washing with sterile deionised water. Ascospores were stored at 1-5°C. Wet mounts were prepared with ascospore and vegetative cells and enumerated by phase microscopy on an Eclipse E200-F (Nikon Instruments Inc.) at 400 x magnification. Images were taken using the NIS-Elements software (Nikon Instruments Inc.). The sporulation rate at each timepoint was calculated as a percentage of ascospores to vegetative cells.

Heat resistance of ascospores

The heat resistance of *S. cerevisiae* ascospores was expressed as a D_{60}-value (the time required at 60°C for a decimal reduction in recoverable ascospores). D_{60} was calculated from the time required to decrease live spores from N_0 (the initial amount of ascospores) to N_x, where x is the time in minutes of heat inactivation.

As described by Rachon et al (2022), spore crops were removed from cold storage and 1.5 mL centrifuged at 3000 x g for 5 minutes in sterile 2 mL Eppendorf tubes. The supernatant was discarded, and the pellet resuspended in the test liquid and analysed by the capillary tube method. Here, soda glass capillary tubes (G119/02, Fisher Scientific, UK) were inoculated with 50 µL of test liquid inoculated with at least 1 x 10^7 CFU/mL yeast ascospores, heat sealed at both ends and placed in a water bath (Grant T100-ST5, UK). Heat inactivation experiments were performed at 60 ± 0.05°C, (confirmed using a reference thermometer - Fisher Scientific, Model 15-077-8). Every 10 minutes, three replicate, capillary tubes were removed from the water bath and cooled in ice water (unless otherwise stated). Immediately after cooling, the test liquid was washed from each tube with 450 µL quarter strength Ringer's solution (Oxoid, UK). Samples were serially diluted and used within 15 minutes. Viable cells were enumerated by spread plating 100 µL on YM agar and incubating aerobically for 10 days at 27 ± 0.5°C. The limit of detection was ≤ 100 CFU/mL. D-values, 95% Confidence Intervals (CI), standard error and coefficient of determination (R^2) were calculated using MiniTab 21 software.

Heat resistance of ascospores sporulated at different temperatures

Four crops of ascospores were sporulated at 25, 27 or 30 ± 0.5°C for 10 days, with each experiment in triplicate. This enabled the comparison of the heat resistance of yeast ascospores immediately after (T0) and two weeks after cropping (T14). Student T-tests to establish significant difference were performed on crops D_{60} values at T0 and T14.

Shelf life of ascospores

Three replicates of *S. cerevisiae* BRYC 501 were recovered and sporulated at 25, 27 and 30 ± 0.5°C before storage in sterile deionised water at 1-5°C for 120 days. At 0, 14, 30, 60, 90 and 120 days the heat resistance of yeast ascospores suspended in pH 4.0 McIlvaine's buffer was established on all three stocks in triplicate. The average D_{60}-value and standard error, 95% confidence interval were calculated in MiniTab 21.

Recovery conditions for ascospores after heat inactivation

To establish which nutrient agar was ideal for yeast growth after heat inactivation, six media were investigated for the recovery of viable cells. Ascospore recovery on Wallerstein Laboratory Nutrient agar (WLN, CM0309 Oxoid, UK), Malt Extract agar (MEA, CM0059 Oxoid, UK), Sabouraud Dextrose agar (SDA CM0041, Oxoid, UK), Potato Dextrose agar (PDA, NCM0018A Neogen, UK), and Yeast Extract Peptone Glucose agar (YEPG) were compared to the recovery of heat treated ascospores on Yeast and Mould agar (YM, CM0920 Oxoid, UK). The composition of these agars is reported in Table 3. Yeast cells were grown and cropped as described above. Serial dilutions and spread plating were carried out on all six agars after heat treatment in McIlvaine buffer (pH 4.0), alcohol free beer and lager. All experiments were performed in triplicate. Timing of heat inactivation was based on preliminary screening of each matrix (data not shown). All plates were incubated at 27 ± 0.5°C and quantification of colonies in relation to recovery of colonies on YM agar was determined after 3, 5, 7 and 10 days. Relative standard error was calculated using Microsoft Excel.

Table 3.

Culture media.

Components (g/L)	YM	WLN	MEA	SDA	PDA	YEPG
Yeast extract	3	4	-	-	-	5
Malt extract	3	-	30	-	-	-
Potato extract	-	-	-	-	4	-
Glucose	10	50	-	40	20	20
Peptone (Tryptone)	5	(5)	5	10	-	10
Agar	20	15	15	15	15	20
Other*	-	1	-	-	-	-
Final pH ± 0.2	6.2	5.5	5.4	5.6	5.6	6.4

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Results

Effect of temperature on the sporulation of ascospores

Sporulation was observed at all three temperatures with the average sporulation rate and standard deviation presented in Figure 1. No spores were detected at the time of inoculation, day zero or on day one. On day two, spores were detected at 25, 27 and 30°C with 40, 30 and 60% of ascospores to total cells. Ascospores grown at 27°C achieved a sporulation rate of 90% on day five. Sporulation at 90% at 25 and 30°C required eight and nine days. Polynomial (2nd order) trendlines were attributed to sporulation rate of *S. cerevisiae* BRYC 501 at 25 and 27°C, with a coefficient of determination (R^2 values) of >0.96, although the trendline for the sporulation rate at 30°C gave an R^2 of 0.85. The lowest sporulation at 92% was found at 30°C, however these spores had the highest rate before plateauing on the eighth day. The maximum sporulation was 98% with spores at 27°C, whilst these spores showed the slowest initial rate of sporulation. All morphologies of ascospores - single spores, dyads, triads and quatrads - were detected in all samples but were not enumerated individually.

Heat resistance of ascospores sporulated at 25, 27 and 30°C

Crops of *S. cerevisiae* BRYC 501 ascospores were grown at 25, 27 and 30°C and heat resistance determined in McIlvaine's buffer (pH 4.0) on cropping and after 14 days. Yeast sporulated at 30°C had a low heat resistance with average D_{60} of <3 min at both crop and after 14 days (data not shown). Initial data for ascospores cropped at 25 and 27°C showed variance in both the heat resistance at cropping and after 14 days. To establish an appropriate range, four separate measurements of three crops were established over a three week period. The D_{60} of crops were assessed in McIlvaine's buffer (pH 4.0) and the average D_{60} per crop and standard deviation were calculated (Table 4). The results showed yeast ascospores sporulated at 25°C gained heat resistance after 14 days with the exception of Trial C (Trial A: p = 0.006, Trial B: p = 0.013, Trial C: p = 0.159, Trial D p = 0.0002). When ascospores were sporulated at 27°C there was no significant difference in heat resistance after 14 days with the exception of Trial D (Trial A: p = 0.066, Trial B: p = 0.101, Trial C: p = 0.392, Trial D: p = 0.03).

Figure 1.

Effect of temperature on the sporulation of *S. cerevisiae* BRYC 501.

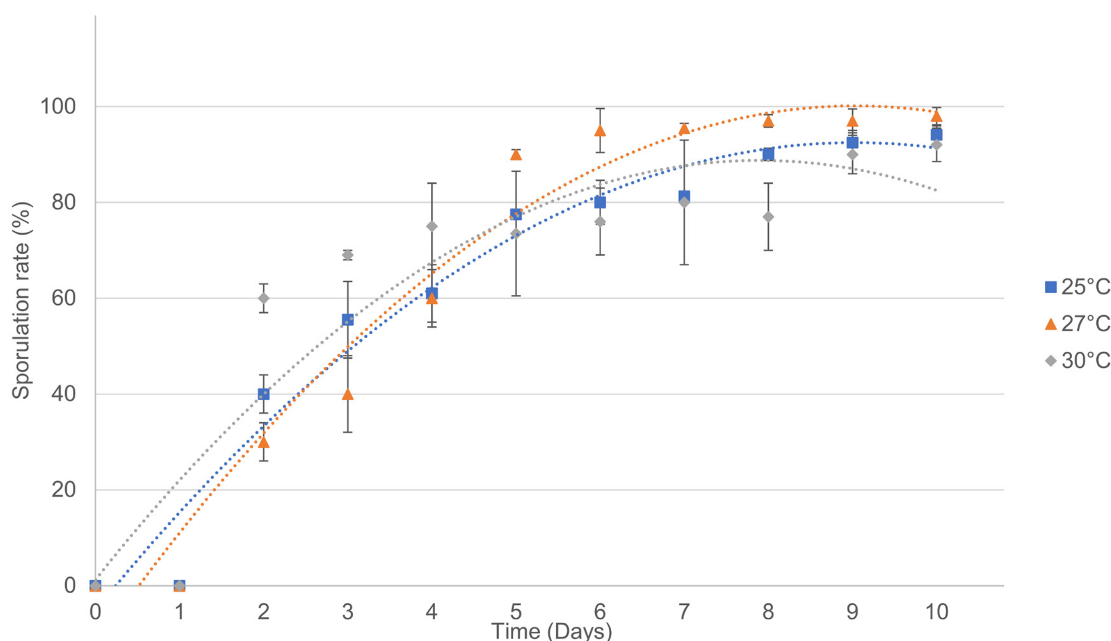


Table 4.

Effect of sporulation temperature of *S. cerevisiae* BRYC 501 ascospores on D_{60} in McIlvaine's buffer pH 4.0 at day 0 and 14.

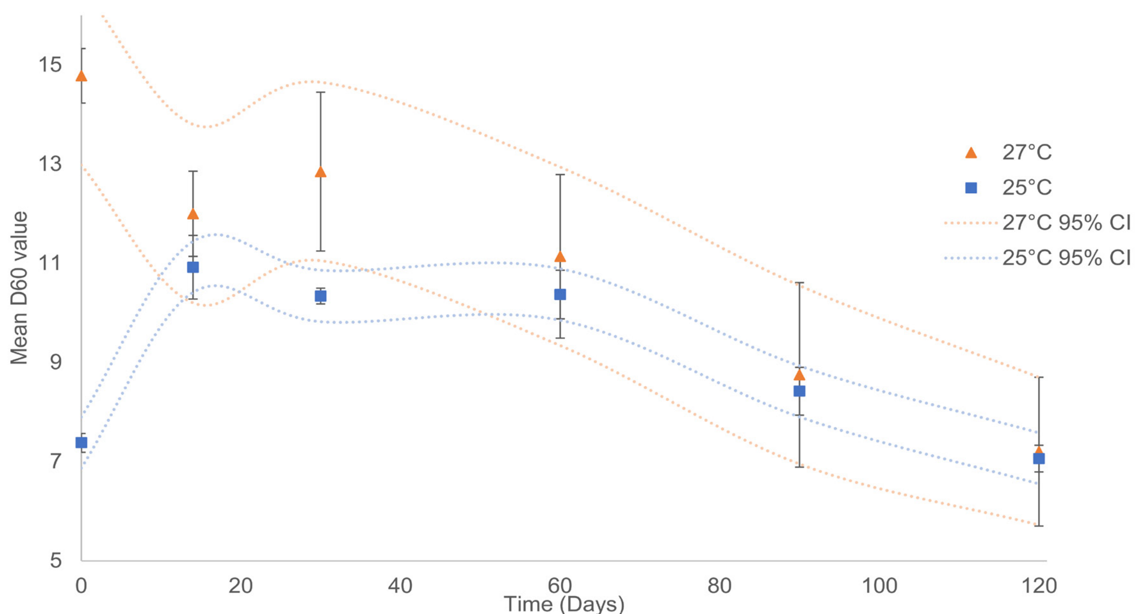
Each value is a mean and standard deviation of three experiments. Each experiment consisted of three replicates. *Indicates significant difference ($p<0.05$) between day 0 and day 14 D_{60} values.

Temp (°C)	Trial	D60 (min) Day 0	D60 (min) Day 14
25	A	5.3 ± 0.1*	7.8 ± 0.6*
	B	6.6 ± 0.4*	7.6 ± 0.1*
	C	8.8 ± 0.3	9.0 ± 0.3
	D	7.4 ± 0.1*	10.9 ± 0.4*
	Mean	**7.0 ± 1.5***	**8.8 ± 1.5***
27	A	6.6 ± 0.1	7.5 ± 0.6
	B	7.9 ± 0.5	8.2 ± 0.4
	C	9.0 ± 0.6	9.0 ± 0.4
	D	14.4 ± 0.3*	12.0 ± 0.9*
	Mean	**9.6 ± 3.6**	**9.2 ± 2.0**

Figure 2.

***S. cerevisiae* BRYC 501 ascospores sporulated at 25°C (blue square) and 27°C (orange triangle) D_{60} - values in McIlvaine buffer (pH 4.0) over 120 days whilst stored in sterile deionised water at 1-5°C.**

Each point is a mean of three individual parallel crops investigated in triplicate. Standard deviation is shown by error bars and 95% CI for both experiments are shown in dotted lines.



Shelf life of ascospores

To establish how long ascospores of *S. cerevisiae* BRYC 501 in sterile deionised water would retain heat resistance, one crop (Trial D) at both temperatures was assessed to establish shelf life. Ascospores were grown at 25, 27 and 30°C with heat inactivation experiments performed in McIlvaine buffer (pH 4.0) after storage in sterile deionised water for 0, 14, 30, 60, 90 and 120 days. The D60 values, standard deviation and 95% confidence intervals are plotted in Figure 2. Ascospores grown at 30°C were not investigated due to their low heat resistance. The heat resistance of spores grown at 25°C showed an increase in heat resistance over 14 days, while the heat resistance of spores grown at 27°C showed a decrease in heat resistance over 14 days. The ascospores grown at 25°C increased from a D_{60} of 7.4 to 10.9, while at 27°C spores decreased from D_{60} of 14.8 to 12.0. Both crops exhibited plateauing for D_{60} values from day 14 to 60. One-way ANOVA was used but no statistically significant change in heat

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resistance was found for either ascospore crop (25°C, days 14 to 60, p = 0.310; 27°C days 14 to 60, p = 0.395). After 60 days, both crops exhibited reduced heat resistance with a decrease in D_{60} after 90 and 120 days.

Recovery media for heat treated ascospores

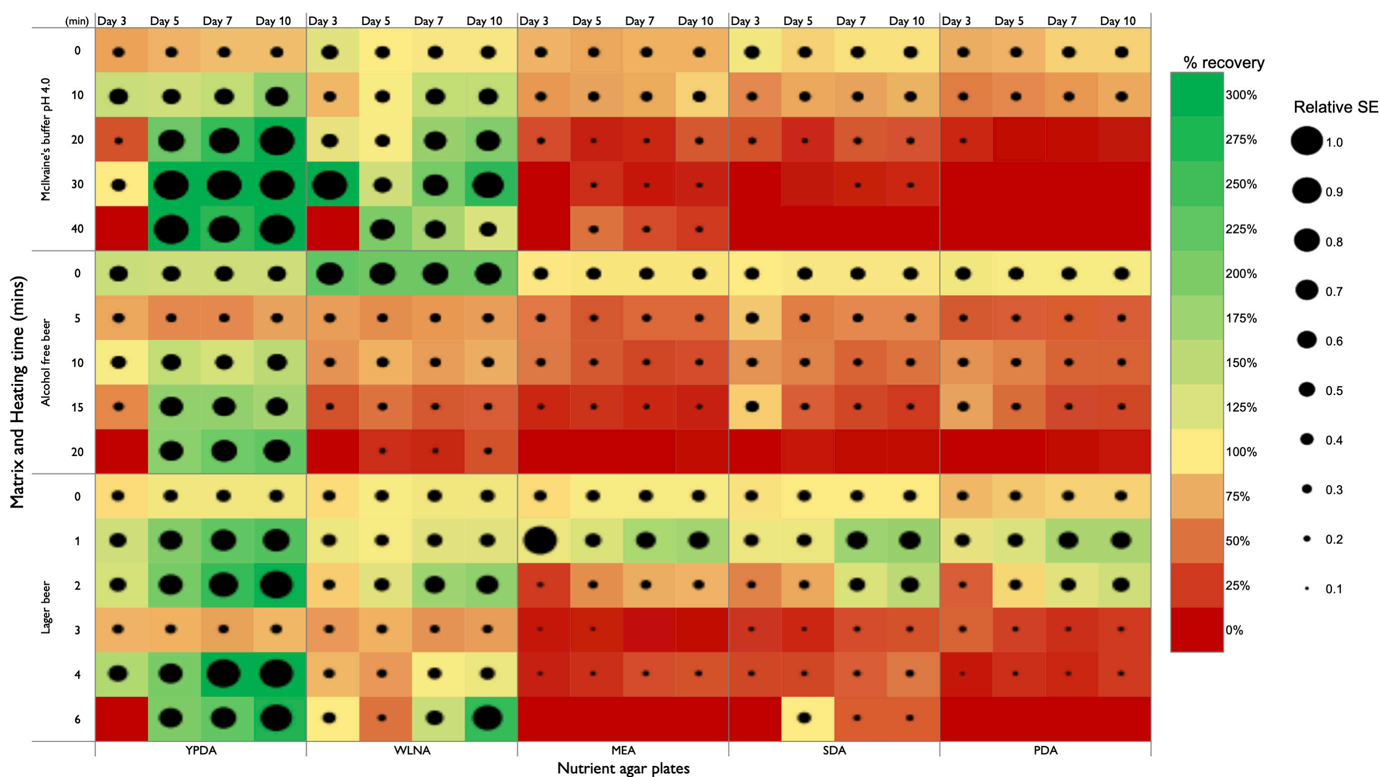
To investigate the optimal agar for recovery, five different media were assessed to recover heat treated and sub-lethally damaged ascospores. Recovery was measured at all heat treatment time points and after incubation of 3, 5, 7 and 10 days. The relative recoveries were compared to the recovery of heat-treated *S. cerevisiae* BRYC 501 ascospores on YM agar - previously used as the recovery media of choice (Rachon et al. 2018, 2021, 2022). The recovery of ascospores and relative standard error are reported in Figure 3. Recovery of ascospores was compared at each timepoint (including T0 samples) of a heat inactivation experiment in lager beer, alcohol free beer, and McIlvaine's buffer (pH 4.0).

Timepoints for heat inactivation differed between products as both hop bitter acids and alcohol content reduce the D-value of yeast ascospores (Rachon et al 2022). The data reported here shows that YEPG exhibited the greatest recovery of yeast ascospores in all three matrices with WLN second and YM the third best nutrient agar to recover yeast ascospores. The nutrient agars - MEA, SDA and PDA – delivered < 75% the recovery of YM. Generally, recovery of ascospores from YEPG in each matrix were >200% the recovery of YM. The T0 recovery of yeast cells on all agars was similar, with only YEPG and MEA recovering yeast cells from buffer on day 3 and 5 showing more than a 25% reduction. In buffer, YEPG showed less recovery of yeast ascospores than YM and WLN agar at T0 before heat treatments at all days of incubation. YEPG showed a better recovery of treated ascospore cells after ten minutes of heat treatment with 150% relative recovery to YM. However, heat resistance at 20, 30, and 40 minutes was > 215%, with incubation for 7- and 10 days showing > 250% recovery and some reaching 500%

Figure 3.

Heatmap of the recovery of *S. cerevisiae* BRYC 501 ascospores.

Recovery of ascospores on YM agar after incubation for 3, 5, 7 and 10 days at 27°C – green: high recovery; yellow: similar recovery; red: low recovery. The relative standard error is shown by the size of the black circle.



recovery when compared to YM. In alcohol free beer, YEPG again showed poor recovery at day 3 in most heating timepoints although T0 recovery was 140% that of YM. After incubation for 5, 7 and 10 days, YEPG showed recoveries of more than 130%, with the exception of the 5 minute heat treatment samples. In trials with lager beer, YEPG recovered heat treated ascospores at a higher percentage than the other agars, with recoveries typically (except the three-minute heat treatment) exceeding 130%.

Discussion

The sporulation of *S. cerevisiae* BRYC 501 ascospores at three different temperatures did not follow the trend of sporulation at a higher temperature resulting in greater heat resistance (Conner and Beuchat 1987; Bayne and Michener 1979; Melly et al. 2002). It is noted that spores were only grown for 10 days and that greater heat resistance may be achieved by continuing the incubation of ascospores. Spores grown at 30°C were extremely heat sensitive while spores grown at 25 and 27°C were similarly heat resistant. Growth at 30°C represents a higher temperature than used in previous work for the formation of ascospores (Table 1). This approach resulted in the quicker formation of yeast ascospores (over a one to two day period) and a greater variation in spore formation compared to 25°C and 27°C. Although, there are no reports on the effect of temperature on the sporulation of yeast ascospores, previous research on mould ascospore production showed higher variation during sporulation at higher temperatures (Damialis et al. 2015).

Although sporulation was most effective at 27°C, the spores grown at 25°C had a D_{60} - value in buffer (pH 4.0) with a lower mean standard deviation (8.8 ± 1.5 minutes) after 14 days storage (Table 4). The previously reported D_{60} values for *S. cerevisiae* BRYC 501 are considerably higher (19.7 minutes) (Rachon et al. 2021). However, this value is less precise having been calculated from the inverse slope of three timepoints (0, 1 and 10 minutes) rather the five timepoints used here (0,10, 20, 30, and 40 minute). Three timepoints is prone to overfitting in a linear model, while five timepoints are more robust. Indeed, calculating the slope for recovered spores from YM and YEPG for 0 and 10 minutes timepoints results in D_{60} values of 16.7 and 44.3.

In this work, the heat resistance of mixed morphologies of spores was investigated and all inactivation curves were linear (data not shown). In the future, it would be useful to investigate the heat resistance of single spores, dyads, triads and quatrads to determine which ascospore morphology is the most heat resistant. This approach would require flow cytometry which may not be an easy or practical solution.

Figure 2 suggests that (i) *S. cerevisiae* BRYC 501 ascospores require a 14 day period in sterile deionised water such that heat resistance stabilises as the spores mature, and (ii) ascospores have a shelf life of up to 45 days after maturation. This poses the question as to what changes occur during storage that allow spores to achieve heat resistance for a month. Although there are no reports for yeast, there are insights from studies with mould ascospores of *Byssochlamy* and *Aspergillus/ Neosartorya* (Bayne and Michener 1979; Beuchat 1986) and bacterial spores of *Bacillus* species (Ghosh et al. 2009).

A defining feature of wet heat resistance is the core water content of spores under heat stress (Melly et al 2002), with both heat resistant mould and bacterial spores having a low (<30%) core water content (Ghosh et al. 2009; Sanchez-Salas et al. 2011). These ascospores, like those previously reported (Snider and Miller 1966), have hydrophobic cell walls composed of mannan and β-glucan (found in vegetative yeast) as well as additional chitosan and tyrosine (specific to spores) (Coluccio et al. 2008), which prevent movement of water in or out of the cell. Zhang et al (2017) reporteds that maturation of the ascus in baker's yeast (*S. cerevisiae*) is responsible for the lower permeability to some dyes. More data is required, including the size of spores at cropping compared to those after 14 days. However, it is new insight that *S. cerevisiae* BRYC 501 ascospores do not lose heat resistance when tested in buffer after 60 days (Figure 2). Splittstoesser et al (1986) suggest – without data – that ascospore crops can be kept for up to nine months without major changes in heat resistance. The data in Figure 2 would not support this suggestion.

Understanding ascospore shelf life will allow more flexibility, ease of preparation, and consistency as using the same crop would enables the comparison

of heat resistance between products. Generally, investigations are concerned with how culture conditions could affect the viability of spores used immediately (Šajbidor et al 1992; Agosin et al 1997). It is possible that the shelf life of *S. cerevisiae* BRYC 501 ascospores could be improved by anaerobic storage under a protective atmosphere (Barnette et al. 1996).

Milani et al (2015) investigated the recovery of *S. cerevisiae* BRYC 501 ascospores on YEPG agar after heat treatment. However, in this work, the ascospores from *S. cerevisiae* were removed from their ascus (Curran and Bugeja 2006) - an approach not used in this work. This approach results in the isolation of homogeneous spores and reduces the inherent stochastic variation in ascospore characteristics from being single, double, triple or quatrad ascospores. The data presented in Table 4 suggests this variation is not significant when measuring ascospore heat resistance. The ascospores used by Milani et al (2015) were recovered from beer after two days at 28°C. The data in Figure 3 shows that two days recovery is too little for heat damaged yeast ascospores to recover and produce colony forming units. Although the recovery is higher at day three than that of YM, the recovery on day five is almost double that after three days.

A higher recovery of ascospores would affect the D-value. A comparison of the three best performing agars (Table 3) shows that YEPG, YM and WLN all contain yeast extract while PDA, SDA and MEA do not. The use of yeast extract supplies amino acids and vitamins to recovering microorganisms. WLN agar was shown to be the third best performing agar behind YEPG and YM. In buffer and in beer, recovery of yeast ascospores on WLN showed less recovery than YEPG but more than YM. However, yeast ascospores recovered in alcohol free beer on WLN showed relatively poor recovery, similar to recovery recorded from PDA, SDA and MEA. Both YEPG and YM have a pH of >6, which may contribute to the recovery of yeast ascospores. Recent research (Plante et al. 2023) shows that as dormant yeast ascospores begin to germinate in the presence of nutrient rich media, with the cytosolic pH increasing from 5.9 to 7.3. This is accompanied by a decrease in the viscosity of the cytosol and an increase in protein solubility. Proteins, especially heat shock proteins, have been shown to assist recovering cells to return to normal growth (Yoo et al. 2022). Further investigations using YEPG agar at different pH levels may provide a better environment for the recovery of sub-lethally heat damaged yeast ascospores.

Conclusions

The data presented here shows that ascospores from *S. cerevisiae* BRYC 501 (ATCC 9080, NCYC 74) are best sporulated at 25°C. Sporulation at 27 or 30°C resulted in spores with greater variation in heat resistance. Furthermore, ascospores grown at 25°C require at least 14 days of storage in sterile deionised water before use. The significant increase in D_{60}- value is notable as these cells can be used for validation of the 'worst case scenario' when assessing heat treatment. *S. cerevisiae* BRYC 501 ascospores grown at 25 and 27°C can be stored in sterile distilled water at 1-5°C for up to 60 days, after an initial 14 day period, without significant change in heat resistance. *S. cerevisiae* ascospores exhibited the best recovery on YEPG at 27°C after 10 days following heat treatment experiments in lager beer, alcohol free beer, and buffer. Therefore, it is suggested that YEPG should be the agar of choice for the recovery of yeast cells after heat injury. This insight may be useful in other work on stress response but will require validation.

Author contributions

Christopher Raleigh: conceptualisation, methodology, validation, formal analysis, investigation, resources, data curation, writing (original draft, review and editing), visualisation, project administration and funding acquisition.
Stephen Lawrence: writing (review and editing), supervision.
Grzegorz Rachon: writing (review and editing), conceptualisation, methodology, supervision.

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Conflict of interest

The authors declare there are no conflicts of interest.

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