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Utilisation of Defined Media Towards Evaluating Brewing Ale Yeast Fermentation in Small Scale Batches †

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Abstract: The utilisation of high gravity wort possessing a higher sugar content is frequently used to increase the capacity and efficiency of beer production. In such an environment, osmotic stress, lower nutrient availability, ethanol toxicity and elevated CO_2 concentrations may decrease the sustainability of yeast performance and have a negative impact on the desired end product. Traditionally, apparent attenuation has been used as a standard measure of fermentability. Here, we describe a miniature fermentation assay with a defined fermentation media that could be used to assess fermentability on a small scale, including determination of the original gravity and final gravity of the medium for trials using four yeast strains. The results obtained using the defined media were approximately comparable to those obtained using regular wort in terms of their real attenuation limits and specific gravity, although the defined media showed lower final pH values compared to the wort for several strains. With further optimisation, the mini fermentation process applied to a defined medium could provide the foundation for future analytical research into the brewing process, using techniques such as high-performance liquid chromatography (HPLC) and quantitative polymerase chain reaction (qPCR).

Keywords: *Saccharomyces cerevisiae*; beer; mini fermentation assay; specific gravity

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1. Introduction

Production of ethanol by the yeast is a process of global importance and such processes, productivities and yields are pushed to their maximum possible values leading to cellular stress [1]. In the brewing process, the enzymatic activities that occur through the mashing steps [2] and during carbohydrate degradation [3] are well understood. However, the understanding of sugar utilisation by yeast remains largely based on interpreting observations from different brewing batches [4]. With the advancement of contemporary high end analytical instruments comes the opportunity to gain a quantitative insight into factors affecting the fermentation process, impacts of the brewing medium used, and the performance of difference yeast strains [5].

In most instances, the extent of fermentation is measured using several simple techniques, most common being specific gravity [6], but sometimes through the measurement of pH [7], CO_2 evolution [8] or ethanol concentration [9]. An important derived parameter is the Apparent Attenuation Limit (AAL), calculated as: 100 × (OG – FG) / (OG – 1), where OG = original specific gravity and FG = final apparent specific gravity, and expressed as ºP or ºexcess Gravity [10]. Most importantly, the AAL predicts the percentage of wort (malt sugar) that is converted by the yeast strain to CO_2 and ethanol. As the AAL over-

estimates the proportion of converted sugars due to the lower density of ethanol compared to water, a derivative known as the Real Attenuation Limit (RAL) is commonly used; being calculated as (OG – FG) / (OG - 1) × 81.92 [11].

The brewing process can be described as a batch process where the ingredients are added step by step [12]. Briefly, these include the wort – containing sugars and nutrients – and hops, with the fermentation stage expected to take up to 4 to 20 days. Fermentable sugars that form the main part of wort are particularly maltose and maltotriose. However, non-fermentable sugars, largely branched dextrins, may comprise up to 20 – 50 % of the sugars present, depending on the malt characteristics and mashing processes [13]. Other key nutrients present in the wort are amino acids, peptides, proteins, lipids, vitamins, metal and non-metal ions [14,15]. In the first step of fermentation, glucose and fructose enter the yeast cells through facilitated diffusion, via HXT transporters [16], and are metabolised. The hydrolysis of sucrose into glucose and fructose often occurs outside the cell, but may also occur in the cellular environment, catalysed by β-fructosidase. As high glucose levels (>10 g L^{-1}) suppresses the active process of maltose uptake via the down-regulation of the MALR (maltose regulatory gene) and MALT (maltose transport gene) [17], maltose is only utilised once glucose levels have dropped. Maltotriose is the last fermentable sugar to be utilised [18].

Environmental factors that affect yeast growth rates include ethanol toxicity, pH, water quality, osmotic stress, oxidative stress, and shear stress [14]. Different yeast strains may vary in their susceptibility to these factors. Many of these stressors can be induced, in particular, during high-gravity brewing, where higher levels of carbohydrates are added to the wort in view of producing elevated final ethanol concentrations [19]. Whereas traditional brewing techniques utilise worts comprising between 11 - 12% dissolved solids, high-gravity brewing increases the dissolved solid content to between 16 - 18% [20]. The higher alcohol concentrations achieved after fermentation are then diluted later in the process to achieve the desired alcohol content of the finished product [21]. High-gravity brewing processes are frequently used by many brewing operations to increase brewing capacity (i.e. ethanol production) as well as to reduce labour and material costs [19]. Furthermore, the finished product benefits from improved flavour due to increased production of aroma-active compounds [22], and increased smoothness and greater haze stability due to the more complete consumption of hydrophobic polypeptides during the kettle boil and fermentation stages [23].

The final composition of the wort is derived through a complex series of biochemical interactions between the constituents of the malt, the chemical composition of the mash water, and the physical conditions that are imposed upon the mash (e.g. pH, temperature, time). With respect to the barley used for the malt, the important variables that needs to be considered include the barley variety [24], endosperm composition [25], hordenine levels which in turn affect the activity of α- and β-amylase [26], β-glycan concentration [27], husk retention [2], endosperm wall texture [28], softness of the grain, enzyme levels and colour [29]. Hence the arduous task of obtaining a consistent repeatable model of fermentation is required to commence investigating the degree to which various yeast strains cope with the mentioned environmental stressors of the brewing process [30].

Previous work found that lager yeast ferments were initially faster in a defined medium as compared to a semi-defined medium, but reached the fermentation end-point at a similar time to the defined medium [31]. This is likely due to the enhanced initial availability of nutrients for the yeast compared to the more complex nature of sugars in the semi-defined medium, which must first be broken down into simple sugars prior to fermentation. Other researchers found that the resultant brew from defined media also has a similar final gravity compared to a regular wort medium [30]. Conversely, ale yeasts have been observed to ferment slower in a defined medium, possibly due to a lack of free amino nitrogen (FAN) resulted in incomplete utilisation of maltose, with a slightly higher final gravity [30]. However, it is important to note that the relationship between

fermentation speed and defined media may vary, depending on the exact composition of the defined media.

In contrast to defined media, semi-defined media are where the concentrations of most but not all components are known. This is a cheaper alternative to fully defined media and has been successfully used in large scale trials [30]. Adjustment of the pH and addition of a buffer such as citric acid is commonly required when using semi-defined media such as those that are produced from sugar molasses and yeast extracts [14]. The main drawback to using a semi-defined medium is that some biological heterogeneity may remain, as a result of minor variations in the naturally-derived material.

However, the cost of producing fully defined media remains prohibitive for large-scale fermentation trials. Hence the development and validation of mini fermentation assays, which best mimics the condition found in full-scale fermentation, is essential to advance the current state of research. Relatively few studies have investigated the fermentation performance of yeasts in a small-scale fermentation setting [32].

A miniature fermentation assay designed by Lake et al., (2008) to mimic the commercial brewing process has been previously used for fermentation experiments which has even been adopted by the American Society for Brewing Chemists (ASBC) (method ASBC Yeast-14) [33]. Furthermore, it is worth noting that other researchers have used mini fermentation assays to model the effects of gravity in brewing with success [19,34].

In this study, the primary aim was to develop a defined medium and miniature fermentation assay that mimicked the performance of a full-size fermenter and use this to assess the performance of four yeast strains under high-gravity conditions. The development and validation of a defined medium for brewing purposes should assist future researchers seeking to apply high-end analytical instrumentation to study the biochemical changes throughout the brewing process.

2. Methods

Three industrial ale yeast strains (*Saccharomyces cerevisiae*) were sourced from Wyeast (Kolsh 2565, French Saison 3711, Belgian Abbey 1214). For comparative purposes, a lager yeast (*Saccharomyces pastorianus*; California Lager 2112) was also used in the initial wort fermentation trials. Strains were cultured on malt agar plates (Oxoid brand) with a pH of 5.4 and stored at 21 ºC, with subculturing to fresh malt agar performed every seven days. To minimise the risk of selective pressure leading to chromosomal rearrangements, single nucleotide polymorphisms (SNPs) or development of respiratory deficiencies, future researchers could keep yeast strains as glycerol stocks at -80°C rather than at 25°C.

The pitching rate was optimised for each yeast strain by determining their growth rate in malt extract broth (Difco) over an 84-hour period. Malt extract agar, malt extract broth and saline tablets were sourced from Oxoid. Yeast nitrogen base with amino acids was sourced from Difco. All other reagents were sourced from Sigma-Aldrich Australia.

Fermentation vessels were constructed from 50 mL plastic freestanding centrifuge tubes, with a Pasteur pipette inserted through a rubber stopper to provide an outlet for CO_2 backpressure. Excess CO_2 was vented through a silicon tubing via this outlet, with a sterile air lock preventing air from entering the vessel (Figure 1).

The composition of the fully defined medium that was used for the fermentation trials is shown in Table 1. The composition was adapted from a previously established protocol by Taidi, *et al.* [31], including the addition of a buffer, as preliminary trials indicated that un-buffered medium resulted in a significant reduction in the pH level and an associated stalling of fermentation rates. The fully defined medium comprised of several carbohydrates at concentrations previously determined in wort [35], a phosphate buffer, and a commercial yeast nitrogen base (Difco) of known composition. The maltose (Sigma grade II maltose) selected for this study consists of up to 5 % maltotriose, an amount considered appropriate to use in simulating a model of a wort [31]. The density of the final solution was 1.045 g cm^{-3} (measured using an Anton Paar 34N density meter), while the

pH was 6.5 (Cyberscan pH 11 meter). Following standard brewing practices, the samples were not filtered before measuring their density.

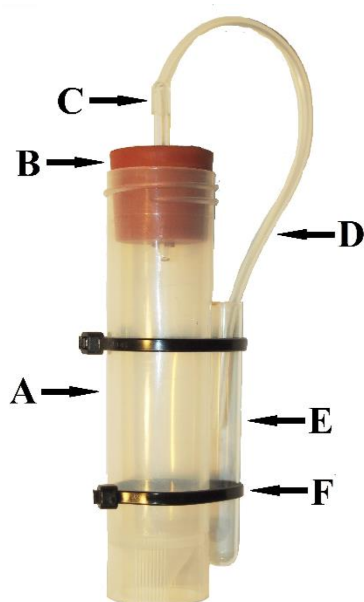

Figure 1. The mini-fermentation vessel used in this study. A: 50 mL centrifuge tube (Starstedt, part no. 62.559), B: stopper (Westlab, part no. 071919-0008), C: pasteur pipette (Sarstedt, part no. 86.1172), D: silicon tubing (Kartell, part no. 3921), E: mini glass test tube (Corning, part no. 99445-12) and F: nylon tie (Jaycar, part no. HP1246).

Table 1. The composition of the fully defined medium used for fermentation trials.

Component	Concentration	Concentration used by Taidi, *et al.* [31]	Typical range in natural wort
Carbohydrates			
Glucose (g L^{-1})	12	10.4	0.47 - 6.48
Fructose (g L^{-1})	8	4.6	ND
Sucrose (g L^{-1})	0.4	3.5	ND
Maltose (g L^{-1})	115	115.5	34.74 - 51.69
Maltotriose (g L^{-1})	5.75	Not specified*	9.13 - 13.87
Dextrin (g L^{-1})	0	0	18.03 - 39.85
Buffer			
Potassium phosphate dibasic (K_2HPO_4) (g L^{-1})	2 g L^{-1}	0 (used 0.625 g L^{-1} citric acid & 0.215 g L^{-1} $CaSO_4{\cdot}2H_2O$)	ND
pH	6.2	5.2	5.2-5.4
Yeast Nitrogen Base			
Ammonium sulphate (g L^{-1})	5	0.1307	ND
Monopotassium phosphate (g L^{-1})	1	0	ND
Magnesium sulphate (g L^{-1})	0.5	0	ND
Sodium chloride (g L^{-1})	0.1	0	ND
Calcium chloride (g L^{-1})	0.1	0	ND
Boric acid (µg L^{-1})	400	0	ND
Manganese sulphate (µg L^{-1})	400	0	ND
Zinc sulphate (µg L^{-1})	400	0	ND
Ferric chloride (µg L^{-1})	200	0	ND
Sodium molybdate (µg L^{-1})	200	0	ND
Potassium iodide (µg L^{-1})	100	0	ND
Copper sulphate (µg L^{-1})	40	0	ND
L-histidine monohydrochloride (mg L^{-1})	10	0	ND
L-D-methionine (mg L^{-1})	20	0	ND
L-D-tryptophan (mg L^{-1})	2	0	ND
Inositol (mg L^{-1})	0.5	0	18-60
Niacin (mg L^{-1})	0.4	0	8-41.36
Pyridoxine HCl (µg L^{-1})	400	0	580-1005
Thiamine HCl (µg L^{-1})	400	0	280-600
Calcium pantothenate (µg L^{-1})	400	0	450-980
Riboflavin (µg L^{-1})	400	0	400-930
p-aminobenzoic acid (µg L^{-1})	200	0	19-96
Folic acid (µg L^{-1})	2	0	100-130
Biotin (µg L^{-1})	2	0	0.85-1.15

References: [36-41].
*The grade of maltose used in the original synthetic wort contained up to 7% maltotriose [31]
ND = no data

For comparative purposes, wort was created from Pilsner malt (Joe White Maltings) using a modified version of the European Brewery Convention (EBC) section 4 malt method 4.6 [42]. The ground malt was processed in a malt bath (Honeyman Automated Solutions) at 65 ºC for 1 hour. After filtering, the density of the filtrate solution was

adjusted to 1.050 g cm^{-3} with deionised water before going through a cold break for 12 hours. This was followed by autoclaving (121 ºC; 20 mins) and centrifuging the filtrate (3300 rpm; 12 ºC; 15 mins) to remove the trub.

Miniature fermentation assays were conducted in autoclaved mini fermenters, each pitched with 36.7 mL of either the wort or synthetic medium, and 400 µL of the respective yeast inoculum. Cell number counts were performed to ensure the initial biomass of yeast inoculum were comparable between different trials and yeast strains. After capping, the airlocks were filled with 1 mL of 50 % ethanol before incubating the mini fermentation vessels at 21 ºC. The airlock was refilled with 50 % ethanol when necessary due to evaporation. Throughout the fermentation trials, the density, pH and temperature were recorded every 24 hours for 9 days, with results recorded from biological triplicates at each time point. It should be noted that after each sampling point, the mini fermenter vessels from which sampling occurred were then discarded, with the remaining replicate vessels used to obtain the data points at further subsequent time points. Whilst the temperature and density were measured using a portable density meter (Anton Paar DMA 35), pH was measured using a pH meter for each sample. To ensure matrix homogeneity, the samples were agitated by hand before measuring their density. As anticipated, flocculation of the yeast cells was observed towards the end of the fermentation trials [43], resulting in a marked change in the opacity of the media. Data were analysed using Microsoft Excel, while graphs were drawn in GraphPad Prism 6.

3. Results

The validation trials using a regular wort fermentation displayed the expected changes in gravity and pH for a full-scale fermentation across the yeast strains. However, there was some difference in the fermentation efficacy between the different strains, as shown in Figure 2 (coloured lines represent different yeast strains). Notably, strain 1214 appeared to be a more efficient fermenter, as its density fell at a rate much faster compared to the other strains (Figure 2), indicating that the fermentation of this strain had reached completion after only ~115 hours, compared to ~200 hours for the other yeast strains. The lager yeast strain included for comparison (California Lager 2112) appeared to be a less efficient fermenter, as its final density was notably higher compared to the ale yeast strains (Figure 2).

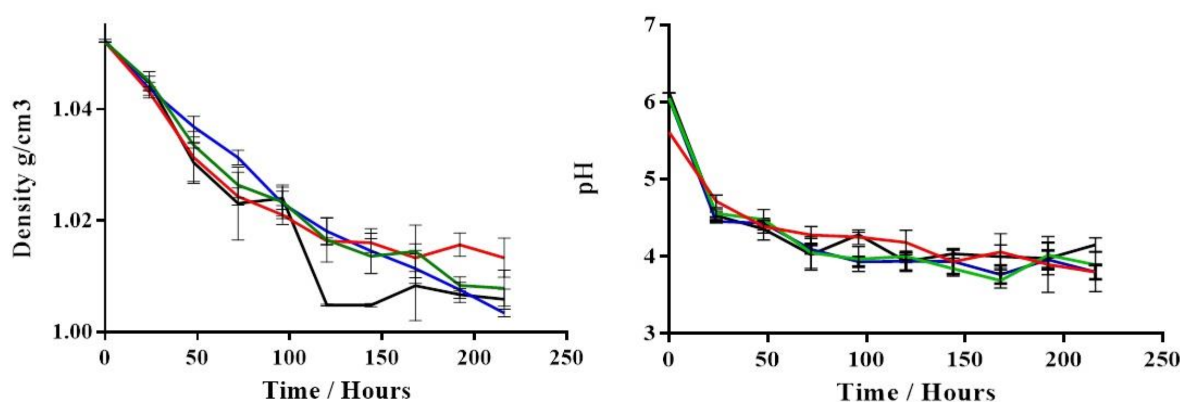

Figure 2. Change in density and pH throughout the fermentation trials using a wort medium. Colours correspond to the yeast strains: green - 2565, blue - 3711, black – 1214. For comparison, the red line shows results from a lager yeast (California Lager 2112). Error bars show standard deviation from independent biological replicates (n=3 for each).

Overall, the change in density (Figure 3) and pH (Figure 4) was somewhat comparable between the defined medium trials and the control trials (using regular wort), indicating relatively similar fermentation conditions between the synthetic medium and regular wort medium. One exception to note is that strain 2565 (Kolsh) showed the final density

of the defined media fermentation to be higher than that of the wort, indicating that less sugars had been fermented by the yeast (mean RAL of 47 %, compared to an RAL of 58 % for the wort fermentation; Table 2). However, the change in pH for this strain (2565) followed a similar trend to that observed during the wort fermentations (Figure 4). For the remaining three strains, the change in pH was similar between the defined media and regular wort for the first 40 hours of fermentation (Figure 4). After this point, the pH continued decreasing in the defined media fermentations, levelling off approximately 70 hours after the fermentation process began, to reach a final pH value of approximately 2.5-3. In contrast, the pH of the wort fermentations began to equilibrate earlier and at a higher pH (approximately 4). This may be indicative of exhaustion of the buffer capacity in the defined media, similar to that previously reported by Taidi, *et al.* [31] in their work with defined media. This suggests that future work optimising the amount of buffer agent in synthetic media is necessary for consistency and optimum performance of this system.

Table 2. Mean apparent attenuation limit (AAL) and real attenuation limit (RAL) following the fermentation of the defined media and regular wort samples. T-test rows show the results of a heteroscedastic t-test between the wort and defined media. Values show mean ± one standard deviation.

Yeast strain	Media	AAL (%)	RAL (%)
2565 Kolsh	Wort	81±7	58±7
	Defined	58±7	47±3
	T-test	NS	NS
3711 French Saison	Wort	98±7	80±5
	Defined	98±6	80±6
	T-test	NS	NS
1214 Belgian Abbey	Wort	92±2	76±2
	Defined	107±3	88±2
	T-test	*	*

* P<0.05; NS – not significant

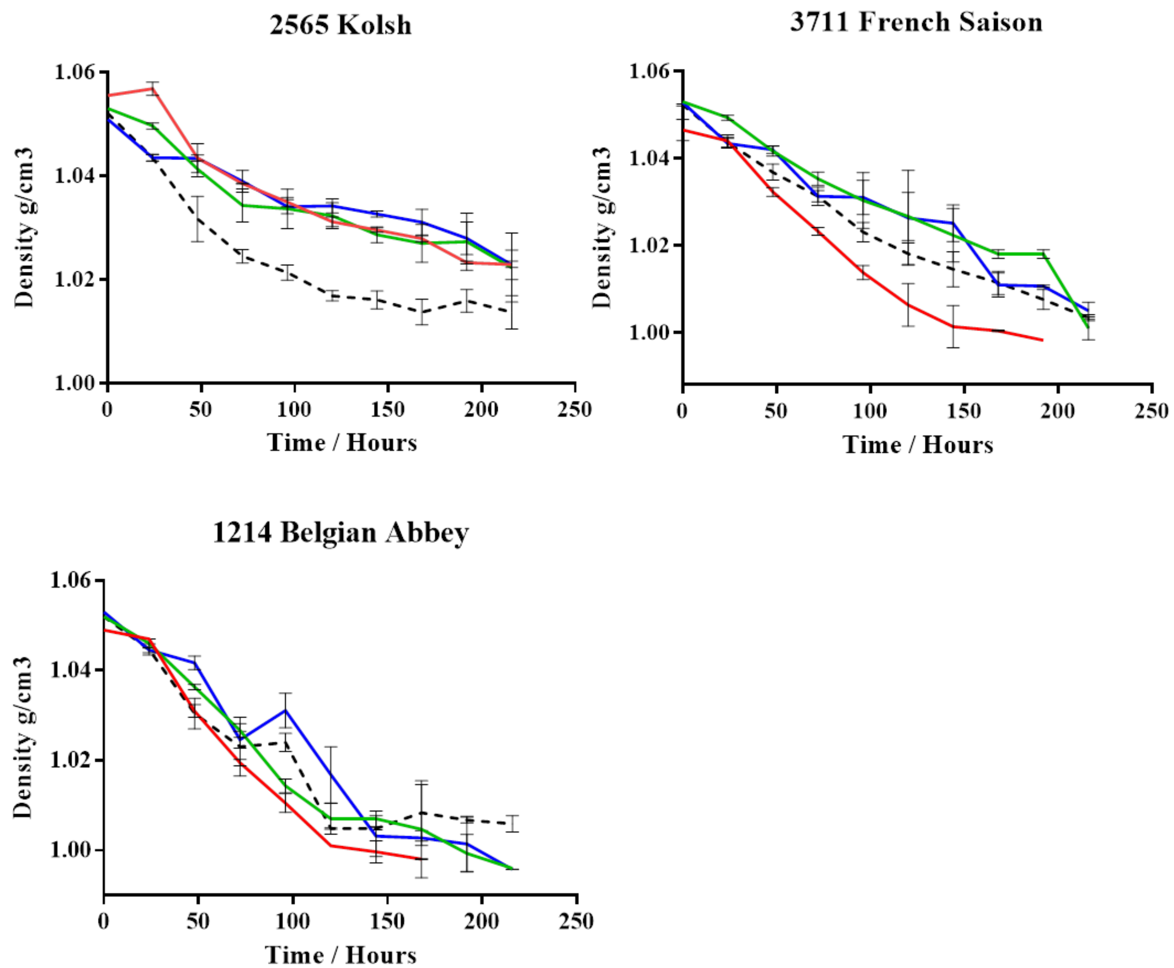

Figure 3. Changes in density throughout the defined medium trials. The coloured lines indicate results from independent trials conducted with the same yeast strain. The black dashed line indicates results from the regular wort fermentation trial. Error bars show results from biological replicates (n=3 for each).

Paired-samples t-tests showed no significant difference (P>0.05) in the AAL or RAL at the final timepoint between the wort and synthetic media for two of the strains, but the AAL and RAL were significantly higher in the defined media for the Belgian Abbey strain (Table 2). The values for RAL were identical for the French Saison strain, indicating that the extent of fermentation was very similar between the synthetic media and wort for this strain.

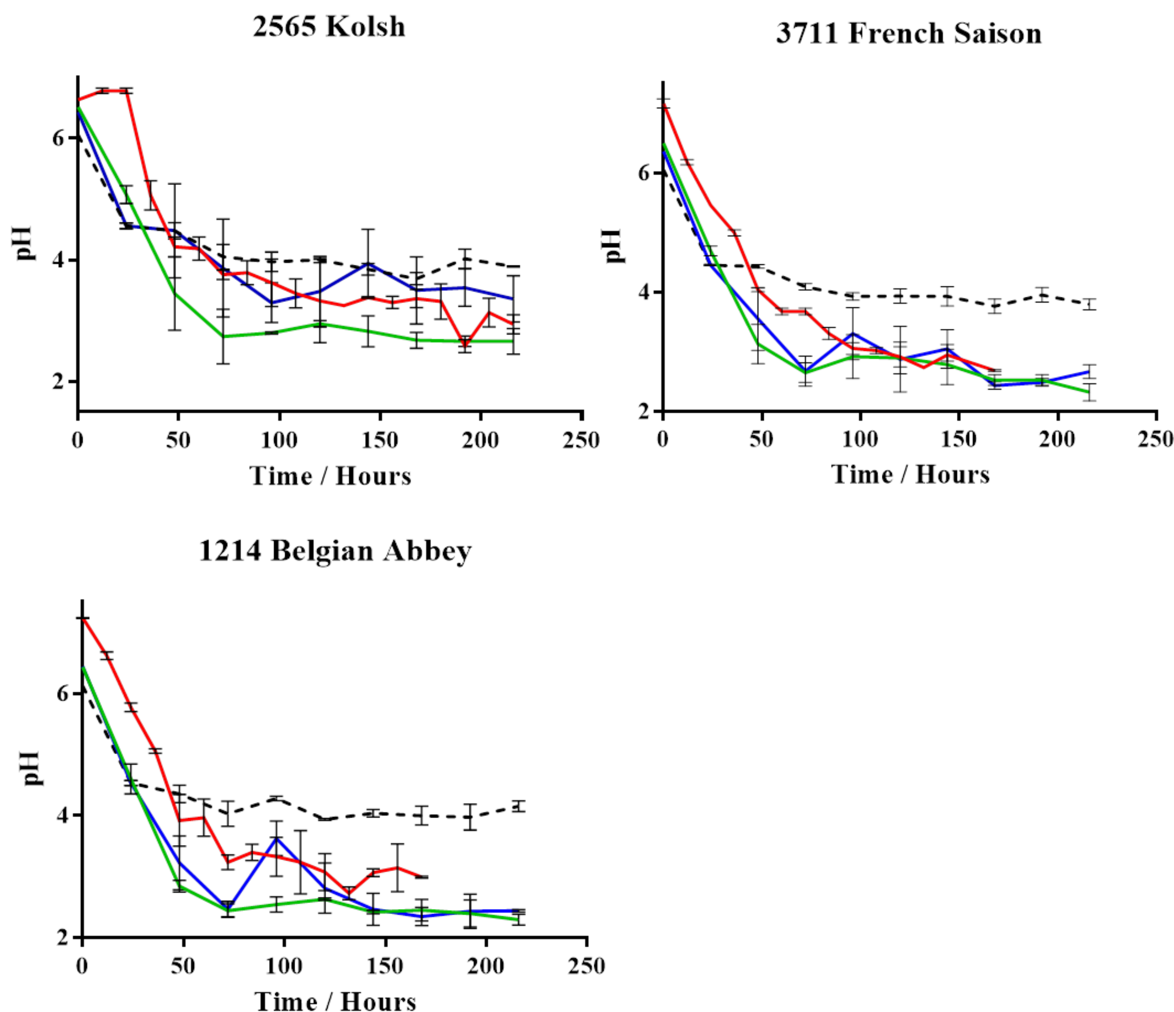

Figure 4. Changes in pH throughout the defined medium trials. The coloured lines indicate results from independent trials conducted with the same yeast strain. The black dashed line indicates results from the regular wort fermentation trial. Error bars show results from biological replicates (n=3 for each).

4. Discussion

The fermentation vessels used in this research were inspired by observations on the full-size fermenters in our brewery as well as a previously described mini fermentation assays [32]. A miniature fermenter vessel should simulate the environment and activities that takes place in a regular scale fermenter as closely as possible, ideally possessing a conical shaped base and an air lock.

In preliminary trials aiming to validate results from the mini fermenter, the reduction in density and pH corresponded with observations from large-scale fermenters. However, some deviations in the operation of the mini fermenters, such as the change in pH for strains 3711 and 1214 (Figure 4), became apparent during the main trials conducted with the defined media.

As outlined below, several issues were noted with the mini fermenters during preliminary trials, resulting in alterations to the methods used. A wort volume of 40 mL was initially used, however, this resulted in an increased back flow through the airlock, producing a powder crust at the top of the airlock, which could potentially lead to and/or exacerbate contamination with other microorganisms, as observed in two preliminary trials. To prevent such unwanted contamination, the media or wort volume was reduced to 36.7 mL, allowing for a full 25 % of the total volume as headspace, as would be the

situation in a full-size fermenter. Additionally, the water initially used in the airlock was replaced with 50 % ethanol to allow for protection from any biological contaminants that may enter the fermenter via this entry point.

Further preliminary trials conducted highlighted the need for a buffering mechanism. Whilst consistent results could be obtained using the malt base with the carbohydrates, when using the yeast nitrogen base the pH dropped from 5 to below 3 within the first 24 hours, due to acidification resulting from the production of CO_2 [44]. This was accompanied by a slowing of the fermentation rate. Hence, 2 g L^{-1} of potassium phosphate dibasic was added as a buffer in subsequent trials. However, despite increasing the pH to 6.2, slightly above the typical range of 5.2-5.4 found in natural wort, the substantially lower pH at the end of some fermentation trials using the synthetic media (Figure 4) indicated that increasing the buffering capacity further may be beneficial in future work. The higher pH in the natural wort medium is likely from nitrogenous basic compounds, such as amino acids, which could potentially provide increased buffering capacity. Including higher concentrations of such compounds may also provide a more accurate simulation of the natural wort.

Since the fermenters were designed as a "one shot system", where each vessel was discarded after it was opened and the relevant measurements taken, this reduced the risk of exposing the vessel contents to the environment and allowing potential microbial contamination which could affect subsequent sampling time points. In addition, the fermentation vessels could be reused, meaning that another trial run could be started while the first was still underway.

One of the primary objectives of this study was to determine how closely defined media and wort fermentations matched each other in performance. In most instances, (see Figures 3 and 4) the changes in density and pH were consistent between the defined media and wort, although some anomalies were noted, most notably in the density of strain 2565 and the pH of strain 3711. Furthermore, differences in the fermentation rates were observed between the yeast strains, indicating a differential fermentation capacity of the various strains.

For example, the growth of strain 2565 (Kolsh) appeared to be hindered in the defined media, as evident by the reduced changes in density (Figure 3). This observation likely suggests that this strain was unable to metabolise the larger sugars (i.e. maltose and maltotriose), possibly due to the presence of insufficient nitrogen thus causing catabolite inactivation of sugar transporters, as highlighted by several previous researchers [45,46]. As the phenotype of this particular strain is typical of an ale yeast [30], this observation is worthy of further investigation and should be a point of consideration for future researchers designing small scale fermentation assays.

For strain 3711 (French Saison), the final density using a defined media was approximately similar to that achieved using a defined media, although the results for one of the biological replicates showed a faster fermentation rate between approximately 100-200 hours and hence resulted in a slightly lower density.

For the Belgian Abbey yeast strain (1214) following fermentation in the defined media, there was little difference between the final density and that of pure water (0.999 g cm^{-3}). This indicates the level of fermentable sugars remaining after the fermentation process was negligible (i.e. the fermentation process had proceeded to completion). This strain would appear to be particularly suited for the defined media and could be of pronounced use to future researchers using small scale fermentation assays. However, this also highlights that addition of non-fermentable sugars to the defined media may be required to provide a more realistic simulation of a wort fermentation (Bamforth, 2003).

In general, the variation of pH was smaller for the wort than that observed for the defined media. Such observation was particularly evident for the Kolsh strain (2565). The slightly lower pH during the later stages of the defined media fermentation trials with this strain may have reduced the viability of the yeast cells and hence reduced its fermentation performance in the defined media.

It is important to note that significant differences remain between the composition of the wort and defined media. In natural wort, the breakdown of starch would produce dextrins such as maltotriose; this source of sugars is evidently absent in the synthetic media. However, this caveat does not preclude the use of defined media in all circumstances, as the primary use of defined media is to allow consistent growing conditions for comparing different strains of yeast or assessing the impact of certain nutritional constituents on growth rates. For more realistic simulation of natural wort, researchers could possibly add small amounts of starch and/or dextrins to the synthetic media, as well using as a fuller complement of amino acids.

Despite this, the results show promise for the future refinement and use of mini-fermentation vessels with defined media for modelling the fermentation capabilities of different yeast strains under highly controlled and uniform conditions. Furthermore, it has the potential to determine which strains had difficulty metabolising the different fermentable sugars present. Such a setup could be used to model the effects of pH on flocculation and fermentation between each strain. Furthermore, future work could validate the present results through the use of reference strains such as *S. paradoxus* type strain (VTT C-09850), *S. cariocanus* type strain (VTT C-15951), lager yeast A15 (VTT A-63015) and ale yeast A62 (VTT A-81062) recently used by Nikulin, *et al.* [47].

Application of more sophisticated analytical techniques would provide greater insight into the biochemical changes that take place throughout the fermentation process of different yeast strains, without requiring the high start-up and running costs associated with a full-scale fermenter. For example, high performance liquid chromatography (HPLC) could quantify the different sugars or their breakdown products present at each time point and hence monitor their usage by different yeast strains (Fox et al., 2001), while qPCR (quantitative polymerase chain reaction) could identify the genes which are differentially expressed in yeast metabolism between fast and slow attenuating yeast strains (Smart, 2003).

5. Conclusion

In the work presented here, a fully defined synthetic media was developed, along with a mini fermentation vessel, which were used to assess differences in the capacity of four brewing yeast strains to utilise the various sugars. The results indicate that yeast strains vary in their capacity to utilise and ferment sugars in part due to their sensitivity to pH, nitrogen content or other variables. Furthermore, significant differences were found between natural wort produced from malt and a defined media. It is possible that other wort constituents not included in the defined media will act in ways beneficial to the yeast, thus causing at least some of the observed differences in growth. However, in general it appears that the miniature fermenter vessels and defined media developed here can be successfully used as a miniature fermentation assay, as long as the results are calibrated and correlated to a conventional standard large-scale fermentation process.

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