The Combined Effects of Oxygen Supply Strategy, Inoculum Size and Temperature Profile on Very-High-Gravity Beer Fermentation by *Saccharomyces cerevisiae*

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**ABSTRACT**

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The timing and concentration of oxygen supply to wort are of particular relevance in industrial beer brewing where tank volumes exceed brewhouse capacity, thereby necessitating fermenter filling in a multiple-brew fashion. A simple technique for accurately controlling dissolved oxygen concentration is presented to model industrial, multi-brew fermentations at bench and pilot scales. This method was employed to identify an effective oxygen supply strategy for batch fermentations conducted with very-high-gravity (VHG) wort. Addition of 25 ppm dissolved oxygen to the fermenting wort, 12 h after inoculation, was the most effective oxygenation strategy and reduced fermentation time by 33% compared to the control conditions. Pilot-scale trials were subsequently conducted to further optimize VHG batch fermentation performance through simultaneous manipulation of key fermentation process parameters, including increased yeast inoculum size, early and increased free-rise timing and temperature, and optimized oxygenation strategy. This approach reduced the time to achieve end of fermentation targets by 34% compared to trials conducted under control conditions. The improved fermentation profile was consistent over three successive inoculations and minimal impact was observed on wort flavor volatiles. Employing the optimized process for VHG batch beer production would be industrially desirable due to the potential for improved process efficiency and cost-savings.

**Key words:** Beer, inoculation, *Saccharomyces cerevisiae*, very high gravity wort, wort oxygenation, temperature.

**INTRODUCTION**

Due to the potential for increased volumetric productivity, reduced capital costs, and lower energy, labor and material costs\(^6\) employing very-high gravity (VHG) wort, defined as substrate having a specific gravity of 18\(^°\)P or higher [degrees Plato (°P) being the recognized term used in the brewing industry to describe the normalized specific gravity of a wort, based on the w/v % of a standard sucrose solution]\(^9,41\) for batch beer production holds great commercial appeal. However, the practice of fermenting very-high-gravity substrates has traditionally been avoided in the brewing industry due, in part, to the potential for increased extract losses, and resultant beers with organoleptic profiles that deviate from those produced at lower gravities.\(^59\) Increasing the carbohydrate content of a given wort also impacts the liquid’s rheological properties (e.g. increases specific gravity, viscosity, and interfacial surface tension) which can, in turn, influence mixing patterns, wort oxygen solubility, and rate of oxygen dissolution. Moreover, high osmotic pressure,\(^1,4,33,49,50,54,58\) the formation of elevated levels of toxic fermentation by-products (e.g. ethanol),\(^11,65\) and limiting yeast nutritional factors\(^9\) have been implicated as key factors contributing to the deleterious results on yeast fermentation performance under very-high-gravity conditions.\(^9,65\) The primary nutrient deficiencies are said to be assimilable nitrogen,\(^34,44,45,47,51\) mineral ions such as magnesium\(^22,56,57,63,64\) and zinc,\(^7,56\) and the various lipid compounds synthesized in the presence of molecular oxygen.\(^21,30,55\) The effects of stress that arise due to the common brewing practice of recycling the yeast over successive fermentation cycles are cumulative and have also been reported to negatively influence VHG fermentation performance due to diminished cell robustness.\(^45\)

The unfavorable effects of yeast stress associated with a very-high-gravity environment can be manifested in the fermentation system in various ways including the occurrence of high dead cell counts, resulting in reduced yeast recycling potential,\(^43\) as well as the formation of so-called ‘invisible haze’ from glycogen.\(^30\) Furthermore, findings reported by D’Amore\(^57\) indicate that ethanol yields for brewing worts of gravities up to 20\(^°\)P were very close to the theoretical targets after 168 h, but as sugar concentrations were increased beyond that level, stuck fermentations were encountered and ethanol productivities fell increasingly short of the theoretical yields, leaving a higher residual extract in the final beer.\(^17\)

Several workers have reported that limitations associated with VHG fermentation can be alleviated using a strategy of meeting fundamental nutrient and micronutrient requirements of the yeast,\(^8,22\) and/or by implementing process changes.\(^17,46\) Optimizing the dissolved oxygen

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sterol biosynthesis. The biosynthesis of sufficient ergosterol, the primary sterol constituent of the fungal plasma membrane, is crucial due to its importance in promoting cell growth, as well as regulating membrane fluidity, permeability, and activity of membrane-bound enzymes and transporters through interactions with various other membrane components. Oxygen has also been shown to play an important role in differentiating mitochondria to fully functional mitochondria. This cellular organelle is reportedly critical for ethanol tolerance in S. cerevisiae, owing to the fact that the mitochondrial matrix has a manganese-containing superoxidase dismutase which actively neutralizes toxic superoxidase radicals.

Other reported roles of oxygen in the yeast cell include participation in biosynthesis of heme (serves as the prosthetic group in cytochromes), regulation of several yeast genes, and ring cleavage of proline.

As the potential for diminished oxygen solubility in high gravity (HG) and VHG worts is amplified due to the increased concentration of dissolved solids, supplying a sufficient quantity of dissolved oxygen to such worts has been shown to play a crucial role in yeast fermentation performance. Conversely, delivering too much oxygen has been reported to contribute to yeast degeneration. The amount of oxygen required by a specific yeast strain is dependant on several factors including the physiological condition of the cells, the inoculation rate, and fermentation conditions.

In addition to the level of oxygen provided, several researchers have established that the timing of supply is critical. O’Connor-Cox and Ingledeuw reported that oxygenating 28°C wort to DO levels at or above air-saturation prior to yeast inoculation did not stimulate fermentation to the same extent as the introduction of low levels of DO at 12 h post-pitching. Under the conditions studied, optimal fermentation performance resulted when oxygen was introduced between 10 and 14 h post-inoculation. Similarly, Younis and Stewart reported that the most stimulatory period for oxygenation, in terms of attenuation, was found to be at the time of initiation of the second yeast cell division. It has been postulated that cells are more efficient at utilizing oxygen for unsaturated lipid biosynthesis following lipid dilution caused by yeast budding and division. Applying oxygen after 1–2 cell divisions reportedly extends the time period before yeast reach minimum lipid levels, thereby increasing the overall number of cell divisions. In turn, this leads to higher attenuation rates. The delayed oxygenation strategy may also be advantageous in that the length of time a wort is exposed to oxygen can be minimized, resulting in the formation of lower levels of superoxide and other radicals.

In industrial brewing environments employing a delayed oxygen provision strategy to optimize VHG fermentation performance would be particularly relevant when combined with the commonly practiced multi-fill approach, wherein a brewery’s tank volumes exceed brewhouse capacity. In such instances, large fermentation vessels must be filled in a stepwise fashion with multiple brews. This allows for a great deal of flexibility in the timing and concentration of oxygen, which can be supplied in-line during specifically timed wort transfer(s) from the brewhouse. It is also of importance to note that the altered fermentation dynamics associated with a multiple-fill strategy have been widely reported to affect cell growth patterns, fermentation rates and organoleptic profiles of the finished beer. This departure in behaviour from that of traditional, single-brew fermentations can be attributed to such factors as vessel geometry, brew size, filling interval, cell pitching rate, and oxygen provision regime. In order to study and optimize the performance of industrial VHG multi-brew fermentations, it is essential to reproduce aspects of this process (including accurate control of the timing and concentration of oxygen supply) for examination at smaller scales.

The literature suggests that, in addition to an optimal oxygen delivery strategy, moderately increasing the yeast inoculation rate can also be an effective tool for enhancing VHG fermentation performance as attenuation commences more rapidly, losses in viability typically observed following inoculation are not evident, the yeast utilize less free amino nitrogen (FAN) and the dependence on nutrients for growth is minimized owing to the fact that the overall level of growth is reduced. Furthermore, fermentations conducted with relatively high inoculation rates have exhibited increased rate and extent of fermentation, as well as higher cell viabilities throughout the fermentation. D’Amore and colleagues reported that the rate and extent of ethanol production increased with increasing inoculum size (note that a correlation exists between the concentration of yeast in suspension and attenuation rates). However, in instances where serial re-pitching is employed, excessive inoculation rates must be avoided as this could negatively impact the long-term cell population viability, as well as fusel alcohol concentrations in the finished beer, due to insufficient growth.

Another process variable that can be manipulated to enhance VHG fermentation performance is the temperature profile. Temperature free-rise is a commonly employed brewing technique whereby the heat naturally generated by fermentation is leveraged in order to accelerate the diacetyl (i.e. 2,3-butanedione) reduction process by increasing the temperature of the fermenting beer by a predetermined value. In order to minimize the impact of this practice on the organoleptic profile of the resultant beer, free-rise typically occurs at the onset of the stationary cell growth phase. However, by implementing a slightly earlier and increased free-rise timing and temperature, the thermal decarboxylation of α-acetolactate to diacetyl (a beer flavour defect at levels exceeding 20 ppb), and subsequent reduction to acetoin may be further accelerated.

A key objective of this study was to establish a technique that could be applied in bench- and pilot-scale fermentations to control both the concentration (between 0.1 and 38 ppm) and timing of the oxygenated wort supply. In this way, the multi-fill approach for industrial batch fer-
mentation could be simulated more accurately for scaled-down studies. This technique was employed to help optimize the oxygen supply strategy for VHG batch fermentations conducted with 22°P lager wort and Saccharomyces cerevisiae (carlsbergensis) strain LCC 2021 from the Labatt Culture Collection. The selection of the most effective oxygen supply regime was based on overall fermentation performance, including extent of yeast growth, fermentation rate and extent, and final diacetyl concentration.

Expanding upon the learnings from the bench scale trials, additional work was performed at the pilot scale with industrial lager yeast strain LCC 2034 to demonstrate how, in nutritionally rich wort, common process parameters such as oxygen supply strategy, inoculum size and fermentation temperature profile can be manipulated to provide a unique, multi-faceted solution for optimizing batch fermentation performance for the production of beer from VHG worts and to produce a robust system that is capable of promoting yeast health over several fermentation cycles.

**MATERIALS AND METHODS**

**Wort collection, supplementation and storage**

**Bench-scale (3-L).** Commercial industrial lager wort, supplied by Labatt Brewing Company Limited (London, ON), containing high-maltose corn syrup (HMCS) as adjunct was aseptically collected from the production brewery. An additional 72 g/L of HMCS (Clearbrew® 43% High Maltose IX Liquid Adjunct; Cargill Inc., Dayton, OH) was added to the wort in order to increase the specific gravity from 17.5°P to 22°P. The wort was also supplemented to target levels of 550 mg/L free amino nitrogen (FAN) with a known quantity of yeast extract powder (BBL Yeast Extract; BD, Sparks, MD), 0.6 ppm zinc (food grade ZnSO₄ ⋅ 7H₂O; Mallinckrodt Baker Inc., Philipsburg, NJ), 200 mg/L magnesium (food grade MgSO₄ ⋅ 7H₂O; Mallinckrodt Baker Inc., Phillipsburg, NJ) and 67 mg/L calcium (CaCl₂; Sigma Chemical Co.).

**Pilot-scale (100-L).** VHG wort (22°P) was produced in the pilot brewhouse of Labatt Brewing Company Limited (London, ON) then aseptically transferred to a 2000-L wort storage vessel (Model WT-20H; Newlands Systems Inc., Abbotsford, BC), where it was cooled and stored at 2°C under a CO₂ counter pressure of 0.5 psi. The wort was then allowed to settle for approximately 8 h, and standardized for solids content by discharging the proteaceous trub plug that had formed at the bottom of the storage vessel. To ensure it possessed sufficient levels of the key nutritional factors required for optimal fermentation performance, the VHG wort was produced with a moderate malt-to-adjunct ratio (*i.e.* 60:40), resulting in a free amino nitrogen concentration of 370 mg/L. Furthermore, the 22°P wort composition was adjusted to 0.6 ppm zinc (food grade ZnSO₄ ⋅ 7H₂O; Mallinckrodt Baker Inc.), 200 mg/L magnesium (food grade MgSO₄ ⋅ 7H₂O; Mallinckrodt Baker Inc.) and 67 mg/L calcium (CaCl₂; Sigma Chemical Co.).

**Yeast**

All bench-scale studies were carried out with an industrial lager brewing strain of Saccharomyces cerevisiae (carlsbergensis) LCC 2021 from the Labatt Culture Collection (LCC). Subsequent trials at the pilot scale were carried out exclusively with industrial lager brewing strain LCC 2034, based on its superior performance under VHG conditions during screening trials to identify an effective yeast candidate (data not shown). Yeast for bench-scale experiments was propagated from cryogenically preserved vials of pure yeast culture (to final batch volumes of 1 L) in the same 22°P wort as used in the trials, and subsequently pelletized according to the methods reported by Petersen and colleagues.32 The desired mass of yeast pellet was weighed and transferred into the target bioreactor, based on the previously established relationship that 1 × 10⁶ cells/mL/°Plato would be achieved by delivering 0.265 g/L/°Plato.32 Cells for pilot-scale fermentations were further propagated (at 21°C) beyond the laboratory scale to a final volume of 90 L in a 200-L pilot-scale propagator equipped for continuous air sparging and temperature control (Model BST 2H; Newlands Systems Inc., Abbotsford, BC). All stages of propagation were conducted in the same wort as was employed during fermentation. Yeast within the 200-L propagation vessel was allowed to multiply until a specific gravity of 6.3°P was achieved, with the goal of cooling the yeast before it reached stationary growth phase. This target specific gravity was selected based on propagation curves conducted at bench-scale (data not shown). Once the target specific gravity was realized, sparging was discontinued, the liquid was rapidly cooled to ~2°C and the yeast was allowed to settle for 12 h. The yeast bed was then harvested into a sterile 20-L canister (Challenger “VI”®; Spartanburg Steel Products Inc., Spartanburg, SC). A uniform sample of yeast slurry was subsequently extracted from the canister for viable yeast cell enumeration and yeast solids analyses. The desired mass of yeast slurry to be transferred into the target pilot-scale bioreactor was calculated based on the previously established relationship that 1 × 10⁶ cells/mL would be achieved by delivering 0.265 g/L of pure yeast.32 Between the time of yeast harvesting and yeast inoculation into the pilot bioreactors (maximum 24 h), the 20-L canisters were stored in a 3°C cold room.

**Gas delivery technique**

The target dissolved oxygen (DO) levels for bench and pilot-scale fermentations were achieved by aseptically combining specific quantities of oxygen-saturated (i.e. 38 ± 1 ppm DO) and desaturated (i.e. ~0 ppm DO) 22°P worts. The required volumes of 22°P oxygen-saturated and desaturated worts to achieve a target DO were determined by simultaneously solving the following equations:

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Initially, the worts were aseptically combined by transferring 3-L quantities of 22°P wort in the Bellco bioreactor spinner jars according to the procedures outlined above. The DO concentration was measured during sparging by pumping 22°P wort through a closed circuit containing the Bellco bioreactor and in-line Orbisphere O₂ Micro Logger (Model 3650, Orbisphere Laboratories, Vesenzaz, Switzerland) using a Masterflex peristaltic pump (Model 7523-20; Cole-Parmer Instrument Co., Vernon Hills, IL) and Easy Load® pump head (Model 900-1146; Cole-Parmer Instrument Co., Vernon Hills, IL) and ¼” ID norprene tubing. Dissolved oxygen values were recorded and stored in the Orbisphere data logger in 15 sec intervals. Once the trial was complete the resultant data was downloaded to a PC using WinLog97 software (as a .txt file) and subsequently converted into an Excel (Microsoft) file to generate the curves.

Oxygenation procedures for pilot-scale (100-L) trials performed with optimized process conditions. The portion of wort to be oxygen-saturated was sparged to a DO level of 0.1 ± 0.03 ppm using the pilot brewery’s in-line gas delivery system during aseptic transfer from the 2000-L wort storage vessel to the target pilot-scale bioreactor. Wort oxygenation was performed in a sterilized 110-L cylindrical portable wort storage vessel (Model BST 1H; Newlands Systems Inc., Abbotsford, BC), equipped with a sintered stainless steel sparger (Series 19000; Zahm & Nagel Co., Holland, NY) located at the vessel base. Wort was sparged with oxygen for 0.5 h prior to the transfer time to ensure saturation (i.e. 38 ± 1 ppm DO) as measured by an Orbisphere O₂ Micro Logger. During sparging 0.04-mL/L of Fermcap-S antifoam was introduced to prevent over-foaming. This led to a DPS concentration in the wort of 7.69 ppm. Once saturated, the oxygenated VHG wort was aseptically transferred from the portable storage vessel to the pilot-scale bioreactor using gas pressure as the driving force.

Pitching protocols and operating conditions for bench-scale (3-L) fermentations

All bench-scale fermentations were carried out in 3-L Bellco (working volume) bioreactor spinner flasks (model 2-45; Bellco Glass Inc., Vineland, NJ) with magnetic stirring capabilities. One litre (1-L) wort volumes were oxygenated/nitrogenated in 2-L (capacity) Nalgene polypropylene heavy-duty bottles (model EW-06025-50; Cole-Parmer, Anjou, QUE) containing a magnetic stir bar, and fitted with Nalgene filling/venting caps and platinum-cured silicone tubing. All vessels were set up to accommodate aseptic liquid transfer, gas exhaust, and gas sparging.

For each of the oxygen provision scenarios studied, the specific volumes of wort to be combined following oxygen-saturation/desaturation were aseptically dispensed into the appropriate bioreactor/heavy-duty bottle configuration, as prescribed by Equations 1 and 2, and equilibrated to 14.5°C. As dictated by the oxygen provision scenario, each wort volume was subsequently sparged with either 99.993% oxygen (Ultra High Purity 4.3; Praxair Canada, Mississauga, ON) or 99.5% nitrogen (Model 75-72-V157; Parker Balston nitrogen generation system, USA) using a gas-delivering, deflected point, non-coring septum penetration needle with standard hub and 304 stainless steel cannula (18G × 12″; Popper & Sons Inc., New Hyde Park, NY). Worts were sparged for 0.5 h to ensure saturation and the line pressure was held constant at 18 psi during gas delivery. To facilitate the mass transfer of gas into solution during the saturation process, the wort was constantly mixed by magnetic stir bar/impeller using a magnetic stirrer (Model 4657; Cole-Parmer Instrument Co., Vernon Hills, IL) adjusted to set point 5. Once saturated, the worts were aseptically combined by transferring the contents of heavy duty bottle(s) to the designated bioreactor using gas pressure as the driving force.

Dissolved oxygen concentration versus time curves were generated in duplicate for oxygen and nitrogen by sparging 3-L quantities of 22°P wort in the Bellco bioreactor spinner jars according to the protocols outlined above. The DO concentration was measured during sparging by pumping 22°P wort through a closed circuit containing the Bellco bioreactor and in-line Orbisphere® O₂ Micro Logger (Model 3650, Orbisphere Laboratories, Vesenzaz, Switzerland) using a Masterflex peristaltic pump (Model 7523-20; Cole-Parmer Instrument Co., Vernon Hills, IL) with an Easy Load® pump head (Model 900-1146; Cole-Parmer Instrument Co., Vernon Hills, IL) and ¼” ID norprene tubing. Dissolved oxygen values were recorded and stored in the Orbisphere data logger in 15 sec intervals. Once the trial was complete the resultant data was downloaded to a PC using WinLog97 software (as a .txt file) and subsequently converted into an Excel (Microsoft) file to generate the curves.

Pitching protocols and operating conditions for bench-scale (3-L) fermentations

All bench-scale fermentations were carried out in 3-L Bellco (working volume) bioreactor spinner flasks (model 2-45; Bellco Glass Inc.) with magnetic stirring capabilities, and set up for anaerobic fermentation conditions and sampling under a positive-pressure headspace as described by Petersen and colleagues. During fermentation the spinner jars were situated (partially submerged in 3.5” of water) in a re-circulating utility bath fitted with a six-position, high-powered magnetic driver. To control temperature the water bath assembly was set up in a Psycrotherm controlled environment wall incubator (New Brunswick Scientific Co. Inc., Edison, NJ). All bench-scale fermentations were conducted isothermally at 14.50 ± 0.05°C and mixing was carried out using a magnetic impeller set to stir at 33 rpm. End of fermentation was considered to be achieved when the specific gravity of the fermenting wort became stable and did not demonstrate any further decrease for 12 hours.

Timing of initial cell division (3-L bench-scale). Bench-scale fermentations, inoculated with 9.24 × 10⁷ cells/mL, were carried out to establish the timing of initial cell division in oxygen-saturated and de-saturated 22°P worts. In subsequent bench-scale fermentations this would be the time at which delayed oxygen would be supplied. Bioreactor contents were monitored for viable cell concentration in 3 to 6 h intervals for a period of 24 h.

Trials to determine the most effective oxygen supply strategy (3-L bench-scale). Screening trials were carried out once-over at the 3-L scale, using the gas delivery technique previously described, to identify the most effective oxygen supply strategy for batch fermentations with yeast strain LCC 2021 conducted in 22°P lager wort. The most effective oxygen supply regime was selected based on overall fermentation performance, including extent of

\[ \text{DO}_i V_f = \text{DO}_{NW} V_{NW} + \text{DO}_{OW} V_{OW} \]  

where

\[ V_f = V_{NW} + V_{OW} \]  

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\[ V_f = V_{NW} + V_{OW} \]
yeast growth, fermentation rate and extent, and final diacetyl concentration. The following oxygen provision scenarios were examined:

i. The fermentation conducted in Bioreactor B was designated as the control, and was supplied with 25 ppm of DO at 0 h (i.e. immediately prior to inoculation). This oxygen provision scenario was achieved by combining 2 L of oxygen-saturated wort (Note: DO concentration ~38 ppm) with 1 L of nitrogen-saturated wort in Bioreactor B. This wort was then immediately inoculated with \(3.08 \times 10^7\) cell/mL (see Fig. 1a) and fermented (anaerobically) until the end of fermentation.

ii. Bioreactor A served as the feed jar for Bioreactors C and D and was charged with 3-L 22°P wort, which was subsequently sparged to saturation with nitrogen (i.e. DO concentration ~0 ppm). The wort was then inoculated with \(9.24 \times 10^7\) cell/mL and fermented (anaerobically) for a period of 12 h (see Fig. 1b).

iii. The contents of Bioreactor C were supplied with 25 ppm of DO at 12 h only. This was achieved by transferring 1-L of well-mixed, yeast-laden fermenting wort from Bioreactor A, which had been fermenting for a period of 12 h, to Bioreactor C which contained 2-L of oxygen-saturated wort (see Fig. 1b). The fermentation was then allowed to proceed (anaerobically) to completion.

iv. The contents of Bioreactor D were supplied with 12.5 ppm of DO at 12 h only. This was achieved by transferring 1-L of well-mixed, yeast-laden fermenting wort from Bioreactor A, which had been fermenting for a period of 12 h, to Bioreactor D which contained 1-L of oxygen-saturated wort plus 1-L of nitrogen-saturated wort (see Fig. 1b). The fermentation was then allowed to proceed (anaerobically) to completion.

v. Bioreactor F served as the feed jar for Bioreactor E and was charged with 1-L of nitrogen-saturated wort combined with 2-L of oxygen-saturated wort, to arrive at a DO concentration of 25 ppm. This wort was subsequently inoculated with \(9.24 \times 10^7\) cell/mL, and fermented (anaerobically) for a period of 12 h (see Fig. 1c).

Fig. 1. Summary of bench-scale oxygenation and pitching procedures for bench-scale trials to identify an optimized oxygen delivery strategy for \(S.\) cerevisiae strain LCC 2021 in 22°P wort. a: Bioreactor B (25 ppm DO supplied at 0 h); b: Bioreactors C (25 ppm DO supplied at 12 h) and D (12.5 ppm DO supplied at time 12 h); c: Bioreactor E (25 ppm DO supplied at 0 and 12 h). All bioreactors had 3-L working volumes.
vi. The contents of Bioreactor E were supplied with 25 ppm of DO at 0 h (i.e. immediately prior to inoculation) and 25 ppm of DO at 12 h post-inoculation. This was achieved by transferring 1 L of well-mixed, yeast-laden fermenting wort from Bioreactor F, which had been fermenting for a period of 12 h, to Bioreactor E which contained 2 L of oxygen-saturated wort (see Fig. 1c). The fermentation was then allowed to proceed (anaerobically) to completion.

Pitching protocols and operating conditions for pilot-scale (100-L) fermentations

All pilot-scale fermentations were carried out in 150-L cylindrical conical pilot bioreactors (Model FV-1H; Newlands Systems Inc., Abbotsford, BC) equipped with dimpled glycol cooling jackets and PLC-based temperature sensing and controlling systems. During fermentation, mixing resulted solely from the convective gas-lift action created during CO2 evolution.

Pilot-scale (100-L) fermentations conducted with optimized process conditions. The 2000-L wort storage vessel CO2 counter pressure was increased to 2.0 psi from 0.5 psi, in order to utilize pressure as the driving force for transferring 22°P wort from the storage vessel to the target pilot-scale bioreactor. A 33-L quantity of VHG wort (i.e. initial transfer volume) was aseptically transferred, through a series of 1” and 2” ID beer hoses, from the 2000-L wort storage vessel to the target pilot-scale bioreactor. The flow rate of the wort was controlled by manipulating a manual Keystone butterfly valve (Model 250; Romatec, Sarnia, ON) located at the bioreactor’s stand-pipe inlet. During transfer, the wort passed through a pilot-scale plate heat exchanger (custom made; Fischer Engineering, Germany) wherein the hot stream was 20°C city water. In this way, the wort temperature was increased from 2°C to a target temperature of 14.5°C. Once 33 L of wort had entered the bioreactor, the wort flow from the 2000-L vessel was discontinued and the quantity of yeast slurry to achieve the desired cell concentration was aseptically introduced, using positive pressure, through the 150-L bioreactor’s drain line from the (well shaken) 20-L Challenger "VI"® yeast storage canister. Subsequently, a 67-L quantity of 22°P wort (i.e. secondary transfer volume) was aseptically transferred from the 2000-L wort storage vessel to a sterilized 110-L cylindrical portable wort storage vessel, equipped with a 0.22-μm Opticap 4” Durapore hydrophobic capsule filter, as well as a 1” (diameter) × 4” (length) sintered stainless steel sparger (Series 19000; Zahn & Nagel Co., Holland, NY) located at the base of the vessel. Eleven and one-half hours after inoculation of the bioreactor with yeast, the wort within the 110-L cylindrical portable storage vessel was sparged with 99.993% oxygen until a dissolved oxygen concentration of 22 ppm was achieved, as measured by an Orbisphere® O2 Micro Logger. The quantity of yeast slurry to achieve the desired cell concentration was then aseptically introduced through the 150-L bioreactor’s drain line from the (well shaken) 20-L Challenger “VI”® yeast storage canister using positive pressure.

Pilot-scale fermentations conducted under control process conditions. The aseptic transfer of 100 L of 22°P wort from the 2000-L wort storage vessel to the target 150-L bioreactor was carried out in accordance with the procedures outlined above pertaining to the transfer of the initial wort volume to the bioreactor for optimized fermentations. Once 100 L of wort had entered the bioreactor, the wort flow from the 2000-L vessel was discontinued. A 1/2” (diameter) × 4” (length) stainless steel, porous metal sparger (Type G; Mott Corporation, Farmington, CT), connected to the oxygen tank by reinforced ½” ID reinforced PVC tubing (Cole-Parmer Canada Inc., Anjou, QUE), was then aseptically lowered to the bottom of the bioreactor. The wort was subsequently sparged with 99.993% oxygen until a dissolved oxygen concentration of 22 ppm was achieved, as measured by an Orbisphere® O2 Micro Logger. The quantity of yeast slurry to achieve the desired cell concentration was then aseptically introduced through the 150-L bioreactor’s drain line from the (well shaken) 20-L Challenger “VI”® yeast storage canister using positive pressure.

Pilot-scale fermentations conducted under control process conditions were inoculated with 1 × 106 cells/mL/P (i.e. 2.2 × 107 cells/mL). Oxygen was supplied to the 22°P wort at a rate of 1 ppm/P (i.e. 22 ppm) immediately prior to inoculation. The fermentation temperature was held constant at 14.5°C for 48 h and then increased to 20°C until the end of fermentation.

End of fermentation yeast harvest, storage and recycle. In all pilot-scale trials, once the end of fermentation was achieved, based on the criteria that the wort specific gravity had stabilized and the diacetyl concentration had reduced to a maximum level of 45 ppb, the beer was rapidly cooled to ~2°C to promote yeast settling. After a minimum of 12 h settling time, the yeast bed was subsequently collected using the same method employed for harvesting freshly cultivated yeast from the pilot-scale propagator, quantified and re-pitched within 24 h. Between the time of yeast harvest and yeast inoculation into
the pilot-scale bioreactor, the 20-L yeast canisters were stored in a 3°C cold room. Fermentation trials involving control and optimized process conditions were monitored and evaluated over a series of three successive inoculations wherein the yeast was recycled. Table I summarizes the distinct fermentation conditions employed for the control and optimized pilot-scale trials.

Analytical methods

**Sampling protocol.** Fermentation liquids were sampled, without introducing oxygen, in volumes and frequencies best suited for the analytical requirements throughout the trials. Samples to be processed for chemical analyses were centrifuged at 3500 rpm at 4°C for 20 min (Model RC5C; Sorvall/Dupont, USA). Subsequently, the supernatant was decanted for further processing.

**Dissolved oxygen determination.** Dissolved oxygen was measured with an Orbisphere® O₂ Micro Logger (Model 3650; Orbisphere Laboratories, Vesenzaz, Switzerland). The meter was connected in-line (for bench-scale DO curve generation) and to the target vessel’s sample port (for pilot scale trials). The unit was calibrated prior to each use and the reading (in ppm) was obtained after the value stabilized. Note that the Orbisphere could not be utilized to measure dissolved oxygen during bench-scale fermentations, as there was no way to sterilize the unit for in-line use.

**Microbiological analyses.** Samples were aseptically collected from 3-L bioreactors/pilot-scale bioreactors after 24 h of fermentation, as well as at the end of fermentation. For each sample, a 0.2 mL aliquot was aseptically spread onto MacConkey agar (50 g MacConkey Agar, 1.0 L distilled water) to screen specifically for enteric bacteria. Additionally, for each sample 0.5–2.0 mL aliquots of well mixed sample slurry were poured spread onto MacConkey agar (50 g MacConkey Agar, 1.0 L distilled water) to screen specifically for enteric bacteria. Additionally, for each sample 0.5–2.0 mL aliquots of well mixed sample slurry were poured plated using Nystatin Universal Beer Agar (NUBA; 62.0 g UBA, 750 mL distilled water, 250 mL lager beer, 3 mL Tween® 80, and 0.01 g nystatin), then incubated aerobically and anaerobically. The presence of Nystatin in the NUBA media serves to suppress yeast growth, so that only bacteria present in the sample will colonize the plate. Aerobic NUBA plates were incubated 4 days, and anaerobic NUBA plates for 8 days, all at 28°C. The MacConkey plates were incubated for 3 days at 35°C.

**Yeast cell concentration and viability determination.** Yeast cell concentration and viability determination was carried out according to the ASBC microscopic yeast cell counting technique using a hemocytometer (1/400 mm² open type improved Neubauer ruling pattern; Hauser Scientific, USA) with a hemocytometer cover slip. Viability was ascertained using the ASBC methylene blue staining technique, which capitalizes on the fact that live yeast cells oxidize methylene blue, thereby removing the colour. Dead cells stained blue.

**Specific gravity determination.** As previously mentioned, degrees Plato (°P) is the recognized unit used in the brewing industry to describe the normalized specific gravity of a wort, based on the w/v% of a standard sucrose solution. The specific gravity was measured using a hand-held digitalized portable Density/Specific Gravity/Concentration meter (Model DMA 35N; Anton Paar, Graz, Austria). Samples for analysis were first filtered through a Titan2™ 30 mm, 0.45 μm nylon syringe filter (Sun Sri; Rockwood, TN), and degassed.

**Diacetyl (VDK) determination.** Diacetyl concentration was determined in accordance with the method of the Technical Committee and Editorial Committee of the ASBC. Samples for analysis were first filtered through a Titan2™ 30 mm, 0.45 μm nylon syringe filter, and subsequently degassed. Each sample was then equilibrated to 62°C over a period of 30 min. Quantification of 2,3-butanedione (i.e. diacetyl) and 2,3-pentanedione, were conducted using a Perkin Elmer 8310 Gas Chromatograph equipped with an electron capture detector. A nitrogen stream, flowing at 60 mL/min, was employed as the carrier gas and the sample was passed through a J&W DB-Wax capillary column. The injector temperature was maintained at 125°C, while the detector temperature was set at 175°C. A Perkin Elmer headspace autosampler (model HS40) was employed to facilitate the analysis.

**Free amino nitrogen (FAN) determination.** Samples for analysis were first filtered through a Titan2™ 30 mm, 0.45 μm nylon syringe filter, and subsequently degassed. Free amino nitrogen was measured according to method published by the ASBC using a Perkin Elmer LS50B spectrophotometer with a Tungsten lamp (570 nm wavelength), which measures a colour reaction between ninhydrin and the nitrogen present in the sample. This method detects nitrogen derived from amino acids, ammonia, and to some extent, end group α-amino nitrogen of peptides and proteins.

**Key beer volatiles determination.** Samples for analysis were first filtered through a Titan2™ 30 mm, 0.45 μm nylon syringe filter, and then degassed. Beer volatiles including acetaldehyde, ethyl acetate, isobutanol, isoamyl acetate, 1-propanol and isoamyl alcohol were measured by the headspace method according to the method of the Brewery Convention of Japan, using n-butanol as the internal standard. A Hewlett Packard 5890 Gas Chromatograph equipped with a FID, a Hewlet Packard 7994E headspace autosampler and a J & W DB-Wax capillary column was utilized. The injector temperature was set at 200°C and the detector temperature at 220°C. The oven temperature was initially set at 40°C for 5 min, and sub-

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Table I. Summary of pitching conditions for pilot-scale trials conducted with strain LCC 2034 and 22°P wort

<table>
<thead>
<tr>
<th>Fermentation conditions</th>
<th>Cell inoculation ratea (cell/mL)</th>
<th>Target DO concentration (ppm)</th>
<th>Timing of oxygen supply</th>
<th>Fermentation temperature profile</th>
<th>Successful fermentation cycles involving yeast recycling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control process</td>
<td>2.2 × 10⁷</td>
<td>22</td>
<td>Immediately prior to inoculation</td>
<td>14.5°C for 48 h, then increase to 20°C</td>
<td>3</td>
</tr>
<tr>
<td>Optimized process</td>
<td>3.1 × 10⁷</td>
<td>25</td>
<td>12 h post-inoculation</td>
<td>14.5°C for 35 h, then increase to 21°C</td>
<td>3</td>
</tr>
</tbody>
</table>

a Based on 100-L wort volume.
sequently ramped up to 200°C, at a rate of 10°C per min. Subsequently, the temperature was further ramped to 220°C at a rate of 50°C per min, and held for 5 min. The 6 mL/min helium carrier gas was supplemented with a helium stream at 30 mL/min. A hydrogen stream at 50 mL/min, along with an air stream at 300 mL/min, were employed to support the flame. The GC cycle time for a sample loop of 1 mL was 40 min.

Ethanol determination. Samples for analysis were first filtered through a Titan2™ 30 mm, 0.45 µm nylon syringe filter, and then degassed. Ethanol concentration was determined according to the method published by the American Society of Brewing Chemists. Samples were combined with a 5% (v/v) isopropanol internal standard, followed by the injection of 0.2 mL of the mixture into a Perkin Elmer 8500 gas chromatograph equipped with a flame ionization detector (FID) and Dynatech autosampler. A Chromosorb 102, 80–100 mesh support packing was employed with helium, flowing at 20 mL/min, as the gas carrier, injector temperature of 175°C, detector temperature of 250°C and column temperature of 185°C.

Ethanol productivity determination. Ethanol Productivity (EP) was calculated according to the following equation:

\[
EP = \frac{\text{EtOH}_f - \text{EtOH}_i}{t}
\]

where

\[
\text{EP} = \text{ethanol productivity (g ethanol/L/h)}
\]
\[
\text{EtOH}_i = \text{initial ethanol concentration (g/L)}
\]
\[
\text{EtOH}_f = \text{final ethanol concentration in (g/L)}
\]
\[
t = \text{fermentation cycle time (hours)}
\]

RESULTS AND DISCUSSION

Dissolved oxygen versus time calibration curves (3-L bench-scale)

Fig. 2 illustrates the dissolved oxygen versus time calibration curve for oxygen in 22°P wort at 14.5°C. Under the conditions studied, it took approximately 20 min to achieve maximum saturation of the wort. It is also interesting to note that once the oxygen supply to the wort was turned off at 30 min, the dissolved oxygen concentration dissipated at a relatively slow rate. In terms of nitrogen saturation of the wort (see Fig. 2) it was found that the dissolved oxygen concentration of the nitrogen-saturated wort reduced to a level of approximately 0.07 ppm within 20 min. Complete anaerobiosis of the wort could not be achieved with the set-up and nitrogen generation system available. Similar results were previously reported by Lodolo.

It has been well established that it becomes progressively difficult to oxygenate worts as the specific gravity is increased. Elevated concentrations of dissolved sugars and/or other substances in the substrate have been found to negatively impact on oxygen solubility. Furthermore,
the rate of oxygen dissolution is significantly influenced by the rheological characteristics (e.g. density, viscosity and surface tension) of the fermenting wort. Increases in the above mentioned properties, brought on by a higher dissolved solids concentration, cause an increase in oxygen bubble size with resultant decreases in gas hold-up and interfacial area (i.e. a) available for oxygen transfer.\textsuperscript{60} Indeed, in fermentation trials conducted at the pilot-scale it was not possible to oxygenate 22°P wort beyond a level of 9 ppm in a single pass of the wort through the pilot brewery’s existing in-line oxygen delivery system. Yet, this system was capable of achieving dissolved oxygen levels in excess of 20 ppm in a wort possessing a specific gravity of 17.5°P in a single pass. Clearly, as the implementation of VHG brewing practices become increasingly popular in industrial brewing, the need for more efficient in-line oxygen delivery equipment is also intensified.

**Timing of initial cell division (3-L bench-scale)**

Cells pitched into nitrogen-saturated and oxygen-saturated worts were observed to be actively budding by 6 h post-inoculation. This indicates that, under the conditions studied, the duration of the lag phase was independent of the initial wort DO concentration. However, the occurrence of budding was not as extensive throughout the cell populations as might be expected in fermentations inoculated with lower cell concentrations. Due to limited oxygen availability, it was not surprising that the proportion of cells that multiplied during the 24 h was observed to be particularly low in the nitrogen-saturated wort, which was unable to support even one full division of the cell population. This was reflected in the viable cell concentrations present at the 24 h mark (see Fig. 3). However, the cells observed to be actively budding at the 6-h mark, both in the nitrogen- and oxygen-saturated worts, had achieved complete cell division by 12 h post-inoculation. Therefore, this was the timing selected for the application of delayed oxygenation in subsequent trials at both the bench and pilot scales.

**Determination of the most effective oxygen supply strategy (3-L bench-scale)**

Fig. 4 illustrates that the peak cell concentrations were similar for all fermentations where 25 ppm DO was supplied (i.e. Bioreactors B, C and E). However, cells present in trials performed with 12.5 ppm DO (i.e. Bioreactor D) did not multiply to the same extent as those exposed to 25 ppm DO, indicating that under the conditions studied an oxygen provision of 12.5 ppm was not sufficient to generate the required growth.

As depicted in Fig. 5, Bioreactor C (i.e. 25 ppm DO at 12 h) demonstrated a somewhat lower fermentation rate for the first 12 h after dilution with oxygenated wort. Ultimately however, this system achieved full attenuation of
the fermenting wort within in the shortest period of time (i.e. 96 h). This represents a 33% reduction in overall attenuation time compared to the control fermentation (i.e. Bioreactor B supplied with 25 ppm at 0 h), which required 146 h to achieve the same result. The rapid rate of fermentable carbohydrate reduction observed in Bioreactor C may, in part be explained by the fact that the contents of this bioreactor achieved the highest peak cell concentration. These results are in agreement with findings published by O'Connor-Cox and Ingledew and Lodolo where it was reported that a delayed oxygenation approach is more stimulatory to yeast growth and fermentation performance than oxygen supplied immediately prior to yeast inoculation. Fig. 5 also reflects that the contents of Bioreactor E (i.e. 25 ppm DO supplied at 0 and 12 h) were the second fastest to fully attenuate the wort. The performance of the fermentation conducted in Bioreactor E may indicate that no added benefit can be realized by providing DO to aerobically grown cells both at onset of fermentation and at the timing of the initial division. On the contrary, the results indicate that early oxygen provision may have been detrimental to the robustness of the fermentation system. The accelerated attenuation and slightly more complete fermentation behaviour (see Table II) observed in Bioreactors C and E may also be attributed to the fact that the cells that received oxygen after the initial cell division experienced a longer period of growth than the cells that were exposed to oxygen only upon pitching. It has been reported that metabolically active cells ferment wort up to 33 times faster than non-growing cells.

Literature indicates that the DO demand of yeast can be minimized when oxygen is introduced at the optimal time. O'Connor-Cox and Ingledew reported that 7.5 ppm DO introduced at 12 h post-inoculation was sufficient to ferment a 28°P wort (to approximately 8°P). Furthermore, increasing the level of DO supplied at the optimal time served to further stimulate the fermentation rate, but had no impact on viable cell profile or extent of fermentation. Conversely, in the current study the cells present in Bioreactor D (i.e. 12.5 ppm DO supplied at 12 h post-inoculation) demonstrated sluggish fermentation behaviour due to insufficient cell growth and lower metabolism. The discrepancy may be due to strain differences, as the additional oxygen allowed for complete attenuation, which was not achieved in the earlier study. The supplemental nutrients introduced to the VHG wort and the increased pitching rate employed in the current study may help to further explain the improved rate and extent of fermentation observed.

Table II illustrates that Bioreactors B, C and E all achieved similar end of fermentation ethanol concentrations. Table II further demonstrates the end of fermentation diacetyl concentrations for the bench-scale trials conducted to identify an optimized oxygen delivery strategy. It is apparent that under the conditions studied the diacetyl levels did not reduce to an acceptable level (i.e. the

![Fig. 4. The effect of oxygen supply strategy on viable cell concentration over the course of fermentation with strain LCC 2021 in 22°P wort at 14.5°C. Bioreactor B = 25 ppm DO at 0 h; Bioreactor C = 25 ppm DO at 12 h; Bioreactor D = 12.5 ppm DO at 12 h; Bioreactor E = 25 ppm DO at 0 and 12 h. Arrow denotes dilution of fermenting wort with oxygenated wort at 12 h post-inoculation.](image)
equivalent of 20 ppb in a beer containing 5% (v/v) ethanol). Even when the beers were warm aged at 14.5°C for several days beyond the time of final attenuation, the diacetyl levels remained elevated (data not shown). The higher than expected diacetyl concentrations observed upon attenuation could be attributed to the laboratory conditions employed, including the constant mixing and lack of a temperature free-rise, and could be remedied at a larger scale. From Table II it is also evident that the control (i.e. Bioreactor B) demonstrated the lowest end of fermentation diacetyl concentration, which may be related to the observations that the contents of this bioreactor achieved the peak cell concentration almost a full 24 hours earlier and attenuated more slowly than the cells present in Bioreactor C and E (refer to Figs. 4 and 5). In this way, compared to the contents of Bioreactor C and E, the cells present in the control fermentation would have experienced prolonged stationary growth thus leading to an extended period of diacetyl reduction.

In addition to the above results, it should also be noted that no wort or beer spoiling contaminants were detected in microbiological samples collected from the bench-scale bioreactors after 24 h of fermentation or at the end of fermentation.

As previously mentioned, the delayed oxygen strategy employed in the current study would tie in well with current industrial practices for breweries that employ multibrew fermenters, wherein the brewery’s bioreactor volumes exceed brewhouse capacity. In this situation, the entire yeast quantity could be introduced into the first (un-oxygenated) brew. Delayed oxygen provision could be achieved by introducing oxygen in-line during specifically

![Fig. 5. The effect of oxygen supply strategy on fermenting wort specific gravity over the course of fermentation with strain LCC 2021 in 22°P wort at 14.5°C. Bioreactor B = 25 ppm DO at 0 h; Bioreactor C = 25 ppm DO at 12 h; Bioreactor D = 12.5 ppm DO at 12 h; Bioreactor E = 25 ppm DO at 0 and 12 h. Arrow denotes dilution of fermenting wort with oxygenated wort at 12 h post-inoculation.](image)

<table>
<thead>
<tr>
<th>Oxygen supply strategy</th>
<th>Wort attenuation time (h)</th>
<th>Final ethanol concentration (g/L)</th>
<th>Final concentration (ppb)</th>
<th>Final concentration normalized to 5% (v/v) ethanol (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioreactor B – 25 ppm DO at 0 h</td>
<td>146</td>
<td>84.3</td>
<td>106.3</td>
<td>49.77</td>
</tr>
<tr>
<td>Bioreactor C – 25 ppm DO at 12 h</td>
<td>96</td>
<td>84.7</td>
<td>126.6</td>
<td>58.94</td>
</tr>
<tr>
<td>Bioreactor D – 12.5 ppm DO at 12 h</td>
<td>+172</td>
<td>82.1</td>
<td>172.7</td>
<td>82.95</td>
</tr>
<tr>
<td>Bioreactor E – 25 ppm DO at 0 and 12 h</td>
<td>120</td>
<td>84.5</td>
<td>133.1</td>
<td>62.14</td>
</tr>
</tbody>
</table>
timed wort transfers beyond the initial, yeast-inoculated, brew. Delayed oxygenation for single-brew or quick-filling industrial fermenters could be addressed either by i) employing in-tank oxygen sparging of the fermenter contents at the optimal time or ii) introducing oxygen in-line during an optimally-timed transfer of fermenting wort from one vessel to another.

**Fermentation performance comparison for control and optimized process condition trials at the 100-L pilot scale**

As depicted in Fig. 6 although the VHG fermentations employing optimized process parameters demonstrated significantly higher peak viable cell concentrations, a slightly lower degree of viable cell growth was actually achieved compared to that observed in control trials (e.g. a 4.3 fold increase versus a 4.6 fold increase for the third successive pitching under optimized and control conditions, respectively). Due to the 40% higher inoculum size employed in the optimized fermentations, it is likely the 22°P wort employed in these trials was unable to nutritionally support the same degree of growth as that observed in the control fermentations. Interestingly, although the peak viable cell concentrations occurred after approximately 48 h of fermentation in both the control and optimized fermentations, only the cells present in the optimized process trials immediately proceeded to fall rapidly out of suspension. Conversely, the cells present in the control fermentations demonstrated a more prolonged period of peak viable cell concentration, followed by a slower rate and lesser degree of cell settling. An analogous trend was observed in the dry weight biomass concentration profiles (data not shown). The differences observed in the yeast flocculation patterns between the control and optimized process fermentations may have been influenced by such factors as inoculation rate, gas-lift mixing patterns, timing and extent of nutrient limitation and by-product formation.35

Figs. 7 and 8 illustrate that significantly improved rates of specific gravity reduction and diacetyl reduction were achieved in the optimized fermentations compared to VHG control fermentations. As depicted in Fig. 7 the fermentations conducted with optimized process parameters always arrived at the final specific gravity (i.e. 3.4°P) and diacetyl (i.e. 42 ppb) targets within 109 h. In contrast, the control fermentations were unable to achieve the same degree of attenuation as the fermentations conducted with altered process parameters, only reducing to a specific gravity of 3.9°P even after 175 h of fermentation time had elapsed. It is also interesting to note that although the timing of peak diacetyl concentration occurred after 48 h of...
fermentation in all instances, the control fermentations demonstrated higher peak concentrations (i.e. 850 ppb versus 650 ppb) and slower rates of diacetyl reduction compared to fermentations carried out under optimized conditions (see Fig. 8). The slower diacetyl reduction rates observed in the control trials cannot be attributed to differences in pH, as the same feed wort was employed in both sets of trials and the pH profiles over the course of fermentation were very similar (data not shown). However, due to the elevated inoculum size employed in the trials conducted under optimized conditions, it is feasible that the period of Group A amino acid uptake in this instance was shortened. Note that during the uptake of Group A amino acids, yeast produces valine (i.e. a Group B amino acid). Therefore, fermentations employing optimized conditions may have experienced a shorter period of valine anabolism and consequently less diacetyl production, as α-acetolactate (i.e. the precursor of diacetyl) is a by-product of valine synthesis. Furthermore, by implementing an earlier and increased free-rise timing and temperature, the thermal decarboxylation of α-acetolactate to diacetyl, and subsequent reduction to acetoin may have been accelerated.

It is apparent from Table III that the fermentations conducted with optimized process parameters demonstrated significantly improved ethanol productivities compared to the control counterparts (i.e. 37% improvement). This improvement in ethanol productivity can be primarily attributed to the reduction in time required to complete fermentation, as final ethanol concentrations, averaged over three serial fermentation cycles, were comparable (refer to Table III). It is also interesting to note that the average percentage of FAN utilized over three fermentation cycles was slightly higher in the optimized process trials, perhaps due to the elevated cell population present. In terms of consistency, the trials conducted with optimized process conditions demonstrated less variability in final ethanol concentration, FAN utilization and ethanol productivity over three serial fermentation cycles (refer to Table III).

Contrary to the widely reported problem of excessive flavour volatile production associated with VHG beer production (attributed to factors such as the excessive gas-lift mixing and higher temperatures associated with this type of fermentation), Table IV illustrates that the average concentrations of key flavour volatiles measured at

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Fig. 7. Wort specific gravity reduction and ethanol formation profile comparisons for the third successive fermentation of pilot-scale fermentations conducted with strain LCC 2034 in 22°P wort. ∅ = Control process specific gravity; ● = Optimized process specific gravity; □ = Control process ethanol concentration; ▲ = Optimized process ethanol concentration. Control fermentations were conducted with the following process conditions: Inoculation rate = 2.2 × 10^7 cell/mL; oxygen delivery strategy = 22 ppm DO prior to inoculation; temperature profile = 14.5°C then increased to 20°C at 48 h post-inoculation. Optimized fermentations were conducted with the following process conditions: Inoculation rate = 3.08 × 10^7 cell/mL; oxygen delivery strategy = 25 ppm DO prior to inoculation; temperature profile = 14.5°C then increased to 21°C at 35 h post-inoculation. Arrows denote dilution of fermenting wort with oxygenated wort at 12 h post-inoculation.
the end of fermentation (normalized to 5% (v/v) ethanol) were all within the expected ranges for commercially produced lager beers. These results were consistent with findings reported by D’Amore\textsuperscript{17} who successfully fermented a 25\textdegree P wort to produce a beer with very similar flavour volatile concentrations as one generated from a 16\textdegree P wort. The lower than expected levels of key flavour volatile compounds may have been influenced by such factors as the C:N ratio of the wort and the presence of elevated levels of yeast cell mass.\textsuperscript{10} Of particular importance, the end of fermentation concentrations of acetaldehyde and diacetyl, both considered beer defects when present in excessive concentrations, were below the flavour threshold in both the control and optimized process trials. One key difference noted when comparing average end of fermentation flavour metabolite values for the two sets of trials, however, was in the average acetaldehyde levels. The average end of fermentation concentration in the optimized process trials was 44\% lower than that observed in the control trials. As the presence of increased levels of acetaldehyde can be an indicator of yeast stress,\textsuperscript{38} this may indicate that the cells exposed to the optimized process conditions were impacted by the harsh VHG environment to a lesser degree than those exposed to the control conditions. It is also interesting to note that the concentrations of fusel alcohols (\textit{i.e.} isobutanol, isoamyl alcohol and 1-propanol) present in the optimized trials were only slightly higher than those observed in the controls. As the formation of fusel alcohols are related to the degree of yeast multiplication, this finding further substantiates that yeast growth in the optimized fermentations was not significantly compromised by the altered process parameters. It should also be noted that the slightly elevated end of fermentation fusel levels observed in the trials conducted under optimized conditions are consistent with the observed increase in FAN consumption, as all of the fusels specified are by-products of the catabolic valine pathway from \textit{\textalpha}-ketoisovaleric acid.\textsuperscript{18,52} Overall, the key flavour metabolites were not negatively influenced by the altered process conditions.

**Table III.** Comparison of final ethanol concentration, FAN utilization and ethanol productivity means over three serial fermentation cycles for pilot-scale trials with strain LCC 2034 in 22\textdegree P wort.

<table>
<thead>
<tr>
<th>Fermentation conditions</th>
<th>Final ethanol (g/L)</th>
<th>FAN utilization\textsuperscript{a} (%)</th>
<th>Ethanol productivity (EP)\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control process</td>
<td>82.61 ± 0.89</td>
<td>62 ± 4</td>
<td>0.52 ± 0.10</td>
</tr>
<tr>
<td>Optimized process</td>
<td>82.74 ± 0.81</td>
<td>71 ± 3</td>
<td>0.71 ± 0.05</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Percent FAN Utilization = (Initial FAN conc.– Final FAN conc.)/Initial FAN conc. * 100%  
\textsuperscript{b}See Equation 3 for definition of ethanol productivity

\textbf{Fig. 8.} Diacetyl profile comparison for the third successive fermentation of pilot-scale trials conducted with strain LCC 2034 in 22\textdegree P wort. □ = Control process parameters (inoculation rate = 2.2 × 10\textsuperscript{7} cell/mL; oxygen delivery strategy = 22 ppm DO prior to inoculation; temperature profile = 14.5\textdegree C then increased to 20\textdegree C at 48 h post-inoculation); ● = Optimized process parameters (inoculation rate = 3.08 × 10\textsuperscript{7} cell/mL; oxygen delivery strategy = 25 ppm DO prior to inoculation; temperature profile = 14.5\textdegree C then increased to 21\textdegree C at 35 h post-inoculation). Arrow denotes dilution of fermenting wort with oxygenated wort at 12 h post-inoculation.
Please note that no wort or beer spoiling contaminants were detected in microbiological samples obtained from the pilot-scale bioreactors after 24 h of fermentation or at the end of fermentation.

**CONCLUSIONS**

In summation, a simple technique for accurately controlling dissolved oxygen concentration at pilot and bench scales was presented to facilitate the reproduction of fermentation behaviour in industrial multi-brew tanks. This process was designed to allow for a great deal of adaptability in terms of wort addition timing and DO concentration, and could be applied to simulate any oxygen supply strategy in multi-brew vessels. The technique developed was employed to identify an effective oxygen provision strategy for batch fermentations with strain LCC 2021 in 22\(^{3}\)P lager wort. The introduction of 25 ppm DO 12 h post-inoculation improved fermentation significantly better than the supply of oxygen immediately prior to inoculation. Under the conditions studied, the delayed oxygen provision approach resulted in a 33% improvement in attenuation time compared to control results. The results concur with previously published works\(^{39,40}\) which demonstrated that, compared to oxygen supply at the onset of fermentation, a delayed oxygenation strategy led to more efficient yeast growth and fermentation performance. This study further revealed that a DO concentration of 12.5 ppm introduced at 12 h post-inoculation was insufficient to fully ferment the wort under the conditions evaluated, suggesting that oxygen sparging (rather than air saturation) is necessary. Additional work would be required to substantiate the bench-scale trial findings and to establish the period of time during which oxygen could be optimally supplied under the conditions studied.

The current work also demonstrated that for batch beer fermentations conducted under VHG conditions in nutritionally rich wort, a multi-variable approach of manipulating standard fermentation process parameters, including increased inoculum size, early and increased free-rise timing and temperature, and delayed and increased supply of oxygen, can successfully enhance and sustain the fermentative power of yeast over several successive fermentation cycles involving serial re-pitching of the yeast. In this way, a 34% reduction in overall fermentation time, and significantly improved ethanol productivity were attained without compromising the final concentrations of key flavour volatiles. All of the strategies introduced in this work could easily be implemented in an industrial brewery setting. This approach could be further extended to benefit the fuel ethanol, wine, spirit and industrial alcohol industries.

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**REFERENCES**


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**Table IV.** Comparison of key flavour metabolite values from pilot-scale trials conducted with strain LCC 2034 in 22\(^{3}\)P wort to reported literature values.

<table>
<thead>
<tr>
<th>Key flavour metabolite</th>
<th>Sensory impact</th>
<th>Typical concentration in beer(^{43})</th>
<th>Flavour threshold(^{43})</th>
<th>Trials with control process conditions</th>
<th>Trials with optimized process conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diacetyl (ppb)</td>
<td>butter, butterscotch</td>
<td>8–60(^{b})</td>
<td>7–20</td>
<td>12.70 ± 1.50</td>
<td>11.59 ± 1.00</td>
</tr>
<tr>
<td>Acetaldehyde (mg/L)</td>
<td>sour, green apple</td>
<td>2–15</td>
<td>25</td>
<td>10.21 ± 1.96</td>
<td>5.70 ± 1.46</td>
</tr>
<tr>
<td>Ethyl acetate (mg/L)</td>
<td>fruity, solvent-like</td>
<td>8–42</td>
<td>21–30</td>
<td>19.94 ± 0.88</td>
<td>21.42 ± 1.65</td>
</tr>
<tr>
<td>1-Propanol (mg/L)</td>
<td>pungent</td>
<td>3–16</td>
<td>600–800</td>
<td>10.94 ± 0.56</td>
<td>12.68 ± 0.41</td>
</tr>
<tr>
<td>Isobutanol (mg/L)</td>
<td>alcohol</td>
<td>5–20</td>
<td>100–200</td>
<td>14.41 ± 1.02</td>
<td>17.78 ± 1.12</td>
</tr>
<tr>
<td>Isoamyl acetate (mg/L)</td>
<td>banana, pear</td>
<td>0.8–6.6</td>
<td>0.6–1.2</td>
<td>1.49 ± 0.10</td>
<td>1.56 ± 0.07</td>
</tr>
<tr>
<td>Isomyl alcohol (mg/L)</td>
<td>alcohol</td>
<td>30–70</td>
<td>70</td>
<td>55.84 ± 1.38</td>
<td>62.44 ± 0.39</td>
</tr>
</tbody>
</table>

\(^{a}\) EOF = end of fermentation  
\(^{b}\) Although a limited diacetyl flavour is desirable in some ales, any degree of diacetyl flavour in lager is generally regarded as a defect.\(^{62}\)
52. Petersen, E. E., Margaritis, A., Stewart, R. J., Pilkinson, P. H. and Mensour, N. A., The effects of wort valine concentration on


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