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# Enhancing fermentation performance through the reutilisation of wine yeast lees

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## ABSTRACT

Extensive research has been dedicated to elucidating the role of various nitrogen sources, nitrogen concentrations and the timing of addition when modulating grape must fermentation using yeast. The wine industry invests substantial resources in both the vineyard and the winery to provide adequate nitrogen concentrations for fermentation. This approach ensures optimal yeast performance during fermentation and minimises the risk of negative sensory attributes associated with poor ferment nutrition. In addition to wine, the winemaking process produces a substantial quantity of nutrient-rich biomass, a poorly explored resource that, if appropriately recycled, could be used to support the nutrient requirements of other winery fermentations. This study explored the feasibility of using processed yeast lees generated during alcoholic fermentation as a nutrient supplement in subsequent fermentations. Three lees treatment options were assessed: accelerated autolysis, enzymatic lysis and mechanical lysis. The ability of these treatments to achieve complete lysis of yeast cells and release amino acids and trace elements is reported. The addition of processed lysates into grape juice was shown to improve fermentation timeframes and influence the production of yeast-derived fermentation volatile compounds in a dose-dependent manner. This study demonstrates that recycling spent lees from winery waste is feasible and provides some strategies for extracting nutrients from winery waste.

**KEYWORDS:** Yeast lees, accelerated autolysis, organic nitrogen, fermentation

## INTRODUCTION

The availability of yeast assimilable nitrogen (YAN), primarily from inorganic ammonium salts and amino acids, significantly influences the kinetics of grape must fermentation by *S. cerevisiae* (Gobert *et al*., 2019). YAN concentrations vary significantly across grape musts originating from diverse grapevine cultivars, geographical locations and viticultural management techniques (Rapp and Versini, 1995; Ribéreau-Gayon *et al*., 2006; Schreiner *et al*., 2018; Stines *et al*., 2000). In general, yeasts require around 140 mg/L of YAN to meet their basic metabolic requirements for the synthesis of proteins and the production of sufficient biomass to complete fermentation (Beltran *et al*., 2005; Bely *et al*., 1990; Henschke and Jiranek, 1993; Linda, 1999). Insufficient YAN concentrations reduce biomass yields and lower fermentation rates, increasing the risk of a slow and/or stuck fermentation (Alexandre and Charpentier, 1998; Linda, 1999; Salmon, 1989; Varela *et al*., 2004). To counteract YAN deficiencies, winemakers commonly supplement musts with inorganic nitrogen salts such as diammonium phosphate (DAP) and, less commonly, organic nitrogen compounds in the form of yeast cell lysates (Bell and Henschke, 2005; Pozo-Bayón *et al*., 2009; Rigou *et al*., 2021).

Aside from direct impacts on biomass production and fermentation kinetics, the precise composition and concentration of YAN can modulate several specific aspects of yeast secondary metabolism (Albers *et al*., 1996; Bell and Henschke, 2005; Garde-Cerdán and Ancín-Azpilicueta, 2008). The production of non-volatile compounds, including glycerol, succinic acid, α-ketoglutaric acid and malic acid, are directly impacted by the nitrogen source and concentration (Albers *et al*., 1996; Camarasa *et al*., 2003; Vilanova *et al*., 2007). However, the relationship between nitrogen content and the production of volatile compounds by yeast during wine fermentation is more complex. It is affected by several factors, including the type and concentration of nitrogen available and the specific class of volatile compound in question (Gobert *et al*., 2019). For example, amino acids have been shown to directly impact the production of higher alcohols, esters and volatile acids (Bell and Henschke, 2005; Torrea *et al*., 2011; Vilanova *et al*., 2007), while initial YAN availability influences the production of higher alcohols via the Ehrlich pathway (Crépin *et al*., 2017; Rollero *et al*., 2017).

With sufficient available nitrogen, large quantities of biomass can be generated during alcoholic fermentation, which will ultimately settle as lees at the bottom of the fermentation tank along with inorganic matter and grape-derived components (e.g., tartaric acid, polyphenols) (Pérez-Bibbins *et al*., 2015b). The management of these fermentation lees varies across wineries, although it is generally disposed of as waste, processed into low-value streams (capturing residual alcohol via distillation) or integrated into vineyards as compost (De Iseppi *et al*., 2020). However, rather than representing a low-value waste stream, the yeast fraction of wine lees represents a valuable nutritional resource that, when appropriately processed, can serve as a rich source of peptides, amino acids, minerals, vitamins, lipids and polysaccharides (De Iseppi *et al*., 2020; Jacob *et al*., 2019). Recent studies have investigated the potential use of wine lees as a nutritional supplement for the microbial production of various industrially relevant compounds, including xylitol, citric acid and lactic acid (Bustos *et al*., 2004a; Bustos *et al*., 2004b; Salgado *et al*., 2009) as well as a source of food additives such as β-glucans and mannoproteins (Varelas *et al*., 2016). Furthermore, a preliminary study has shown the potential benefits of utilising lees as a supplement for grape must fermentation (Rojas *et al*., 2021).

This study explores the viability of utilising wine yeast lees obtained from completed alcoholic fermentations as a nutritional supplement for subsequent grape must fermentations. Our investigation involves evaluating various lysis methodologies and determining appropriate addition quantities to enhance fermentation performance, specifically emphasising practical approaches that can be easily implemented in a winery setting. Through the nutrient recycling approaches explored in this work, we hope to reduce winemaker dependence on external sources of nutrients and thereby contribute to the sustainability goals of the global wine industry.

## MATERIALS AND METHODS

### 1. Production of yeast lees

Fermentations were performed at 17 °C using 0.2 µm sterile filtered Chardonnay grape juice in 10 L sealed Schott bottles under continuous stirring (100 rpm) and sparged with air at a rate of 1 mL/min. *S. cerevisiae* strain AWRI 796 was obtained from the AWRI culture collection and was grown for 24 h in YPD (2 % glucose, 2 % peptone and 1 % yeast) and then for another 24 h in 50/50 grape juice, $H_2O$ before inoculating ($10^6$ CFU/mL) into grape juice. Sugars (glucose + fructose) were monitored daily enzymatically (Hohorst, 1965) with adaptations as described by Vermeir *et al*. (2007). Once dry (< 2 g/L of residual sugars), ferments were cold-settled for 7 days at 4 °C, after which the wine was racked, and the lees were carefully extracted into a separate Schott bottle. The dry matter (DCW g/L) was determined by filtering 2 mL of the lees lysate through a 0.22 µm membrane (Pall Life Sciences Corporation, Melbourne, Australia) and drying at 120 °C using a moisture balance until constant weight was obtained (Model AMB50, Inscale Measurement Technology Ltd., Sussex, UK). The lees were then corrected to 10 % (DCW/v) using Milli-Q water. Two different juices were used to produce yeast lees and their general chemical composition is available in Table S1 (Juice A and Juice C).

### 2. Preparation of yeast lysates

#### 2.1 Accelerated autolysis

The yeast lees suspension 10 % (DCW/v) was adjusted to 3 % (w/v) NaCl as an autolysis promoter as previously described (Liu *et al*., 2008; Sugimoto, 1974) and adjusted to pH 5.5 with NaOH to increase the yield of the lysates (Champagne, 1999). The suspension was then incubated with

continuous stirring (100 rpm) at 55 °C for 48 h to induce the autolytic process. After incubation, autolysing enzymes were heat-inactivated at 100 °C for 10 min. Lysates were then pH corrected to 3.5 with HCl. $SO_2$ was added to the suspension to a total of 100 mg/L, in the form of $K_2S_2O_5$, to prevent microbial spoilage of the lysate.

### 2.2 Enzymatic hydrolysis

The yeast lees suspension 10 % (DCW/v) was adjusted to pH 7.0 using NaOH, after which a commercial food-grade Alcalase (serine endoprotease) (Novozymes, Australia) was added at 0.4 % (w/w) and incubated with continuous stirring (100 rpm) at 55 °C for 48 h. After incubation, the enzyme was heat-inactivated at 100 °C for 10 min and then pH corrected to 3.5 with HCl. $SO_2$ was added to the suspension to a total of 100 mg/L, in the form of $K_2S_2O_5$, to prevent microbial spoilage of the lysate.

### 2.3 Mechanical lysis

Mechanical disruption was performed directly on the yeast lees suspension 10 % (DCW/v) using a high-pressure homogeniser (HPH) (Avestin Emulsiflex C5). The yeast lees suspension was passed three times through the HPH at 172 Mpa. $SO_2$ was added to the suspension to a total of 100 mg/L, in the form of $K_2S_2O_5$, to prevent microbial spoilage of the lysate.

## 3. Characterisation of yeast lysates

### 3.1 Amino acids and YAN analysis

Quantification of individual amino acids was performed on 0.45 µm filtered lysates by Metabolomics Australia (Adelaide, Australia), as previously described by (Boughton *et al*., 2011), using a derivatisation technique with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) and analysed by LC-MS/MS (ESI (+) ionisation). As previously described, YAN concentrations were determined by Affinity Labs (Adelaide, Australia) (Bruce and Christian, 1998; Bergmeyer and Beutler, 1990).

### 3.2 Trace elements analysis

Determination of metals by ICP-MS was performed by Affinity Labs (International Organization for Standardization 17025 accredited laboratory, Adelaide, SA, Australia). Samples were extracted in 2 mL $HNO_3$ + 0.5 mL $H_2O_2$ for 90 min at 90 °C in screw-capped 50 mL polypropylene tubes (Environmental Express). Samples were diluted to a consistent volume of 20 mL with Milli-Q water and mixed thoroughly. Undissolved particles were allowed to settle, and then analysis was performed using a Perkin Elmer Nexion 350D ICP-MS with the KED mode, as previously described (Wheal and Wilkes, 2021).

## 4. Fermentation trials

The impact of lysate supplementation on fermentation performance was assessed by adding 10 mL of accelerated autolysis and Alcalase-treated lees as 10 % (DCW/v) suspensions to 90 mL (10 % (v/v) addition) of two different Chardonnay grape musts (Juice A and Juice B, Table S1) in 100 mL fermentation vessels. To assess the optimal lysate addition proportion, accelerated autolysis and Alcalase-treated lees were incorporated at 1 %, 2 % and 5 % (v/v) into 100 mL Schott bottles containing Chardonnay grape must (Juice A, Table S1). This is equivalent to adding 100, 200 and 500 mg of dry yeast lysates into the 100 mL ferments. For experiments utilising yeast lees obtained from a winery, lysates were incorporated at 2 % and 5 % (v/v) into 100 mL Schott bottles containing Chardonnay grape must (Juice A, Table S1). Control ferments without the addition of yeast lysates were included in all experiments. All vessels were inoculated with $10^6$ CFU/mL of *S. cerevisiae* strain AWRI 796 and fermented at 17 °C with continuous stirring (230 rpm). The sugar concentration was monitored daily using the methodology described in section 1. Once dry (< 2 g/L of residual sugars), ferments were cold-settled for 7 days at 4 °C and then supernatant samples were taken for the quantification of fermentation-derived volatile compounds as previously described (Onetto *et al*., 2020). All fermentations were performed in triplicate.

## 5. Determination of viable cells

Viable cells were determined by dispensing 50 µL aliquots of serially diluted samples obtained directly from the yeast lysates or fermentation flasks onto YPD (1 % w/v yeast extract, 2 % w/v peptone and 2 % w/v D-glucose), Lysine and modified de Man, Rogosa and Sharpe (MRS, Oxoid) agar plates using an automated spiral plater (WASP 2, Don Whitley Scientific, Australia). The agar plates were incubated at 27 °C for approximately 2–5 days and enumerated using a Protocol 3 colony counter (Synopsis, Don Whitley Scientific, Australia). Following this protocol, the detection limit for an undiluted sample is 20 CFU/mL.

# RESULTS AND DISCUSSION

## 1. Evaluation of the effectiveness of lysing methodologies

Wine lees were processed to lyse the yeast cells and liberate the associated peptides, amino acids, minerals, vitamins, lipids and polysaccharides. Three lysing methods (HPH, accelerated autolysis and enzymatic treatment with Alcalase) were tested using a standardised 10 % (DCW/v) lees suspension. The effectiveness of lees treatment was assessed by quantifying the concentration of bioavailable nutrients released by each process (Table 1). The concentration of viable yeast cells in the lysates was also determined as a second measure of treatment efficacy and to evaluate the potential contribution of processed lees to the overall viable population of an active ferment.

Spectrophotometric determination of total α-amino nitrogen and the total concentration of amino acids, as determined through mass spectrometry, revealed differences among the treatments (Table 1). Accelerated autolysis and enzymatic treatments increased ammonia concentrations in the processed lees by 19 and 56 mg/L, respectively, relative to the control and HPH-treated lees. Accelerated autolysis and enzymatic treatments increased the total amino acid concentrations by

17.7–20-fold relative to the control (Table 1). Small, probably insignificant, differences between accelerated autolysis and enzymatic treatments were observed in the concentrations of the majority of individual amino acids. However, large differences were evident in the concentrations of specific amino acids, notably glutamine and glutamate, suggesting distinct proteolytic activities between the two treatments. These variations are likely due to the diverse enzymatic activities of the > 30 intracellular proteolytic enzymes reported in *S. cerevisiae*, which would be prominent in the accelerated autolysis sample (Achstetter and Wolf, 1985) compared to the specific endo-serine peptidase activity of Alcalase. Nevertheless, the Alcalase treatment resulted in a higher overall release of amino acids, indicating concurrent autolytic and Alcalase activities.

**TABLE 1.** Nutrient characterisation of yeast lysates. All values are in mg/L unless otherwise stated.

| Analyte | Lees control | HPH | Accelerated Autolysis | Alcalase |
|---|---|---|---|---|
| YAN | | | | |
| Alpha Amino Nitrogen | 42 | 145 | 459 | 592 |
| Ammonia | <10 | <10 | 29 | 66 |
| Amino acids | | | | |
| Alanine | 9 | 31.9 | 214.4 | 167.2 |
| Arginine | 7.5 | 19 | 70.9 | 74.7 |
| Asparagine | 7 | 16.6 | 82.6 | 52.9 |
| Aspartic acid | 5.8 | 18.5 | 80.4 | 132.4 |
| Cysteine + Cystine | 2.7 | 2.5 | 7.7 | 9.4 |
| Glutamic acid | 20.6 | 78.1 | 289.2 | 560.4 |
| Glutamine | 7.2 | 29.5 | 48.5 | 5.9 |
| Glycine | 8.4 | 21.5 | 75.2 | 55.7 |
| Histidine | 3.2 | 2.7 | 27.6 | 19.3 |
| Isoleucine | 1.3 | 11.4 | 98 | 134.4 |
| Leucine | 5.2 | 34.2 | 245.1 | 283.2 |
| Lysine | 11 | 23.9 | 91 | 84.8 |
| Methionine | 2 | 9.3 | 60.3 | 61.9 |
| Phenylalanine | 6 | 29.1 | 176.4 | 193.2 |
| Proline | 1.9 | 5.8 | 19.8 | 21.6 |
| Serine | 5.8 | 16.6 | 113.8 | 83.9 |
| Threonine | 4 | 13 | 72.3 | 83.9 |
| Tryptophan | 1 | 4.5 | 46.5 | 33.8 |
| Tyrosine | 3.3 | 11.5 | 100.3 | 85.7 |
| Valine | 2.3 | 15.3 | 120.9 | 165 |
| Trace elements | | | | |
| Calcium | 54 | 58 | 76 | 75 |
| Cobalt (ug/L) | 4.9 | 77 | 11.1 | 9.8 |
| Copper | 0.5 | 1.9 | 7.2 | 5.2 |
| Iron | <0.3 | 0.8 | 0.4 | <0.3 |
| Magnesium | 85 | 131 | 165 | 159 |
| Manganese | <0.3 | 0.3 | 0.4 | 0.4 |
| Nickel (ug/L) | 13.6 | 21,335.1 | 72.4 | 43 |
| Potassium | 838 | 1726 | 2216 | 2056 |
| Sodium | 29 | 35 | 13,814 | 2568 |
| Strontium (ug/L) | 301 | 363 | 516 | 449 |
| Zinc (ug/L) | 1936 | 6276 | 8360 | 8643 |

Among the measured free amino acids, glutamic acid, leucine, alanine and phenylalanine were found to be the most abundant in the accelerated autolysis and enzymatic treatments. The hydrophobic amino acids isoleucine, valine, leucine, tryptophan and tyrosine exhibited the greatest increase in concentration relative to the control (Table 1). The increase in hydrophobic amino acid concentrations is consistent with the known properties of Alcalase, having previously been shown to generate peptides with hydrophobic characteristics (Tacias-Pascacio *et al*., 2020). During fermentation, *S. cerevisiae* will preferentially consume amino acids transported by permeases that are not affected by the nitrogen catabolite repression system, such as glutamate, leucine, serine and phenylalanine, amongst others (Crépin *et al*., 2012; Beltran *et al*., 2004; Jiranek *et al*., 1995). Both the accelerated autolysis and enzymatic treatments released relatively high concentrations of these amino acids, therefore representing a valuable nutrient source for the early stages of fermentation.

Metal concentrations were higher in all treated lees (Table 1). The HPH treatment exhibited an unusually high concentration of nickel and cobalt, which could indicate a potential leakage of these metals from the device into the processed lees suspension. The accelerated autolysis treatment contained high concentrations of sodium, which reflects the addition of sodium chloride as an autolysis enhancer. Additionally, using sodium hydroxide during pH adjustment contributed to the higher sodium concentrations in both the accelerated autolysis and enzyme treatments. Accelerated autolysis and enzyme-treated lees also contained elevated concentrations of copper (14.4- and 10.4-fold increase), zinc (4.3- and 4.5-fold increase) and, to a lesser extent, potassium (2.6- and 2.5-fold increase). The release of these ions may be evidence of cell rupture. Potassium leakage, in particular, has been associated with loss of membrane integrity (Beker and Rapoport, 1987).

Analysis of viable cell counts indicated that the application of HPH was ineffective in completely lysing the cells ($3.83 \times 10^8$ CFU/mL), even after the addition of 100 mg/L of total $SO_2$ to the treated samples. Conversely, no detectable viable cells were found following the accelerated autolysis or enzymatic treatments. Similar findings have been reported in studies evaluating mechanical cell disruption methods like HPH, where the limited release of bio-available nutrients from wine yeast lees was observed (Gottardi *et al*., 2022; Belinda Pérez-Bibbins *et al*., 2015a). Although HPH may be suitable for extracting proteins, trace elements and

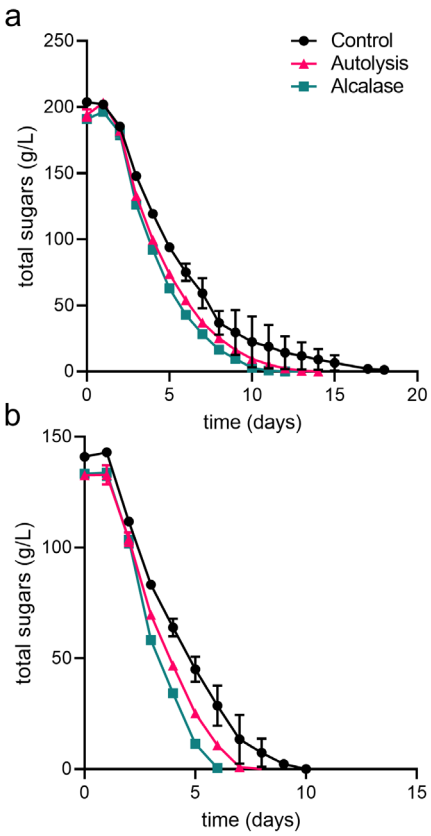


**FIGURE 1.** Effect of lees supplementation on fermentation kinetics.

*S. cerevisiae* strain AWRI 796 sugar consumption kinetics in two Chardonnay grape musts, (a) and (b), supplemented with 10 % (v/v) of two different lees lysates. The control corresponds to the same must without the supplementation of a lees lysate. All data points correspond to the mean of three replicates ± sd. Where error bars are not visible, they are smaller than the points showing the mean value.

cytoplasmic components, it appears unsuitable for re-utilising nutrients from wine lees due to the increased risk of cross-contamination with spoilage microorganisms.

Overall, these results align with previous investigations, highlighting the contribution of both accelerated autolysis and Alcalase treatments in liberating intracellular components during the treatment process (Jacob *et al*., 2019;

**TABLE 2.** The concentration of volatile compounds in two Chardonnay wines supplemented with 10 % (v/v) of lees lysates.

| Volatile compounds | Control Juice A | Control Juice B | Accelerated autolysis Juice A | Accelerated autolysis Juice B | Enzymatic Juice A | Enzymatic Juice B | Unit | Aroma descriptor [b] |
|---|---|---|---|---|---|---|---|---|
| Esters | | | | | | | | |
| Ethyl acetate | 37.7(5.3) | 26.0(12.2) | 50.0(4.0)* | 35.7(1.3) | 54.4(1.1)* | 34.8(0.6) | mg/L | Fruit, ether |
| Ethyl propanoate | 658.1(232.4) | 614.4(367.3) | 393.3(60.5) | 369.5(31.1) | 396.9(21.5) | 253.0(22.3) | ug/L | Plastic |
| Ethyl 2-methylpropanoate | 10.1(3.1) | 12.3(8.3) | 7.4(0.8) | 9.6(1.2) | 7.8(0.6) | 8.4(1.1) | ug/L | Confectionary-raspberry, apple |
| Ethyl butanoate | 230.2(46.7) | 102.3(25.2) | 350.4(33.9) | 171.8(6.8) | 424.1(35.7)* | 214.8(17.1) | ug/L | Confectionary-raspberry, banana |
| Ethyl 3-methylbutanoate | 2.8(1.3) | 3.6(2.0) | 2.7(0.3) | 3.7(0.5) | 3.2(0.2) | 3.1(0.7) | ug/L | Confectionary-musk, apple |
| Ethyl hexanoate | 903.5(302.3) | 353.9(100.8) | 1071.7(142.5) | 443.9(37.0) | 1131.0(112.3) | 540.2(60.4) | ug/L | Tinned apple |
| Ethyl octanoate | 676.8(403.6) | 254.0(167.0) | 1297.2(1131.1) | 682.3(46.5) | 1976.3(357.6)* | 863.7(66.6)* | ug/L | Fruit |
| Ethyl decanoate | 229.0(130.4) | 105.6(94.2) | 1651.2(374.0)* | 569.1(175.8) | 1386.5(79.6)* | 483.7(77.9)* | ug/L | Tropical juice, lemonade |
| | | | | | | | | |
| 2-Methylpropyl acetate | 32.4(9.9) | 13.1(2.8) | 47.0(4.7) | 34.6(2.9) | 53.3(6.1)* | 44.9(7.3) | ug/L | Banana, fruity |
| 2-Methylbutyl acetate | 118.3(64.1) | 24.9(5.3) | 196.5(19.5) | 91.4(3.6)* | 263.6(5.3) | 134.1(18.0)* | ug/L | Banana, fruity |
| 3-Methylbutyl acetate | 2347.8(1621.8) | 349.1(87.7) | 4333.8(402.3) | 1758.4(108.8)* | 5494.4(238.4) | 2535.8(403.9)* | ug/L | Banana |
| Hexyl acetate | 428.3(305.7) | 51.2(28.9) | 749.2(68.8) | 165.9(13.2)* | 843.5(44.2) | 227.2(32.1)* | ug/L | Floral, pineapple |
| 2-Phenyl ethyl acetate | 201.8(77.6) | 101.9(35.2) | 296.7(27.7) | 264.6(23.1)* | 369.3(17.6) | 353.4(39.5)* | ug/L | Floral, rose petal |
| Total | 43.5(7.9) | 28.0(12.3) | 60.4(3.7)* | 40.3(1.6) | 66.8(1.9)* | 40.5(1.2) | mg/L | |
| Higher alcohols | | | | | | | | |
| 2-Methylpropanol | 31.6(11.9) | 22.6(11.0) | 19.5(3.0) | 23.5(1.6) | 18.3(0.3) | 22.6(0.5) | mg/L | Fusel, spiritous |
| 2-Methylbutanol | 68.1(17.4) | 61.5(2.4) | 40.9(7.9) | 43.5(5.2) | 45.2(2.3) | 42.0(3.5)* | mg/L | Nail polish |
| 3-Methylbutanol | 240.1(32.1) | 171.6(27.2) | 189.7(26.0) | 229.0(17.8) | 198.1(5.0) | 216.8(10.4) | mg/L | Cheese, sweat |
| Hexanol | 3.0(0.2) | 1.2(0.1) | 2.3(0.2) | 1.1(0.1) | 2.5(0.0)* | 1.1(0.0) | mg/L | Green, grass |
| 2-Phenyl ethanol | 36.4(8.5) | 45.8(7.4) | 26.4(3.7) | 41.6(3.8) | 28.3(0.4) | 36.6(1.5) | mg/L | Rose |
| Total | 379.4(62.1) | 302.7(36.6) | 278.8(40.5) | 338.8(28.3) | 292.4(7.9) | 319.1(15.3) | mg/L | |
| Volatile acids | | | | | | | | |
| Acetic acid | <LOD (31.4) | <LOD (31.4) | 106.8(6.4)* | 220.3(15.4)* | 107.9(6.2)* | 154.8(20.5)* | mg/L | Vinegar |
| 2-Methylpropanoic acid | 750.4(207.3) | 897.5(417.0) | 538.9(94.8) | 866.3(75.6) | 597.8(14.0) | 664.6(44.1) | mg/L | Cheese, rancid |
| Butanoic acid | 789.6(211.7) | 623.2(218.2) | 891.3(44.1) | 811.1(42.7) | 1103.2(73.4) | 900.2(96.2) | mg/L | Cheese, rancid |
| 2-Methylbutanoic acid | <LOD (400) | 704.6(229.4) | <LOD (400) | 483.9(60.4) | <LOD (400) | 417.7(33.9) | mg/L | Cheese, sweat |
| 3-Methylbutanoic acid | 520.5(317.9) | 815.1(296.0) | 466.8(46.9) | 838.6(76.7) | 567.6(7.0) | 783.3(31.4) | mg/L | Cheese, sweat |
| Octanoic acid | 3.3(1.9) | 1.9(1.3) | 5.8(0.6) | 4.2(0.2) | 6.4(0.7) | 5.0(0.8)* | mg/L | Rancid, harsh |
| Decanoic acid | 771.2(480.1) | 528.6(434.7) | 1452.4(146.2) | 826.2(137.7) | 1582.8(141.3) | 825.2(204.2) | mg/L | Leather, dusty |
| Total | 37.9(1.6) | 36.7(0.7) | 116.3(5.7)* | 228.3(15.5)* | 118.5(7.0)* | 163.4(19.5)* | mg/L | |

Values are mean (sd) of 3 incubation replicates. [b] Odour descriptors obtained from the literature (Siebert *et al.*, 2018; Siebert *et al.*, 2005). LOD: Limit of detection. * = Significant values ($p < 0.05$) within the row according to Dunnett's test.

Liu *et al*., 2016; Takalloo *et al*., 2020). Accelerated autolysis and Alcalase, therefore, represent more compatible technologies for extracting bioavailable nutrients from lees than mechanical disruption. Nevertheless, modifying the conditions for the accelerated autolysis and enzymatic treatments might be required to lower the final sodium concentrations in these lees extracts when translating these techniques for use in a commercial winery context.

## 2. Influence of lees supplementation on fermentation performance and volatile compounds

Extracts obtained from the accelerated autolysis and Alcalase treatments were used to evaluate treated lees as nutrient supplements to alcoholic fermentation because of their higher release of nitrogenous compounds and the absence of measurable viable cells of *S. cerevisiae*. Both extracts were added at 10 % (v/v) into two separate Chardonnay grape musts (Table S1) as 10 % (DCW/v) aqueous suspensions before inoculation with *S. cerevisiae* strain AWRI 796.

The kinetics of sugar fermentation revealed notable differences between treatments (Figure 1). Fermentations supplemented with accelerated autolysis and Alcalase-treated lees finished fermentation (< 2 g/L of residual sugars) four and six days earlier, respectively, than the control ferment for Juice A (Figure 1a) and three and four days earlier, respectively, for Juice B (Figure 1b). The observed

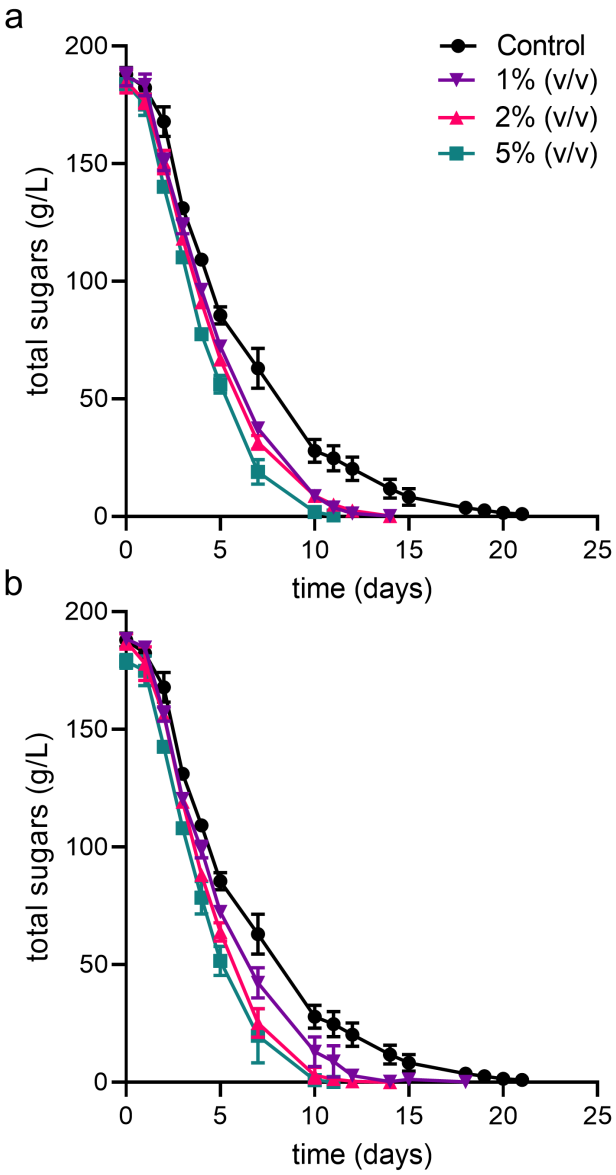


**FIGURE 2.** Effect of lees supplementation proportion on fermentation kinetics.

*S. cerevisiae* strain AWRI 796 sugar consumption kinetics in a Chardonnay grape must supplemented with different proportions of accelerated autolysis- (a) and Alcalase- (b) treated lees. The control corresponds to the same must without the supplementation of a lees lysate. All data points correspond to the mean of three replicates ± sd. Where error bars are not visible, they are smaller than the points showing the mean value.

enhancements in fermentation kinetics could be attributed to the increased YAN, which has been previously shown to reduce fermentation timeframe and rate in a dose-dependent manner (Torrea *et al*. 2011; Gobert *et al*. 2019). Furthermore, supplementation with trace elements such as potassium has also been shown to impact fermentation performance, particularly in deficient musts (Schmidt *et al*., 2011). While vitamins were not quantified here, previous studies have observed a high release of vitamins after autolytic treatments (Jacob *et al*., 2019), which are also known to improve fermentation efficiency in some cases (Ough *et al*., 1989).

In addition to its effects on fermentation time, supplementation with treated lees significantly affected the final concentration of fermentation-derived volatile compounds, including esters, volatile acids and higher alcohols (Table 2). Volatiles fluctuated in a similar pattern as previously reported in Chardonnay grape must supplemented with amino acids (Torrea *et al*., 2011). Several higher alcohols and esters showed inverse and direct relationships, respectively, with the addition of yeast extracts (Table 2). There was evidence that lees treatment of both juices increased the concentration of volatile esters. However, the magnitude of the effect and the number of compounds for which there was evidence of an effect was greater in juices supplemented with Alcalase-treated lees (Table 2). This effect is likely attributed to the higher concentration of amino acids liberated by this processing methodology (Table 1).

A decrease in the concentration of specific higher alcohols was observed, including hexanol in Juice A and 2-methylbutanol in Juice B (Table 2). The reduction in 2-methylbutanol and increased acetate esters is likely due to a decrease in carbon flow through the Ehrlich pathway accompanied by an upregulation of alcohol acyl transferases (Torrea *et al*., 2011; Yoshimoto *et al*., 2002). Hexanol concentration in wine typically reflects the combined effect of grape processing; however, its concentration may be modulated by the yeast-mediated reaction to hexyl acetate (Dennis *et al*., 2012). The reaction to hexyl acetate may, at least in part, explain the decrease in hexanol concentration observed here, although the magnitude of the decrease is small and likely not biologically meaningful, with the ANOVA p-value for the analyte being guided by the small variance observed in the enzymatic treatment. The increased production of medium-chain fatty acid esters after nitrogen supplementation is consistent with previous reports (Saerens *et al*., 2008) and could be explained by an increased synthesis of fatty acids under lees supplementation.

Consistent with the findings of Torrea *et al*. (2011), the concentration of acetic acid was also significantly elevated in fermentations supplemented with accelerated autolysis and Alcalase-treated lees extracts.

## 3. Determination of a lees supplementation threshold

Fermentation trials initially conducted with the supplementation of accelerated autolysis and Alcalase-treated lees demonstrated favourable impacts on fermentation timeframes and significant changes in the concentration of volatile compounds. Nevertheless, in the context of commercial winemaking, adherence to country-specific blending regulations may render 10 % (v/v) lees supplementation infeasible. To address this, experiments were conducted to explore the influence of lower lees supplementation proportions (1 %, 2 % and 5 % (v/v)) on the kinetics of fermentation and the concentration of fermentation-derived volatile compounds.

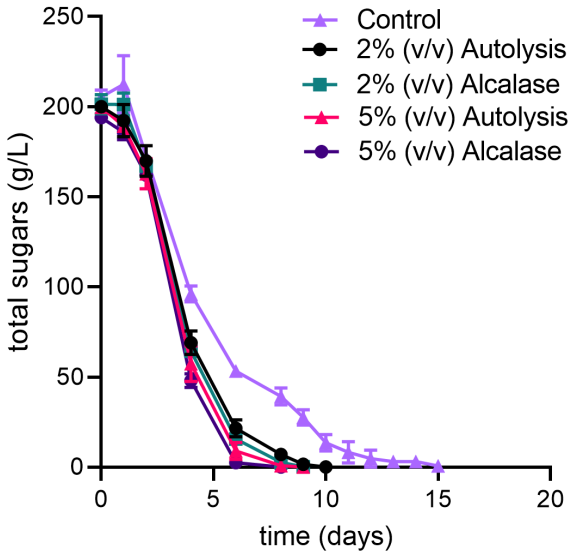


**FIGURE 3.** Effect of winery lees supplementation on fermentation kinetics.

*S. cerevisiae* strain AWRI 796 sugar consumption kinetics in a Chardonnay grape must supplemented with two different proportions of winery post-alcoholic fermentation lees lysed through accelerated autolysis and Alcalase addition. The control corresponds to the same must without the supplementation of a lees lysate. All data points correspond to the mean of three replicates ± sd.

**TABLE 3.** Nutrient characterisation of winery yeast lysates. All values are in mg/L unless otherwise stated.

| Analyte | Lees control | Accelerated autolysis | Enzymatic |
|---|---|---|---|
| YAN | | | |
| Alpha Amino Nitrogen | 116 | 490 | 921 |
| Ammonia | <10 | 27 | 76 |
| Yeast assimilable nitrogen | 124 | 512 | 984 |
| Amino acids | | | |
| Alanine | 56.3 | 241.4 | 329.3 |
| Arginine | 27.0 | 175.9 | 162.5 |
| Asparagine | 79.1 | 140.2 | 171.5 |
| Aspartic acid | 31.7 | 105.2 | 315.7 |
| Cysteine + Cystine | 1.8 | 8.4 | 9.2 |
| Glutamic acid | 41.0 | 173.7 | 443.5 |
| Glutamine | 40.2 | 89.6 | 78.2 |
| Glycine | 11.6 | 63.3 | 66.0 |
| Histidine | 4.8 | 20.4 | 34.4 |
| Isoleucine | 18.0 | 193.2 | 282.5 |
| Leucine | 69.3 | 370.9 | 508.7 |
| Lysine | 34.6 | 278.4 | 241.3 |
| Methionine | 13.4 | 102.4 | 149.7 |
| Phenylalanine | 47.2 | 244.0 | 341.3 |
| Proline | 202.7 | 239.9 | 250.7 |
| Serine | 9.4 | 105.8 | 146.6 |
| Threonine | 7.4 | 113.4 | 157.1 |
| Tryptophan | 7.2 | 54.5 | 89.9 |
| Tyrosine | 17.3 | 144.9 | 210.9 |
| Valine | 22.9 | 223.6 | 285.2 |
| Metals | | | |
| Calcium | 37 | 68 | 60 |
| Cobalt (ug/L) | 2 | 3 | <2.0 |
| Copper | 0.3 | 4.1 | 9.6 |
| Iron | <0.3 | 0.7 | 0.3 |
| Magnesium | 39 | 45 | 40 |
| Manganese | <0.3 | <0.3 | <0.3 |
| Nickel (ug/L) | 10 | 20.7 | 24.1 |
| Potassium | 564 | 1158 | 845 |
| Sodium | 13 | 19,294 | 6017 |
| Strontium (ug/L) | 244 | 1508 | 1321 |
| Zinc (ug/L) | 1694 | 1994 | 1856 |

In agreement with the initial experiments, no detectable viable yeast cells were observed in the extracts after accelerated autolysis and Alcalase treatment. Therefore, any differences between fermentations cannot be due to the direct augmentation of the initial viable yeast concentration. Untreated ferments required 21.3 days (SD 0.45) to complete fermentation, whereas decreased fermentation times were observed in all fermentations supplemented with lees extracts. The magnitude of the change in ferment duration was dependent on the addition of treated lees (Figure 2).

Among the accelerated autolysis treatments, an addition of 5 % (v/v) was the most effective, reducing the mean fermentation time by 11 days (95 % CI, 7.8, 14.5) relative to the control (Figure 2a). Among the Alcalase-treated ferments, a similar decrease in fermentation time was observed, with a mean decrease in fermentation time of 9.9 days. The 2 % (v/v) and 5 % (v/v) Alcalase-treated lees additions were the most stimulatory, with mean decreases in fermentation time of 10.7 days (95 % CI, 7.3, 14.1) and 11.7 days (95 % CI, 8.2, 15.0), respectively. However, there was no evidence ($P = 0.95$) that the 2 % (v/v) and 5 % (v/v) additions differed with respect to each other in their effect on fermentation time (Figure 2b). These results suggest that in a winemaking context, the addition proportion could be adjusted to achieve desired fermentation timeframes or to comply with blending regulations.

The viable cell concentrations were measured four days after inoculation (p-value > 0.05) (Table S2). At that time, large differences in sugar concentration (mean difference; 10.3–31.7 g/L) were already evident between treatments (Figure 2). A mean viable cell concentration of $8.5 \times 10^7$ CFU/mL (SD $6.4 \times 10^6$) was recorded in the untreated control fermentations on day 4. The cell concentrations in the lees-treated ferments ranged from 1–$1.3 \times 10^8$ CFU/mL with a maximum mean difference of $4.4 \times 10^7$ CFU/mL (~52 % increase) recorded for the 2 % (v/v) Alcalase treatments. ANOVA analysis of viable cell concentrations on day 4 provided weak evidence ($P = 0.08$) that 2 % Alcalase-treatments were associated with increased viable cell concentrations. The evidence for the impact of treated lees on viable cell concentrations among the other treatments was even less convincing ($P > 0.2$). We conclude that the addition of treated lees had a minimal effect on viable cell concentrations. Whether this was sufficient to drive the decreases in fermentation time remains an open question.

Several fermentation-derived volatile compounds also exhibited apparent treatment-dependent increases in concentration, however, there was only evidence ($P < 0.05$) for changes in the concentration of 3-methylbutanol and acetic acid across all treatments (Table S3). The metabolism of these fermentation-derived volatiles is known to fluctuate based on the addition of amino acids (Torrea *et al*., 2011). While the additions used in this study demonstrated clear improvements in fermentation performance, they may not have been sufficient to significantly impact the production of fermentation-derived volatiles more frequently associated with positive sensory attributes (e.g., ethyl and acetate esters), as observed with the 10 % addition (Table 2).

### 4. Influence of winery lees supplementation on fermentation performance.

Post-alcoholic fermentation lees generated in a winery are expected to exhibit significant compositional differences from the lees produced in a controlled and sterile laboratory. Winery lees are expected to contain a higher concentration of grape-derived solids and a highly diverse microbial composition, thereby increasing the potential risk of cross-microbial contamination. To address this, post-alcoholic fermentation lees sourced directly from a winery were lysed through either accelerated autolysis or Alcalase addition and assessed for their suitability as a fermentation supplement.

To assess the effects of the treatments against a varied population of wine microbes, yeast and bacteria viable cell counts were monitored before and after treatment to assess the sterilising effectiveness. While a high number of viable cells ($1.74 \times 10^6$ CFU/mL) were observed after plating the untreated lees, no detectable viable cells were observed following either the accelerated autolysis or Alcalase treatment. Although these results are promising, it is essential to conduct further trials to evaluate the effects of these treatments on ferment sanitation. In particular, the control of resilient spoilage microorganisms such as *Brettanomyces bruxellensis*.

In agreement with the initial trials using laboratory-produced lees, accelerated autolysis- and Alcalase-treated winery lees showed an increase in the release of nitrogenous compounds and trace elements (Table 3). Furthermore, Alcalase-treated lees showed a higher concentration of nearly all measured free amino acids. These results confirm that accelerated autolysis and Alcalase treatments are compatible technologies for extracting bioavailable nutrients from winery lees.

Based on the threshold results, treated lees were then incorporated at either 2 % or 5 % (v/v) into a Chardonnay grape must. The sugar consumption kinetics showed a dose-dependent decrease in fermentation duration, with the 5 % Alcalase treatment showing the shortest fermentation times (6.2 days [SD 0.9]), followed by the 5 % accelerated autolysis treatment (6.8 days [SD 1.0]) (Figure 3). Ferments without lees addition required 13.2 days [SD 1.5] to reach a target sugar concentration of 1 g/L. As with previous results, there was no evidence ($P < 0.05$) that processed lees addition changed the viable yeast cell concentration four days after inoculation (Table S4).

## CONCLUSIONS

This study demonstrates the potential of utilising post-alcoholic fermentation wine lees as a nutritional supplement for grape must fermentation. Through accelerated autolysis or enzymatic treatment, significant lysis of yeast cells and subsequent release of amino acids and trace elements were achieved, leading to improved fermentation timeframes and altered fermentation-derived volatile compound production. These findings offer a promising proof-of-concept for nutrient reutilisation. Further research is necessary to assess the practicality and applicability of these treatment methods in a commercial winery.

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## DATA AVAILABILITY

The raw data used to produce Figures 1, 2, 3 and Tables 2 and S4 are available under the dryad repository https://doi.org/10.5061/dryad.h18931zsd.

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