Mannitol bioproduction from surplus grape musts and wine lees

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Abstract: Biological mannitol production at industrial scale is hindered by the elevated costs of fructose-rich feedstocks. Grape must could be an interesting feedstock for this process, due to its large production and to the commercial imbalance caused by unsold wine stocks in producing countries. The feasibility of employing red must and white must for mannitol production was assessed with Lactobacillus fermentum CECT 285, Leuconostoc mesenteroides CECT 8146 and Lactobacillus intermedius NRRL B-3693. By means of experimental design based on response surface methodology (RSM), it was determined that nutrient supplementation could be limited to the addition of manganese and yeast extract, and that grape must should be diluted with water to improve mannitol production. Under optimal conditions, Lb. intermedius NRRL B-3693 produced 68.9 ± 0.57 g/L mannitol from red must and 79.8 ± 0.25 g/L mannitol from white must in 48 h, attaining yields of 0.888 ± 0.014 and 0.895 ± 0.001 mol/mol, respectively. In order to further reduce costs, yeast extract was replaced by wine lees. Red wine lees (RWL) performed better than white wine lees (WWL) as nitrogen source for mannitol production. Grape musts supplemented with manganese and RWL produced 59.4 ± 0.13 g/L mannitol in the case of red must and 65.6 ± 0.06 g/L mannitol in the case of white must in 144 h with Lb. intermedius NRRL B-3693. Therefore, winery surplus and by-products could be used to produce mannitol, thus opening new biorefinery opportunities for rural areas devoted to winegrowing.

Keywords: Mannitol; polyol; grape must; wine lees; Lactobacillales; biorefinery.
1. Introduction

Mannitol (D-mannitol) is a six-carbon alditol which is naturally present in many organisms, like bacteria, yeasts, fungi, algae, lichens and several plants [1]. This polyol has numerous applications in the food and pharmaceutical industries [2–4]. According to Dai et al. [2], the annual consumption of mannitol is approximately 150,000 t. Nowadays, mannitol is mainly produced by chemical synthesis through the hydrogenation of glucose:fructose mixtures (1:1) at high pressure and temperature using Raney nickel as a catalyst, where a mixture of 25.75 mannitol:sorbitol is obtained [2–5]. The technical and economic drawbacks associated with this production route have promoted the research on alternative sources to obtain this polyol, such as extraction from brown algae and Fraxinus trees [2,4], enzymatic routes [2,5] and fermentation with various microorganisms.

Heterofermentative lactic acid bacteria are able to transform fructose, sucrose, inulin and mixtures of fructose-glucose and fructose-sucrose into mannitol [3,4]. These microorganisms ferment hexoses through the 6-phosphogluconate/phosphoketolase pathway under anaerobic conditions, yielding equimolar amounts of lactic acid, ethanol, CO₂ and ATP. However, higher ATP amounts can be obtained if acetate is produced instead of ethanol, but this causes a NAD⁺ deficit that can be solved by reducing electron acceptors such as fructose, thus producing mannitol [1]. Therefore, mannitol is produced as a result of fructose reduction during the fermentation of hexoses. Mannitol yields depend on the type and proportion of sugars fermented. For a typical mixture of 2 mol fructose and 1 mol glucose, the maximum theoretical yield is 0.67 mol mannitol per 1 mol total sugar, or 1 mol mannitol per 1 mol fructose [1,4]. The main disadvantage of mannitol production with lactic acid bacteria using fructose is the cost of the feedstock, a fact which conditions the viability of this fermentation route.

The use of sugar-rich wastes or by-products could constitute a very interesting option to overcome the economic difficulties related to mannitol production by lactic acid bacteria. Sugarcane molasses [6–9], cashew apple juice [10], carob syrup [11], chicory hydrolysate [12], corn fibre hydrolysate [13], Jerusalem artichoke [14] and apple juice [15] have been assessed so far for mannitol production with very variable results. Recently, surplus grape must has been successfully tested as a feedstock for erythritol production (a four-carbon polyol) [16]. Grape must contains 180-220 g/L total sugars, consisting of fructose and glucose in a ratio of roughly 1:1 [17,18]. Although fructose:glucose ratios of 2:1 or 1:0 are preferable for bacterial mannitol production [1,13], surplus grape must could be an interesting feedstock for this process, due to its large volumetric production and to the commercial imbalance and economic problems caused by unsold wine stocks in producing countries [16]. Global wine production attained 29,200 ML in 2018 [19]. Spain ranked third in worldwide wine production during the campaign 2019, by manufacturing about 3,400 ML wine and 604 ML grape juice and must [19,20]. The price of bulk grape must reached barely 0.3037 €/L in Spain in 2019 [21].

In this work, the feasibility of using red grape must and white grape must for mannitol bioproduction was assessed for the first time. Special attention was paid to strain selection, reduction on nutrient supplementation and cost savings during the fermentation stage, including the employment of other winery by-products (i.e. wine lees) as nitrogen source for microorganisms. The aim of this research is the development of a reliable method for mannitol production which can be applied in the near future in winery-related biorefineries.

2. Material and Methods

2.1. Description of grape musts

Grape musts were provided by the Oenological Station of Castile and Leon - ITA CyL (Rueda, Spain). Red grape must (variety Garnacha) was obtained in September 2019 and white grape must (variety Verdejo) was collected in September 2020, and both of them were kept at -20 °C until use. Grape musts were chemically characterised according to Paniagua-García et al. [22]. Red must had a density of 1.10 g/mL and contained 125 g/L glucose, 119 g/L fructose, 0.034% (w/w) total Kjeldahl nitrogen and 0.73 g/L total phenolic compounds. White must had a density of 1.09 g/mL and contained 111 g/L glucose, 116 g/L fructose, 0.069% (w/w) total Kjeldahl nitrogen and 0.15 g/L total phenolic compounds. Their composition in terms of anions and cations is shown in Table 1.

2.2. Wine lees

In some experiments, wine lees were used as nitrogen source for bacterial fermentation, as an alternative to the use of yeast extract. Two types of
wine lees collected in September-November 2020 at the Oenological Station of Castile and Leon - ITACYL (Rueda, Spain) were assessed. Red wine lees (RWL) had a density of 1.05 g/L and contained 9.93% (w/w) ethanol, 1.22% (w/w) total Kjeldahl nitrogen and 1.51 g/L phenolic compounds. White wine lees (WWL) had a density of 1.03 g/L and contained 8.18% (w/w) ethanol, 0.68% (w/w) total Kjeldahl nitrogen and 0.43 g/L phenolic compounds. More information about their chemical characterisation is given in Table 1.

Wine lees (RWL and WWL) were autoclaved in an ultrasound bath (Elma Schmidbauer GmbH, Singen, Germany) for 5 min to inactivate residual vinification yeasts. Afterwards, they were sonicated for 30 min at 330 W and 80 KHz in an Elasonic P 180 H ultrasound bath (Elma Schmidbauer GmbH, Singen, Germany) to provoke cell disruption and release cytoplasmic contents. These treated wine lees were used as nitrogen sources and added to grape musts as explained in section 2.7.2.

Table 1. Contents of anions and cations in grape musts and wine lees.

|          | Red must | White must | Red wine lees (RWL) | White wine lees (WWL) |
|----------|----------|------------|--------------------|----------------------|
| Chloride (mg/L) | 29       | 9          | 31                 | 16                   |
| Fluoride (mg/L)  | 28       | 28         | 19                 | 54                   |
| Phosphate (mg/L) | 185      | 617        | 1180               | 477                  |
| Nitrate (mg/L)   | 1        | 18         | 13                 | 14                   |
| Nitrite (mg/L)   | <1       | <1         | <1                 | <1                   |
| Sulphate (mg/L)  | 49       | 141        | 334                | 334                  |
| Bromide (mg/L)   | <1       | <1         | <1                 | <1                   |
| Sodium (mg/L)    | 3        | 7.63       | 21                 | 14.4                 |
| Potassium (mg/L) | 1447     | 1962       | 7560               | 13184                |
| Calcium (mg/L)   | 143.5    | 82.3       | 251.2              | 213.0                |
| Magnesium (mg/L) | 81.9     | 78.8       | 185.6              | 92.9                 |
| Iron (mg/L)      | <0.1     | 2.06       | 18.7               | 1.29                 |
| Manganese (mg/L) | 0.16     | 0.76       | 3.63               | 4.47                 |
| Zinc (mg/L)      | 0.20     | 0.63       | 5.43               | 2.25                 |
| Copper (mg/L)    | <0.04    | 1.74       | 11.2               | 5.13                 |

2.3. Strain cultivation

Three species of lactic acid bacteria were assessed for mannitol production. The strains Lactobacillus fermentum CECT 285 (= NCIMB 6991) and Leuconostoc mesenteroides CECT 8146 (= NCIMB 6992) were purchased from the Spanish Collection of Type Cultures CECT (Paterna, Spain), whereas the strain Lactobacillus intermedius NRRL B-3693 was obtained from ARS Culture Collection NRRL (Peoria, IL, USA).

For bacterial reactivation, Lb. fermentum CECT 285 and Leuc. mesenteroides CECT 8146 were grown on Man-Rogosa-Sharpe agar (Fluka, Sigma-Aldrich, Buchs, Switzerland) supplemented with Tween 80 (1 mL/L) in Petri dishes at 37 and 30 °C, respectively, under anaerobic conditions. Regarding Lb. intermedius NRRL B-3693, it was grown following a modification of the method described by Saha [6]. In a solid medium containing 50 g/L glucose, 5 g/L yeast extract, 10 g/L peptone, 0.056 g/L MnSO₄·H₂O, 2 g/L ammonium citrate tribasic, 0.205 g/L MgSO₄·7H₂O, 2 g/L Na₂HPO₄ and 20 g/L agar, at 37°C under anaerobic conditions. All the strains were incubated for 24-48 h until the formation of colonies. Afterwards, for all the species, one colony was inoculated into 50 mL of Man-Rogosa-Sharpe broth (Fisher Scientific SL, Madrid, Spain) under a N₂ atmosphere in 100-mL rubber-capped bottles, which were incubated for 24 h at 150 rpm and 37 °C (except for Lec. mesenteroides CECT 8146, whose temperature was 30 °C) in an Infors HT Ecotron orbital shaker (Infors AG, Bottmingen, Switzerland). Then, 1.5 mL of each of the three bacterial suspensions were mixed with 0.4 mL glycerol and preserved at -80 °C.

2.4. Inocula preparation

For preliminary tests of strain selection and for nutrient optimisation experiments, 50 mL of Man-Rogosa-Sharpe broth were placed in a 100-mL glass bottle. These solutions were inoculated with a loopful of microbial cells from the cryopreserved stock of each strain and were capped with a rubber septum. Gaseous N₂ was injected into the headspace of the bottles for 5 min to create anaerobic conditions. The bottles were incubated in an Infors HT Ecotron orbital shaker for 24 h at 150 rpm and 37 °C (CECT 285 and NRRL B-3693) or 30 °C (CECT 8146) until a cell density of about 1·10⁹ cell/mL was attained.

2.5. Fermentation tests for strain selection

In a first stage, the three bacterial strains were compared in order to select the most appropriate one for mannitol production for each of the two grape musts tested. Consequently, red must and white must were supplemented with 10 g/L yeast extract, 0.1 g/L MgSO₄·7H₂O and 0.05 g/L MnSO₄·H₂O and they were sterilised at 121 °C for 15 min. Then, the pH was adjusted to 6.0 with concentrated aqueous solutions of NaOH (2 M and 50 % w/w) and finally 5 g/L CaCO₃ were added as a pH buffer. Fermentation experiments were performed in 100-mL glass bottles containing 50
mL of the abovementioned grape must broths. The bottles were seeded with 3% (v/v) of the Man-Rogosa-Sharpe inocula described in section 2.4, they were capped with rubber septa and gaseous N₂ was bubbled into the broth for 5 min to create anaerobic conditions. Then, the bottles were incubated at 100 rpm and 37 °C (CECT 285 and NRRL B-3693) or 30 °C (CECT 8146) in an Infors HT Ecotron orbital shaker for 72 h. All experiments were performed in triplicate.

2.6. Optimisation of nutrient addition during fermentation

After selecting the most appropriate strain for each type of grape must, an optimisation process was carried out aiming at improving mannitol production by analysing the most adequate nutrient supplementation. A central composite design including response surface methodology (RSM) was applied in order to determine the most suitable concentration values of total initial sugars, yeast extract, MgSO₄·7H₂O and MnSO₄·H₂O (four independent variables) in order to maximise mannitol concentration, mannitol yield and mannitol profit factor (three response variables) (see section 2.8 for calculations). The experimental design consisted of 31 experimental runs including sixteen cube points, seven cube central points, eight axial points and one replicate. The value ranges for each independent variable are shown in Table 2.

Table 2. Minimum and maximum ranges for the independent variables in the RSM experimental design. Note: The different concentrations of initial sugars were obtained by diluting grape musts with distilled water.

|                      | Minimum | Maximum |
|----------------------|---------|---------|
| Total initial sugars | 49 (red) / 45 (white) | 244 (red) / 227 (white) |
| Yeast extract (g/L)  | 0       | 20      |
| MgSO₄·7H₂O (g/L)     | 0       | 0.2     |
| MnSO₄·H₂O (g/L)      | 0       | 0.1     |

Red and white musts containing the nutrients described in Appendix A (Table A.1 and Table A.9) were inoculated with the selected strain and fermented for 72 h according to the conditions provided in section 2.5. Final glucose, fructose, mannitol, acetic acid, lactic acid and ethanol concentrations were measured, and response variables were calculated and employed as input for the RSM model. Response surfaces were calculated for each grape must and the resulting equations were applied to estimate the optimal concentration values of total initial sugars, yeast extract, MgSO₄·7H₂O and MnSO₄·H₂O to obtain the highest mannitol concentration, yield and profit factor. Afterwards, the mathematically estimated optimal points were validated by performing fermentation experiments in triplicate. More details on the experimental design can be found in Appendix A.

2.7. Strategies for cost reduction on mannitol production from grape musts

2.7.1. Use of grape musts as growth media for inocula

After establishing the optimal nutrient concentrations by RSM, various fermentation strategies were evaluated in order to simplify the process and/or reduce costs. The possibility of preparing bacterial inocula in dilute grape must instead of in Man-Rogosa-Sharpe medium was assessed. Liquid growth media based on grape musts were prepared by diluting red or white must with distilled water until a sugar concentration of 50 g/L was attained. Then, diluted red must was supplemented with 7.48 g/L yeast extract and 0.047 g/L MnSO₄·H₂O, whereas white must was supplemented with 7.54 g/L yeast extract and 0.088 g/L MnSO₄·H₂O (Note: yeast extract and MnSO₄·H₂O concentrations correspond to the optimal values explained in section 3.2). These media were seeded with *Lb. intermedius* NRRL B-3693 and cultivated as explained in section 2.4, in order to be used as inocula for fermentation media. The inoculum prepared in red must was used to ferment a broth of red must containing 155.3 g/L sugar, 7.48 g/L yeast extract and 0.047 g/L MnSO₄·H₂O; whereas the inoculum prepared in white must was used to ferment a broth of white must containing 175.7 g/L sugar, 7.54 g/L yeast extract and 0.088 g/L MnSO₄·H₂O (Note: these nutrient concentrations correspond to optimal values in section 3.2). Samples were fermented in triplicate for 72 h as described in section 2.5. Control fermentations of red and white must inoculated with Man-Rogosa-Sharpe inocula were performed simultaneously.

2.7.2. Use of wine lees as nitrogen source in fermentation media

Inocula of *Lb. intermedius* NRRL B-3693 were prepared in diluted red or white must as explained in section 2.7.1. Fermentation media of red or white must did not have the same composition in terms of initial sugars, yeast extract/wine lees and MnSO₄·H₂O as...
described in section 2.7.1. The equivalences between yeast extract and wine lees (RWL or WWL) were calculated to obtain the same total nitrogen (TN) amounts, taking into account that the commercial yeast extract (Fluka) had 11% (w/w) TN. Therefore, fermentable red must was prepared with 155.3 g/L sugar, 7.48 g/L yeast extract (or 67 g/L RWL, or 121 g/L WWL) and 0.047 g/L MnSO₄·H₂O; whereas fermentable white must contained 175.7 g/L sugar, 7.54 g/L yeast extract (or 68 g/L RWL, or 122 g/L WWL) and 0.088 g/L MnSO₄·H₂O. Fermentations were carried out in duplicate under the conditions described in section 2.5, taking samples every 24 h in order to monitor the effect of wine lees on fermentation time. In addition, control fermentations without additional nutrients (yeast extract, RWL, WWL and MnSO₄·H₂O) were also performed. It was considered that fermentations were finished when the increase in mannitol concentration was lower than 2 g/L in a 24-h period.

2.8. Analyses of fermentation broths

Cell density was measured by counting raw samples in a Bürker chamber with a phase-contrast microscope, as described elsewhere [23].

For chemical determinations, fermented samples were filtered through a nylon syringe filter with 0.20 μm pore (Agilent Technologies, Santa Clara, CA, USA) prior to analysis. The concentrations of glucose, fructose and mannitol were analysed by HPLC-RID with an Agilent 1200 HPLC equipment (Agilent) provided with a precolumn Micro-Guard Carbo P (Biorad, Hercules, CA, USA), a column Agilent Guard P (Biorad) and a refractive index detector (RID) G1362A (Agilent). The mobile phase was milli-Q water at a flow rate of 0.6 mL/min. The column temperature was set at 80 °C and the injection volume was 20 μl. The concentrations of acetic acid, lactic acid and ethanol were measured as proposed by [24].

The efficiency of fermentation processes was studied based on different control parameters, such as mannitol concentration (Cₘ, g/L), mannitol yield (Yₘ, mol/mol), glucose consumption (ΔG), fructose consumption (ΔF), total sugar consumption (ΔS), mannitol productivity [Qₘ, g/(L·h)] and mannitol profit factor (πₘ, g/L), according to the following equations:

\[ ΔG = \frac{C_{Gi} - C_{Gf}}{C_{Gi}} \]  
(Eq. 1)

\[ ΔF = \frac{C_{Ff} - C_{Fi}}{C_{Fi}} \]  
(Eq. 2)

\[ ΔS = \frac{(C_{Gi} + C_{Gf}) - (C_{Gf} + C_{Ff})}{C_{Gi}} \]  
(Eq. 3)

\[ Y_M = \frac{m_M}{m_{Gf} - m_{Ff}} \]  
(Eq. 4)

\[ Q_M = \frac{C_M}{t} \]  
(Eq. 5)

\[ π_M = C_M \times ΔF \]  
(Eq. 6)

where letters i and f represent initial and final times, respectively; Cᵢ, Cᵣ and Cᵣ are the concentrations (g/L) of mannitol, glucose and fructose, respectively; mᵢ and mᵣ are the masses (mol) of mannitol and fructose, respectively; and t represents time (h). Yield values (Yₘ) can sometimes be misleading if fructose consumption is low (Eq. 4). In order to avoid that problem, the profit factor (πₘ) was also considered, as it combines in a single figure mannitol production and efficiency in fructose consumption [24].

2.9. Statistical analysis

Response surface methodology (RSM) was applied for optimisation tests by using the software Minitab 16 (Minitab Inc., State College, PA, USA). Comparisons among treatments were analysed with a one-way ANOVA and the Tukey HSD test employing the software Statistica 7 (StatSoft Inc., Tulsa, OK, USA); differences were considered significant when p < 0.05. Curve fitting was performed with SigmaPlot 11 (Systat Software Inc., San José, CA, USA).

3. Results and discussion

3.1. Initial strain selection

Undiluted grape musts (with total sugar concentrations of 225-250 g/L) were used to compare the three tested bacterial strains. Fermentation time was set at 72 h, based on preliminary tests (data not shown).

Red grape must. The highest mannitol concentration was achieved by *Lb. intermedius* NRRL B-3693 (70.5 ± 5.32 g/L), although there were no significant differences among strains for this parameter (Table 3). *Leuc. mesenteroides* CECT 8146 obtained the best mannitol yield (1.076 ± 0.024 mol/mol; p < 0.05), but this value was strongly influenced by its poor fructose consumption. In general, glucose consumption (~25%) and fructose consumption (~50-60%) were low for all the strains with red grape must, although *Lb. intermedius* NRRL B-3693 showed slightly better results for fructose consumption than the others (Table 3).
Productivity values were moderate, in the range of 0.86-0.98 g/(L·h), due to the duration of the fermentation process. \textit{Lb. intermedius} NRRL B-3693 exhibited the highest profit factor ($\eta_m$, 43.3 ± 6.58 g/L), being statistically superior to \textit{Lb. fermentum} CECT 285 (Table 3). In spite of the scarce significant differences among strains, \textit{Lb. intermedius} NRRL B-3693 was selected to perform further optimisation experiments with red grape must, since it offered the highest mannitol concentration and profit factor, and it proved to be able to consume fructose under the tested conditions.

**White grape must.** The strain \textit{Lb. intermedius} NRRL B-3693 was significantly superior ($p < 0.05$) to the others in terms of mannitol concentration (79.4 ± 0.86 g/L), fructose consumption (72.5 ± 0.16 %), total sugar consumption (50.5 ± 0.02 %), productivity [1.103 ± 0.012 g/(L·h)] and profit factor ($\eta_m$, 57.6 ± 0.61 g/L) (Table 3). Accordingly, \textit{Lb. intermedius} NRRL B-3693 was also the strain of choice to perform further experiments with white grape must.

In all cases, biomass development was successful, with bacterial densities of about $10^9$ cells/mL for the three strains, which indicates the adequacy of the feedstocks for microbial growth. In general, mannitol production, glucose consumption and fructose consumption were higher for white must than for red must, a fact which could be due to compositional differences between both feedstocks (Table 1). Sugar consumption (especially glucose) was deficient for the three strains in red and white grape musts. This might be related to an excessive concentration of initial sugars, which provokes substrate inhibition. This hypothesis will be tested in the RSM optimisation experiment, where various sugar concentrations will be assessed (~50-250 g/L) for both grape musts (section 3.2).

In the present work, grape musts were only supplemented with a nitrogen source (yeast extract), Mg$^{2+}$ and Mn$^{2+}$, because these are the conventional nutritive requirements for mannitol production with vegetal-derived feedstocks [8,10,12,14,15], instead of using richer and more expensive nutrient combinations for lactic acid bacteria, such as Man-Rogosa-Sharpé’s nutrients [7]. In fact, as demonstrated with this strain screening, the standard addition of 10 g/L yeast extract, 0.1 g/L MgSO$_4$·7H$_2$O and 0.05 g/L MnSO$_4$·H$_2$O to grape musts resulted in a successful mannitol generation by the three tested strains (Table 3), which corresponds roughly to 1.1 g/L TN, 9.85 mg/L Mg$^{2+}$ and 16.26 mg/L Mn$^{2+}$. However, a fine-tuning of nutrient concentrations for each specific feedstock could lead to higher mannitol concentrations, more efficient sugar consumption or cost reduction on supplement addition.

Although \textit{Lb. intermedius} NRRL B-3693 will be used for further tests in this work – as justified above – the three strains showed good performances for mannitol generation. These strains belong to the most common wild bacteria used for mannitol production [3,4]. The species \textit{Leuc. mesenteroides} can produce dextran (a mucilaginous polysaccharide) from sucrose, but not from glucose or glucose-fructose mixtures [25]. As a consequence, no dextran formation was observed in the present experiment with strain CECT 8146, which simplifies downstream purification processes if this species were to be used industrially for mannitol production from grape musts.

**Table 3.** Fermentation parameters for mannitol production from undiluted grape musts with three different bacterial strains after 72 h (average ± standard deviation). Nutrient supplementation: 10 g/L yeast extract, 0.1 g/L MgSO$_4$·7H$_2$O and 0.05 g/L MnSO$_4$·H$_2$O. Different letters between brackets (a-b for red must, and A-C for white must) indicate the existence of significant differences ($p < 0.05$) between strains for a given parameter.

| Parameter          | Red must  |           |           |           | White must |           |           |
|--------------------|-----------|-----------|-----------|-----------|------------|-----------|-----------|
|                    | CECT 285  | NRRL B-3693 | CECT 8146 | CECT 285  | NRRL B-3693 | CECT 8146 |           |
| Cu (g/L)           | 62.0 ± 0.46 (a) | 70.5 ± 5.32 (a) | 70.1 ± 2.52 (a) | 72.2 ± 0.68 (A) | 79.4 ± 0.86 (B) | 65.5 ± 0.46 (C) |
| $\eta_w$ (mol/mol) | 0.996 ± 0.006 (a) | 0.956 ± 0.016 (a) | 1.076 ± 0.024 (b) | 0.974 ± 0.013 (A) | 0.925 ± 0.011 (B) | 1.017 ± 0.012 (C) |
| $\Delta G$ (%)     | 25.6 ± 0.24 (a) | 25.0 ± 2.28 (a) | 25.2 ± 0.77 (a) | 30.5 ± 0.32 (A) | 28.0 ± 0.14 (B) | 28.3 ± 0.51 (B) |
| $\Delta F$ (%)     | 51.7 ± 0.57 (a) | 61.3 ± 4.92 (b) | 54.1 ± 2.18 (b) | 62.6 ± 0.47 (A) | 72.5 ± 0.16 (B) | 54.4 ± 0.93 (C) |
| $\Delta S$ (%)     | 38.3 ± 0.31 (a) | 42.6 ± 3.55 (a) | 39.2 ± 1.38 (a) | 46.7 ± 0.08 (A) | 50.5 ± 0.02 (B) | 41.5 ± 0.72 (C) |
| Cu [g/(L·h)]       | 0.861 ± 0.006 (a) | 0.979 ± 0.074 (a) | 0.973 ± 0.035 (a) | 1.003 ± 0.009 (A) | 1.103 ± 0.012 (B) | 0.910 ± 0.006 (C) |
| $\eta_m$ (g/L)     | 32.0 ± 0.57 (a) | 43.3 ± 6.58 (b) | 37.9 ± 2.75 (b) | 45.2 ± 0.48 (A) | 57.6 ± 0.61 (B) | 35.6 ± 0.84 (C) |
| Biomass (x10$^6$ cells/mL) | 1.24 ± 0.11 (a) | 1.16 ± 0.06 (a) | 0.84 ± 0.10 (b) | 1.17 ± 0.18 (A) | 1.23 ± 0.04 (A) | 0.77 ± 0.03 (B) |
Newly discovered yeasts have been recently proposed as alternative microorganisms to transform glucose or glycerol into mannitol [5]. Although fungal production of mannitol is known since the 1960s, promising results have been obtained lately, with values in the range of 50-100 g/L mannitol in 3-7 days [26,27].

3.2. Optimisation of nutrient supplementation

The optimal nutrient concentrations (sugars, yeast extract, MgSO$_4$·7H$_2$O and MnSO$_4$·H$_2$O) were mathematically calculated via RSM for both grape musts. A fixed fermentation time of 72 h was established in order to guarantee the completion of the process even for those samples with the highest initial sugar concentrations. The thirty-one fermentation experiments produced variable mannitol concentrations, both for red must (Table A.1) and white must (Table A.9). These data were employed to create model equations for each response variable (C$_m$, Y$_m$, π$_m$), based on the input values of independent variables (sugars, yeast extract, MgSO$_4$·7H$_2$O and MnSO$_4$·H$_2$O). Then, the equations were used to calculate the optimal values of the independent variables which maximised the values of response variables. Further details about RSM analysis and determination of optimal points can be found in Appendix A.

Red must. Mannitol concentrations in the range of 25.8-73.7 g/L were observed in the RSM experiment (Table A.1). Other secondary metabolites, such as lactic acid, acetic acid and ethanol were also produced (Figure A.1); interestingly, there was a strong correlation between mannitol and acetic acid concentrations ($r^2 = 0.9751$), a fact which is in agreement with mannitol metabolic route, where equimolar amounts of these molecules should be produced [1]. Although a similar correlation phenomenon should be expected for lactic acid and mannitol, exceptions were found for fermentations with low initial sugar concentrations (Figure A.1). Regarding the estimation of optimal conditions, several solutions were proposed by the RSM model (Table A.8), but those providing medium-high mannitol concentrations, while reducing reagent addition and saving grape must, were prioritised. According to these estimations, an optimal point established at 155.3 g/L initial sugars, 7.48 g/L yeast extract, 0.00 g/L MgSO$_4$·7H$_2$O and 0.047 g/L MnSO$_4$·H$_2$O would theoretically produce 68.3 g/L mannitol, with a yield of 0.885 mol/mol and a profit factor of 64.5 g/L. This optimal point was validated experimentally, obtaining 66.0 ± 0.21 g/L mannitol, a yield of 0.881 ± 0.005 mol/mol, and a profit factor of 63.4 ± 0.44 g/L; with a glucose consumption of 35.1 ± 0.30 % and a fructose consumption of 96.0 ± 0.54 % (Table A.8). Although the mathematical model had slightly overestimated the responses, this experimental result implies an improvement with regard to the previous non-optimised conditions for red grape must fermentation with Lb. intermedius NRRL B-3693 (Table 3), because, under optimised conditions, similar mannitol concentrations were obtained ($p = 0.2241$), while reducing nutrient addition, saving red must (i.e. grape must was diluted with 36% v/v water) and achieving higher sugar consumption values ($p < 0.05$). It is worth noting that it was possible to rule out the addition of MgSO$_4$·7H$_2$O for the fermentation, and this might be due to the mineral composition of red must, which contains sufficient magnesium for bacterial needs (Table 1).

White must. Mannitol concentrations were in the range of 214.7-71.2 g/L for the thirty-one RSM experiments (Table A.9). Lactic acid, acetic acid and ethanol were produced in lower amounts (Figure A.4). As observed for red must, a positive linear correlation existed between mannitol and acetic acid concentrations for white must ($r^2 = 0.9944$), while polynomial relations seemed to exist for lactic acid or ethanol versus mannitol (Figure A.4). The experimental conditions of run 24 of the RSM were similar to those employed for strain comparison in section 3.1; however, 71.2 g/L mannitol were produced in run 24 (Table A.9), whereas 79.4 g/L had been obtained in the previous experiment (Table 3). This could imply an important effect of inter-day variability, a fact which cannot be ignored if future industrial applications of the process are envisaged. Regarding the fine-tuning of nutrient addition, the most cost-effective solution was selected among the estimations made by the RSM model (Table A.16). Following these estimations, an optimal point established at 175.7 g/L initial sugars, 7.54 g/L yeast extract, 0.00 g/L MgSO$_4$·7H$_2$O and 0.088 g/L MnSO$_4$·H$_2$O, would theoretically lead to a fermentation with 67.0 g/L mannitol, a yield of 0.845 mol/mol and a profit factor of 63.0 g/L. This optimal point was validated experimentally, obtaining 67.6 ± 0.67 g/L mannitol, a yield of 0.817 ± 0.005 mol/mol, and a profit factor of 57.1 ± 0.85 g/L; with a glucose consumption of 31.5 ± 0.44 % and a fructose consumption of 84.4 ± 0.44 % (Table A.16). Despite the overestimation of π$_m$ by the RSM model, the optimised nutrient conditions enabled
cost savings, because white grape must was diluted with 23% v/v water, and the concentration of yeast extract – the most expensive reagent – could be reduced from 10 to 7.54 g/L. In this case, it was also possible to exclude the addition of MgSO₄·7H₂O, as observed previously with red must, thanks to the natural content of magnesium in white must (Table 1).

A general trend towards low sugar consumption was observed as initial sugar concentrations increased (Table A.1, Table A.9), thus supporting the abovementioned hypothesis of a substrate inhibition effect. Sugar consumption started to drop when initial sugar concentrations exceeded 150-160 g/L for both grape must types (Table A.1, Table A.9).

3.3. Cost reduction on mannitol production

The feasibility of using dilute grape musts as growth media for bacteria instead of complex nutrient media was confirmed. For red must, 72.7 ± 0.33 g/L mannitol were obtained employing grape-must inoculum and 69.3 ± 0.82 g/L with Man-Rogosa-Sharpe inoculum; whereas for white must, 80.0 ± 0.81 g/L were produced using grape-must inoculum and 69.9 ± 0.24 g/L with Man-Rogosa-Sharpe inoculum (after 72 h fermentation). In fact, fermentation results were significantly better (p < 0.05) when inocula were prepared in grape musts than in Man-Rogosa-Sharpe medium, both for red and white must (Table A.17). This can be due to bacterial adaptation to a medium with a fructose:glucose ratio similar to that of the subsequent fermentation broth. Another relevant issue was the remarkable difference in mannitol concentration for white must depending on the inoculum nature. Therefore, the inter-day variability mentioned in section 3.2 for white must fermentation could have been related to the state of the inoculum; a fact which was not detected for red must.

Regarding the effect of different nitrogen sources on mannitol production, it was observed that yeast extract was the most effective compound, followed by RWL and WWL (Table 4, Figure 1), both for red must and white must. The kinetics of mannitol production were successfully fitted to the Hill equation in all the cases (Figure 1, Table A.18). Biomass development was also strongly affected by the type of nitrogen source employed (Table 4). In addition, fermentation times were clearly shorter when working with yeast extract (48 h) in comparison to RWL or WWL (144 h) (Figure 1); which could be due to difficulties experienced by bacteria in accessing the nitrogen present in wine lees, a fact that might be improved by applying a different or more severe treatment to wine lees than the one explained in section 2.2. However, the presence of potential bacterial inhibitors in wine lees cannot be discarded.

In the case of red must, the use of yeast extract led to the production of 68.9 g/L mannitol, whereas the use of RWL, WWL or no nutrients produced mannitol concentrations which represented, respectively, 86.3%, 74.3% and 49.9% of that obtained with yeast extract (Table 4). Similarly, in the case of white must, the use of yeast extract enabled the production of 79.8 g/L mannitol, whereas the use of RWL, WWL or no nutrients resulted in mannitol concentrations which corresponded, respectively, to 82.2%, 72.5% and 36.9% of that obtained with yeast extract (Table 4).
Table 4. Fermentation parameters for mannitol production with *Lb. intermedius* NRRL B-3693 using different nitrogen sources (average ± standard deviation). Notes: YE: yeast extract; RWL: red wine lees; WWL: white wine lees.

| Grape must | Initial sugars (g/L) | Yeast extract (g/L) | Lees (g/L) | MnSO$_4$·H$_2$O (g/L) | t (h) | C$_M$ (g/L) | Lactic acid (g/L) | Acetic acid (g/L) | Ethanol (g/L) | $Y_M$ (mol/mol) | $\pi_M$ (g/L) | $\Delta G$ (%) | $\Delta F$ (%) | $\Delta S$ (%) | Q$_M$ [g/(L·h)] | Biomass (x10$^9$ cells/mL) |
|------------|----------------------|---------------------|------------|------------------------|-------|--------------|-------------------|------------------|--------------|----------------|----------------|----------------|----------------|----------------|----------------|-------------|
| Red + YE   | 155.3                | 7.48                | -          | 0.047                  | 48    | 68.9 ± 0.57  | 16.4 ± 0.32      | 11.1 ± 0.16      | <0.20        | 0.888 ± 0.014 | 65.7 ± 0.05  | 35.2 ± 1.18 | 95.4 ± 0.72 | 65.5 ± 0.95 | 1.435 ± 0.012 | 1.19 ± 0.03 |
| Red + RWL  | 155.3                | -                   | 67.4       | 0.047                  | 144   | 59.4 ± 0.13  | 14.1 ± 0.20      | 9.62 ± 0.15      | 3.17 ± 0.02  | 0.875 ± 0.012 | 47.4 ± 0.35  | 38.0 ± 1.18 | 79.8 ± 0.78 | 58.9 ± 0.98 | 0.413 ± 0.001 | 0.78 ± 0.07 |
| Red + WWL  | 155.3                | -                   | 121        | 0.047                  | 144   | 51.2 ± 0.33  | 12.1 ± 0.08      | 8.19 ± 0.06      | 5.31 ± 0.03  | 1.009 ± 0.001 | 35.0 ± 0.41  | 31.3 ± 2.16 | 58.4 ± 0.37 | 50.0 ± 0.12 | 0.355 ± 0.002 | 0.59 ± 0.11 |
| Red        | 155.3                | 0                   | -          | 0                      | 144   | 34.4 ± 3.13  | 8.41 ± 0.52      | 5.81 ± 0.20      | <0.20        | 0.948 ± 0.003 | 15.2 ± 2.71 | 21.9 ± 1.01 | 44.1 ± 1.93 | 33.0 ± 1.26 | 0.239 ± 0.022 | 0.52 ± 0.04 |
| White + YE | 175.7                | 7.54                | -          | 0.088                  | 48    | 79.8 ± 0.25  | 19.4 ± 0.6        | 12.8 ± 0.10      | <0.20        | 0.895 ± 0.001 | 74.6 ± 0.36  | 41.3 ± 0.39 | 93.6 ± 0.16 | 68.6 ± 0.11 | 1.662 ± 0.005 | 1.55 ± 0.05 |
| White + RWL| 175.7                | -                   | 68.0       | 0.088                  | 144   | 65.6 ± 0.06  | 15.8 ± 0.03      | 10.4 ± 0.05      | 3.27 ± 0.03  | 0.986 ± 0.000 | 48.4 ± 0.08  | 37.2 ± 0.18 | 73.8 ± 0.04 | 56.4 ± 0.06 | 0.456 ± 0.000 | 0.78 ± 0.05 |
| White + WWL| 175.7                | -                   | 122        | 0.088                  | 144   | 57.8 ± 1.53  | 14.1 ± 0.52      | 9.29 ± 0.33      | 5.43 ± 0.08  | 0.976 ± 0.020 | 38.5 ± 1.26  | 33.0 ± 0.71 | 66.5 ± 0.41 | 50.8 ± 0.12 | 0.402 ± 0.011 | 0.69 ± 0.01 |
| White      | 175.7                | 0                   | -          | 0                      | 144   | 29.5 ± 2.15  | 7.70 ± 0.49      | 5.30 ± 0.38      | <0.20        | 0.948 ± 0.093 | 9.48 ± 0.45  | 18.1 ± 0.09 | 32.2 ± 0.81 | 25.4 ± 0.38 | 0.205 ± 0.015 | 0.47 ± 0.04 |
Nevertheless, mannitol concentrations produced with wine lees were relatively high (60-66 g/L mannitol with RWL, 50-60 g/L mannitol with WWL) if compared to control fermentations without any nutrient supplementation (29-35 g/L mannitol) (Table 4). Therefore, wine lees are interesting nitrogen sources for mannitol production, although adequate treatment methods and operation conditions need to be developed to facilitate nitrogen release and reduce fermentation times.

The detection of ethanol in fermentation broths supplemented with RWL and WWL (Table 4) is due to the natural presence of ethanol in these winery wastes and not to the metabolic activity of bacteria, since ethanol concentrations remained stable over time.

3.4. Potential of grape must as a feedstock for mannitol production

In recent years, mannitol has been experimentally produced from various alternative agri-food feedstocks (Table 5), such as chicory, Jerusalem artichoke, cashew, carob, apples or sugarcane molasses, in order to reduce substrate costs. Due to the metabolic reasons exposed in section 1, the ideal fructose:glucose (F:G) molar ratio for mannitol production is 2:1; because two thirds of all sugars (corresponding to fructose) are reduced to mannitol, whereas one third of sugars (corresponding to glucose) are fermented into organic acids, ethanol and CO₂. If the F:G ratio is greater than 2:1, the excess fructose can be used for fermentation together with glucose. On the contrary, when F:G ratios are 1:1, theoretically all fructose is reduced to mannitol, half of the glucose is fermented and the other half of the glucose is in excess and remains unconsumed.

Under optimal F:G conditions of 2:1 or greater, mannitol concentrations of ~60 g/L have been obtained with chicory hydrolysate and apple juice [12,15]. The addition of pure glucose or fructose to vegetal feedstocks in order to reach the desired F:G ratio has also been reported, attaining mannitol values of 85-180 g/L depending on the initial sugar concentration [6,12,14]. Gomaa and Rushdy [9] reported mannitol values as high as ~200 g/L from sugarcane molasses, but this result should be considered cautiously, because they stated that their molasses contained important amounts of lactose, which could be an analytical error. Regarding agri-food feedstocks containing approximate F:G ratios of 1:1, they typically produce lower mannitol concentrations, in the range of 18-44 g/L (Table 5), such as those obtained with cashew apple juice, sugarcane molasses or carob syrup [7,8,10,11].

Therefore, the use of grape musts – a feedstock with an F:G ratio of 1:1 – provides mannitol concentrations of ~70-80 g/L, which are superior to those of other agri-food feedstocks with similar or greater F:G ratios (nota bene: excluding those to which external glucose or fructose sources were supplemented). In addition, grape must itself can be used for the stage of bacterial propagation. This opens a new business opportunity for the winery sector.

However, certain challenges should be addressed before mannitol production from grape musts can be performed on an industrial scale. The use of wine lees as an alternative nitrogen source seems promising and it could avoid the dependence on expensive yeast extract; hence, their pretreatment and application methods should be improved. Moreover, as grape must composition can vary depending on the cultivar variety and weather conditions in a particular year, nutrient optimisation should be performed for each type of grape must and nitrogen source employed, in order to maximise mannitol production and reduce reagent costs. Furthermore, cell immobilisation or continuous operation modes could reduce process and nutrient costs [28,29]. It must be highlighted that the presence of non-consumed glucose in the final grape-must fermentation broth (about 50 g/L) can suppose a drawback for downstream processes of mannitol recovery and purification. A second and separated fermentation stage with a microorganism that consumes glucose and fructose but not mannitol, such as Saccharomyces cerevisiae, might be a solution for this problem. In addition, the debris of exhausted S. cerevisiae could be a potential nitrogen source for the previous stage of mannitol production. All these hypotheses could be the object of future scientific works.
Table 5. Bacterial production of mannitol from alternative agri-food feedstocks at laboratory scale. Notes: F: fructose; G: glucose; B: batch; SSF: simultaneous saccharification and fermentation.

| Carbon source                  | Strain                                               | Total sugars (g/L) | F:G | Fermentation type | Time (h) | Mannitol (g/L) | Lactic acid (g/L) | Acetic acid (g/L) | Ethanol (g/L) | $Y_M$ (mol/mol) | $\Delta S$ (%) | $Q_M$ [g/(L·h)] | Reference |
|--------------------------------|------------------------------------------------------|--------------------|-----|-------------------|----------|----------------|-------------------|------------------|---------------|----------------|----------------|----------------|-----------|
| Chicory-derived inulin        | Leuc. pseudomesenteroides CTCC G123 | 112                | 7.82:1 | B                 | 52       | 64.6           | 23.5              | -                | -             | 0.69           | -              | 1.24      | [12]     |
| hydrolysate                   |                                                      |                    |      |                   |          |                |                   |                  |               |                |                |           |          |
| Sugarcane molasses + fructose | Lb. intermedius NRRL B-3693                         | 150                | 4:1  | B                 | 22       | 104.8          | -                 | -                | -             | -              | >95        | -         | [6]      |
| syrup                         |                                                      |                    |      |                   |          |                |                   |                  |               |                |                |           |          |
| Sugarcane molasses (inverted) | Lb. brevis NM101-1, LM-3 (mutant)                   | 447.62             | 2.37:1 | B                  | 24       | 198.95         | 103.78            | 96.86            | -             | -              | 51         | -         | [9]      |
| Apple juice                   | Leuc. citreum TR116                                  | 120                | 2.2:1 | B                 | 48       | 61.6           | 12.64             | 8.51             | -             | 0.96           | 83           | 1.28      | [15]     |
| Cashew apple juice            | Leuc. mesenteroides TR154                            | 120                | 2.2:1 | B                 | 48       | 35.78          | 7.05              | 6.14             | -             | 0.94           | 56           | 0.75      | [15]     |
| Carob syrup                   | Leuc. fructosum NRRL B-2041                          | 92                 | 0.93:1 | B                | 24       | 43.7           | -                 | -                | -             | 1.06           | 91 (F) / 76 (G) | 1.82      | [11]     |
| Red grape must                | Lb. intermedius NRRL B-3693                          | 155.3              | 0.95:1 | B                 | 48       | 68.9           | 16.4              | 11.1             | <0.2         | 0.888          | 95 (F) / 35 (G) | 1.44      | This work |
| White grape must              | Lb. intermedius NRRL B-3693                          | 175.7              | 1.05:1 | B                 | 48       | 79.8           | 19.4              | 12.8             | <0.2         | 0.895          | 94 (F) / 41 (G) | 1.66      | This work |
4. Conclusions

Mannitol can be efficiently produced from red and white grape musts by using conventional wild bacterial strains. Moreover, grape must can be used for microbial propagation before the fermentation stage. Nutrient requirements for an efficient mannitol production with these feedstocks can be limited to the addition of an organic nitrogen source and a manganese salt. Under optimal conditions, using yeast extract as a nitrogen source, about 70-80 g/L mannitol can be produced in 48 h. When wine lees are used as nitrogen sources, about 50-66 g/L mannitol are obtained in 144 h. Thus, grape musts and wine lees are promising substrates for a cost-effective production of biomannitol, which implies new business opportunities for winemakers and opens the possibility of economic revitalisation in winegrowing rural areas by the implementation of biorefineries that use winery surplus and by-products.

Appendix A

Appendix A contains supplementary information about the optimisation of nutrient concentrations for fermentation (via RSM) and about the effects of the nature of inocula and the use of wine lees on mannitol production.

Contributors

MH-V designed the experiments, analysed and processed data, and wrote the manuscript. JG-C designed the experiments and reviewed the manuscript. AP-G analysed data and reviewed the manuscript. RD-A conceived the idea and reviewed the manuscript. All authors have approved the final article.

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APPENDIX A

Mannitol bioproduction from surplus grape musts and wine lees

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Optimization of nutrient concentrations for the fermentation

A central composite design including response surface methodology (RSM) was applied to determine the most adequate values for initial sugars, yeast extract, MgSO₄·7H₂O and MnSO₄·H₂O (four independent variables) in order to maximize mannitol concentration, yield and profit factor (response variables). The experimental design consisted of 31 experimental runs including sixteen cube points, seven cube central points and eight axial points. The value ranges for each independent variable are shown in Table 2. The different conditions tested and their corresponding experimental responses are provided in Table A.1 (red must) and Table A.9 (white must).

For red must, the thirty-one fermentation experiments produced variable mannitol amounts, ranging from 25.8 to 73.7 g/L (Table A.1). The central replicate points (runs 6, 9, 12, 13, 17, 26 and 31 in Table A.1) generated mannitol concentrations of 66.8-68.2 g/L. The statistical analyses of the data showed that one of the independent variables (MgSO₄·7H₂O) was not statistically significant due to its high p values (Tables A.2, A.3 and A.4). Therefore, this variable was removed and the model was recalculated (Tables A.5, A.6 and A.7). Contour plots of these models are represented in Figure A.2 (complete model) and Figure A.3 (model without MgSO₄·7H₂O). Both models (complete and simplified) were used to calculate optimal points to improve response variables according to the following rules: maximise Cₘ in a range of 68.0-75.0 g/L; achieve a Yₘ of 0.90 mol/mol within a valid range of 0.84-0.96 mol/mol; and maximise πₘ in a range of 64.0-69.0 g/L (Note: these limits were established based on the experimental responses of Table A.1). The proposed optimal points are provided in Table A.8 and they were validated experimentally by performing fermentations of red grape must with the nutrients indicated in Table A.8, while the rest of fermentation conditions corresponded to those described in sections 2.5 and 2.6. In all four cases, the model had overestimated the responses, as shown by the values of experimental data (Table A.8). In any case, Optimal Point R4 was selected based on economic reasons, because it provided acceptable mannitol concentrations (66.0 ± 0.21 g/L), while reducing reagent addition and saving grape must. Therefore, optimal nutrient conditions for red grape must were established at 155.3 g/L total sugars, 7.48 g/L yeast extract and 0.047 g/L MnSO₄·H₂O.

For white must, the thirty-one fermentation experiments produced variable mannitol amounts, ranging from 21.2 to 71.2 g/L (Table A.9). The central replicate points (runs 3, 4, 6, 10, 15, 16 and 17 in Table A.9) generated mannitol concentrations of 58.1-59.5 g/L. The statistical analyses of the data showed that several terms of the model were not statistically significant due to their high p values (Tables A.10, A.11 and A.12). Therefore, the terms with p > 0.50 were removed and the model was recalculated (Tables A.13, A.14 and A.15). Contour plots of these models are represented in Figure A.5 (complete model) and Figure A.6 (simplified model). It was observed that high initial sugar concentrations resulted in higher mannitol productions within the studied range. Both models (complete and simplified) were used to calculate optimal points to improve response variables according to the following rules: provide a Cₘ value in a range of 62.0-72.0 g/L; achieve a Yₘ of 0.845 mol/mol within a valid range of 0.800-0.8556 mol/mol; and provide a πₘ value in a range of 58.0-69.0 g/L (Note: these limits were established based on the experimental responses of Table A.9). Among the numerous optimal points estimated by the model which fulfilled the provided calculation rules, three of them were selected based on their estimated Cₘ response and on the possible reduction on reagent costs (Table A.16) and they were validated experimentally by performing fermentations
of white grape must with the nutrients indicated in Table A.16, while the rest of fermentation conditions corresponded to those described in sections 2.5 and 2.6. In all three cases, the model had overestimated the response of $\pi_M$, as shown by the values of experimental data (Table A.16). On the contrary, Optimal Point W3 obtained good estimations for $C_M$ and $Y_M$. In addition, W3 was statistically superior to the other optimal points in terms of glucose and fructose consumption, profit factor and productivity. Moreover, W3 worked with a lower initial sugar concentration, which contributes to cost saving, yet attaining the highest mannitol concentration ($67.6 \pm 0.67$ g/L) of the three evaluated optimal points (Table A.16). Accordingly, optimal nutrient conditions for white grape must were established at 175.7 g/L total sugars, 7.54 g/L yeast extract and 0.088 g/L MnSO$_4$·H$_2$O.
Red grape must

Table A.1. Red grape must. Different conditions tested during the RSM design and their corresponding experimental responses.

| Run | Total initial sugars (g/L) | Yeast extract (g/L) | MgSO$_4$ · 7H$_2$O (g/L) | MnSO$_4$ · H$_2$O (g/L) | C$_{M}$ (g/L) | Y$_M$ (mol/mol) | r$_M$ (g/L) | ΔG (%) | ΔF (%) |
|-----|---------------------------|---------------------|-------------------------|-------------------------|-------------|---------------|-------------|--------|--------|
| 1   | 146                       | 10                  | 0.1                     | 0.1                     | 66.4        | 0.858         | 65.4        | 39.0   | 98.5   |
| 2   | 195                       | 5                   | 0.05                    | 0.025                   | 65.5        | 0.839         | 50.9        | 30.5   | 77.7   |
| 3   | 97                        | 15                  | 0.15                    | 0.075                   | 46.6        | 0.889         | 46.0        | 50.2   | 98.7   |
| 4   | 244                       | 10                  | 0.1                     | 0.05                    | 61.6        | 0.839         | 36.8        | 22.4   | 59.8   |
| 5   | 97                        | 15                  | 0.15                    | 0.025                   | 46.3        | 0.881         | 45.8        | 46.6   | 99.0   |
| 6   | 146                       | 10                  | 0.1                     | 0.05                    | 67.8        | 0.876         | 66.9        | 39.0   | 98.6   |
| 7   | 146                       | 20                  | 0.1                     | 0.05                    | 68.5        | 0.877         | 68.0        | 42.7   | 99.4   |
| 8   | 49                        | 10                  | 0.1                     | 0.05                    | 25.8        | 0.964         | 24.1        | 98.7   | 93.3   |
| 9   | 146                       | 10                  | 0.1                     | 0.05                    | 67.6        | 0.870         | 66.8        | 39.0   | 98.8   |
| 10  | 97                        | 5                   | 0.05                    | 0.025                   | 47.9        | 0.909         | 47.6        | 45.0   | 99.3   |
| 11  | 97                        | 5                   | 0.15                    | 0.075                   | 48.4        | 0.920         | 48.1        | 46.8   | 99.2   |
| 12  | 146                       | 10                  | 0.1                     | 0.05                    | 68.9        | 0.885         | 68.2        | 39.8   | 99.0   |
| 13  | 146                       | 10                  | 0.1                     | 0.05                    | 68.7        | 0.886         | 67.9        | 39.4   | 98.8   |
| 14  | 146                       | 0                   | 0.1                     | 0.05                    | 48.6        | 0.959         | 31.4        | 31.6   | 64.5   |
| 15  | 97                        | 5                   | 0.15                    | 0.025                   | 47.5        | 0.902         | 47.2        | 45.8   | 99.3   |
| 16  | 195                       | 5                   | 0.05                    | 0.075                   | 64.3        | 0.824         | 50.0        | 30.5   | 77.7   |
| 17  | 146                       | 10                  | 0.1                     | 0.05                    | 69.0        | 0.890         | 68.0        | 39.3   | 98.6   |
| 18  | 195                       | 15                  | 0.05                    | 0.075                   | 73.6        | 0.841         | 64.2        | 34.7   | 87.3   |
| 19  | 146                       | 10                  | 0.1                     | 0.05                    | 52.4        | 0.815         | 42.8        | 26.5   | 81.7   |
| 20  | 195                       | 5                   | 0.15                    | 0.075                   | 70.9        | 0.879         | 57.0        | 30.3   | 80.4   |
| 21  | 195                       | 5                   | 0.15                    | 0.025                   | 65.0        | 0.869         | 48.4        | 28.5   | 74.5   |
| 22  | 97                        | 15                  | 0.05                    | 0.075                   | 46.3        | 0.881         | 45.9        | 50.2   | 99.0   |
| 23  | 195                       | 15                  | 0.15                    | 0.025                   | 70.2        | 0.821         | 59.8        | 30.8   | 85.2   |
| 24  | 146                       | 10                  | 0                   | 0.05                    | 69.8        | 0.902         | 68.7        | 37.9   | 98.5   |
| 25  | 97                        | 5                   | 0.05                    | 0.075                   | 47.2        | 0.899         | 46.6        | 45.5   | 98.9   |
| 26  | 146                       | 10                  | 0.1                     | 0.05                    | 67.9        | 0.874         | 67.1        | 37.8   | 98.9   |
| 27  | 97                        | 15                  | 0.05                    | 0.025                   | 47.8        | 0.912         | 47.1        | 46.7   | 98.7   |
| 28  | 195                       | 15                  | 0.15                    | 0.075                   | 73.7        | 0.845         | 64.1        | 34.3   | 86.9   |
| 29  | 146                       | 10                  | 0.2                     | 0.05                    | 69.3        | 0.896         | 68.2        | 38.7   | 98.5   |
| 30  | 195                       | 15                  | 0.05                    | 0.025                   | 72.8        | 0.831         | 63.5        | 32.2   | 87.2   |
| 31  | 146                       | 10                  | 0.1                     | 0.05                    | 68.9        | 0.888         | 68.1        | 39.3   | 98.8   |
Table A.2. Red must. Estimated regression coefficients of mannitol concentration, C_M (g/L). Note: This model includes all the terms.

| Term                                            | Coefficient | Standard error coef. | T     | p    |
|------------------------------------------------|-------------|----------------------|-------|------|
| Constant                                       | -25.96      | 12.120               | -2.142| 0.048|
| Initial sugars (g/L)                           | 0.85        | 0.092                | 9.236 | 0.000|
| Yeast extract (g/L)                            | 1.63        | 0.809                | 2.009 | 0.062|
| MnSO₄·7H₂O (g/L)                               | -49.73      | 80.911               | -0.615| 0.547|
| MnSO₄·H₂O (g/L)                                | 273.11      | 161.823              | 1.688 | 0.111|
| Initial sugars (g/L) * Initial sugars (g/L)   | 0.00        | 0.000                | -10.362| 0.000|
| Yeast extract (g/L) * Yeast extract (g/L)     | -0.09       | 0.023                | -4.038| 0.001|
| MnSO₄·7H₂O (g/L) + MnSO₄·7H₂O (g/L)            | 152.89      | 234.646              | 0.652 | 0.524|
| MnSO₄·H₂O (g/L) + MnSO₄·H₂O (g/L)              | -3460.43    | 938.583              | -3.687| 0.002|
| Initial sugars (g/L) * Yeast extract (g/L)    | 0.01        | 0.003                | 2.298 | 0.035|
| Initial sugars (g/L) * MgSO₄·7H₂O (g/L)       | 0.10        | 0.322                | 0.317 | 0.755|
| Yeast extract (g/L) + MgSO₄·7H₂O (g/L)        | -2.68       | 3.137                | -0.853| 0.406|
| Yeast extract (g/L) + MnSO₄·H₂O (g/L)         | -0.84       | 6.274                | -0.134| 0.895|
| MnSO₄·7H₂O (g/L) + MnSO₄·H₂O (g/L)            | 657.00      | 627.384              | 1.047 | 0.311|

S = 3.13695   PRESS = 897.471
R-square = 96.32% R-square(pred.) = 79.03% R-square(adj usted) = 93.10%

Table A.3. Red must. Analysis of variance of mannitol concentration, C_M (g/L). Note: This model includes all the terms.

| Source                                | df | Sum of squares Seq. | Sum of squares Adjust. | Mean squares Adjust. | F    | p    |
|---------------------------------------|----|---------------------|------------------------|----------------------|------|------|
| Regression                            | 14 | 4122.33             | 294.45                 | 29.92                | 0.000|
| Linear                                | 4  | 2798.76             | 232.69                 | 23.65                | 0.000|
| Initial sugars (g/L)                  | 1  | 2592.93             | 839.42                 | 85.60                | 0.000|
| Yeast extract (g/L)                   | 1  | 151.20              | 39.72                  | 4.04                 | 0.062|
| MnSO₄·7H₂O (g/L)                      | 1  | 0.21                | 3.72                   | 0.38                 | 0.547|
| MnSO₄·H₂O (g/L)                       | 1  | 54.42               | 28.03                  | 2.85                 | 0.111|
| Quadratic                             | 4  | 1246.07             | 311.52                 | 31.66                | 0.000|
| Initial sugars (g/L) * Initial sugars (g/L) | 1  | 960.46              | 1056.31                | 107.35               | 0.000|
| Yeast extract (g/L) * Yeast extract (g/L) | 1  | 141.01              | 160.49                 | 16.31                | 0.001|
| MnSO₄·7H₂O (g/L) + MnSO₄·7H₂O (g/L)   | 1  | 10.84               | 4.18                   | 4.18                 | 0.42  | 0.524|
| MnSO₄·H₂O (g/L) + MnSO₄·H₂O (g/L)     | 1  | 133.76              | 133.76                 | 13.59                | 0.002|
| Interaction                           | 6  | 77.50               | 12.92                  | 1.31                 | 0.307|
| Initial sugars (g/L) * Yeast extract (g/L) | 1  | 51.98               | 51.98                  | 5.28                 | 0.035|
| Initial sugars (g/L) * MgSO₄·7H₂O (g/L) | 1  | 0.99                | 0.99                   | 0.10                 | 0.755|
| Yeast extract (g/L) * MgSO₄·7H₂O (g/L) | 1  | 6.40                | 6.40                   | 0.65                 | 0.432|
| Yeast extract (g/L) * MnSO₄·H₂O (g/L)  | 1  | 7.16                | 7.16                   | 0.73                 | 0.406|
| MnSO₄·7H₂O (g/L) + MnSO₄·H₂O (g/L)    | 1  | 0.18                | 0.18                   | 0.02                 | 0.895|
| MnSO₄·H₂O (g/L) + MnSO₄·H₂O (g/L)     | 1  | 10.79               | 10.79                  | 1.10                 | 0.311|
| Residual error                        | 16 | 157.44              | 157.44                 | 9.84                 |      |
| Lack of fit                           | 10 | 155.31              | 155.31                 | 15.53                | 43.57 | 0.000|
| Pure error                            | 6  | 2.14                | 2.14                   | 0.36                 |      |
| Total                                 | 30 | 4279.78             |                        |                      |      |
Table A.4. Red must. Unusual observations of mannitol concentration, \( C_M \) (g/L). \textit{Note:} This model includes all the terms.

| Obs | Mannitol (g/L) | Fit  | SE Fit | Resid. | Std. Resid.
|-----|----------------|------|--------|--------|------------|
| 7   | 68.450         | 63.942 | 2.396  | 4.508  | 2.23       | R          |
| 14  | 48.620         | 53.902 | 2.396  | -5.282 | -2.61      | R          |
| 19  | 52.350         | 56.736 | 2.396  | -4.386 | -2.17      | R          |

R denotes an observation with a large standardized residue.

Table A.5. Red must. Estimated regression coefficients of mannitol concentration, \( C_M \) (g/L), after removing the variable \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \).

| Term                     | Coefficient | Standard error coeff. | T      | p       |
|--------------------------|-------------|-----------------------|--------|---------|
| Constant                 | -29.70      | 9.144                 | -3.248 | 0.004   |
| Initial sugars (g/L)     | 0.87        | 0.080                 | 10.770 | 0.000   |
| Yeast extract (g/L)      | 1.39        | 0.696                 | 1.998  | 0.059   |
| \( \text{MgSO}_4 \cdot \text{H}_2\text{O} \) (g/L) | 345.31     | 139.201               | 2.481  | 0.022   |
| Initial sugars (g/L) * Initial sugars (g/L) | 0.00       | 0.000                 | -11.215 | 0.000   |
| Yeast extract (g/L) * Yeast extract (g/L) | -0.10      | 0.022                 | -4.417 | 0.000   |
| \( \text{MgSO}_4 \cdot \text{H}_2\text{O} \) (g/L) * \( \text{MgSO}_4 \cdot \text{H}_2\text{O} \) (g/L) | -3525.49   | 872.869               | -4.039 | 0.001   |
| Initial sugars (g/L) * Yeast extract (g/L) | 0.01       | 0.003                 | 2.457  | 0.023   |
| Initial sugars (g/L) * \( \text{MgSO}_4 \cdot \text{H}_2\text{O} \) (g/L) | 0.52       | 0.602                 | 0.862  | 0.398   |
| Yeast extract (g/L) * \( \text{MgSO}_4 \cdot \text{H}_2\text{O} \) (g/L) | -0.84      | 5.868                 | -0.143 | 0.888   |

\( S = 2.93394 \)

PRESS = 727.514

\( \text{R-square} = 95.78\% \)

\( \text{R-square(pred.)} = 83.00\% \)

\( \text{R-square(adjusted)} = 93.97\% \)

A6
Table A.6. Red must. Analysis of variance of mannitol concentration, C_M (g/L), after removing the variable MgSO_4·7H_2O.

| Source                                | df | Sum of squares Seq. | Sum of squares Adjust. | Mean squares Adjust. | F     | p     |
|---------------------------------------|----|---------------------|------------------------|----------------------|-------|-------|
| Regression                            | 9  | 4099.01             | 4099.01                | 455.45               | 52.91 | 0.000 |
| Lineal                                | 3  | 2798.55             | 1011.53                | 337.18               | 39.17 | 0.000 |
| Initial sugars (g/L)                  | 1  | 2592.93             | 998.42                 | 998.42               | 115.99| 0.000 |
| Yeast extract (g/L)                   | 1  | 151.20              | 34.37                  | 34.37                | 3.99  | 0.059 |
| MnSO_4·H_2O (g/L)                     | 1  | 54.42               | 52.97                  | 52.97                | 6.15  | 0.022 |
| Quadratic                             | 3  | 1241.90             | 1241.90                | 413.97               | 48.09 | 0.000 |
| Initial sugars (g/L) * Initial sugars (g/L) | 1  | 960.46              | 1082.74                | 1082.74              | 125.78| 0.000 |
| Yeast extract (g/L) * Yeast extract (g/L) | 1  | 141.01              | 167.94                 | 167.94               | 19.51 | 0.000 |
| MnSO_4·H_2O (g/L) * MnSO_4·H_2O (g/L) | 1  | 140.42              | 140.42                 | 140.42               | 16.31 | 0.001 |
| Interaction                           | 3  | 58.56               | 58.56                  | 19.52                | 2.27  | 0.110 |
| Initial sugars (g/L) * Yeast extract (g/L) | 1  | 51.98               | 51.98                  | 51.98                | 6.04  | 0.023 |
| Initial sugars (g/L) * MnSO_4·H_2O (g/L) | 1  | 6.40                | 6.40                   | 6.40                 | 0.74  | 0.398 |
| Yeast extract (g/L) * MnSO_4·H_2O (g/L) | 1  | 0.18                | 0.18                   | 0.18                 | 0.02  | 0.888 |
| Residual error                        | 21 | 180.77              | 180.77                 | 8.61                 |       |       |
| Lack of fit                           | 5  | 149.90              | 149.40                 | 29.88                | 15.24 | 0.000 |
| Pure error                            | 16 | 31.37               | 31.37                  | 1.96                 |       |       |
| Total                                 | 30 | 4279.78             |                       |                      |       |       |

Table A.7. Red must. Unusual observations of mannitol concentration, C_M (g/L), after removing the variable MgSO_4·7H_2O.

| Obs | Mannitol (g/L) | Fit  | SE Fit  | Resid. | Std. Resid. |
|-----|----------------|------|---------|--------|-------------|
| 7   | 68.450         | 64.170| 2.217   | 4.280  | 2.23        | R       |
| 14  | 48.620         | 54.130| 2.217   | -5.510 | -2.87       | R       |
| 19  | 52.350         | 56.964| 2.217   |-4.614  | -2.40       | R       |

R denotes an observation with a large standardized residue.
Table A.8. Red grape must. Optimal values of nutrient addition calculated according to the RSM models. Estimated and experimental values are shown. Fermentation time 72 h.

| Model  | Initial sugars (g/L) | Yeast extract (g/L) | MgSO₄·7H₂O (g/L) | MnSO₄·H₂O (g/L) | Cₘ (g/L) | Yₘ (mol/mol) | πₘ (g/L) | Lactic acid (g/L) | Acetic acid (g/L) | Ethanol (g/L) | ΔG (%) | ΔF (%) | ΔS (%) | Qₘ [g/(L·h)] | Biomass (x10⁹ cells/mL) |
|--------|----------------------|---------------------|------------------|-----------------|-----------|--------------|---------|------------------|------------------|-------------|--------|--------|--------|-----------|---------------------|
| R1     | Complete             | 172.8               | 8.48             | 0.2             | 0.069     | 74.8         | 0.899   | 69.2             | 69.5 ± 1.34       | 64.7 ± 0.013 | 16.9 ± 0.08 | 11.5 ± 0.10 | 0.36 ± 0.01 | 32.7 ± 0.18 | 93.1 ± 0.52 | 63.0 ± 0.31 | 0.965 ± 0.019 | 1.15 ± 0.07 |
| R2     | Complete             | 172.8               | 8.48             | 0               | 0.069     | > 68.0       | > 0.840 | > 64.0           | 68.3 ± 0.072     | 62.7 ± 1.21  | 16.7 ± 0.14 | 11.4 ± 0.13 | 0.24 ± 0.03 | 31.8 ± 0.55 | 92.2 ± 0.80 | 62.1 ± 0.67 | 0.945 ± 0.010 | 1.21 ± 0.21 |
| R3     | Without MgSO₄·7H₂O   | 168.8               | 11.51            | 0               | 0.058     | 73.4         | 0.867   | 70.2             | 69.0 ± 0.38      | 66.0 ± 0.39  | 16.9 ± 0.02 | 11.5 ± 0.04 | 0.29 ± 0.02 | 32.3 ± 0.40 | 95.6 ± 0.15 | 64.0 ± 0.27 | 0.958 ± 0.005 | 1.19 ± 0.21 |
| R4     | Without MgSO₄·7H₂O   | 155.3               | 7.48             | 0               | 0.047     | 68.3         | 0.885   | 64.5             | 66.0 ± 0.21      | 63.4 ± 0.44   | 16.3 ± 0.02 | 11.0 ± 0.04 | 0.28 ± 0.05 | 35.1 ± 0.30 | 96.0 ± 0.54 | 65.0 ± 0.50 | 0.917 ± 0.003 | 1.17 ± 0.09 |
Figure A.1. Red must. Production of secondary metabolites as a function of mannitol concentration. These data correspond to the RSM optimisation experiments (Table A.1).

Figure A.2. Red must. Contour plots for mannitol production based on the RSM model, including all independent variables.
Figure A.3. Red must. Contour plots for mannitol production based on the RSM model, excluding the independent variable MgSO₄·7H₂O.
White grape must

Table A.9. White grape must. Different conditions tested during the RSM design and their corresponding experimental responses.

| Run | Total initial sugars (g/L) | Yeast extract (g/L) | MgSO₄·7H₂O (g/L) | MnSO₄·H₂O (g/L) | Cₛₛ (g/L) | Yₛₛ (mol/mol) | nₒ (g/L) | ΔG (%) | ΔF (%) |
|-----|--------------------------|---------------------|------------------|----------------|----------|--------------|---------|--------|--------|
| 1   | 136                      | 20                  | 0.1              | 0.05           | 58.1     | 0.871        | 57.8    | 43.2   | 99.6   |
| 2   | 182                      | 5                   | 0.05             | 0.025          | 65.7     | 0.845        | 58.6    | 33.5   | 89.3   |
| 3   | 136                      | 10                  | 0.1              | 0.05           | 59.0     | 0.887        | 58.6    | 42.1   | 99.4   |
| 4   | 136                      | 10                  | 0.1              | 0.05           | 58.8     | 0.883        | 58.5    | 41.4   | 99.5   |
| 5   | 182                      | 15                  | 0.05             | 0.075          | 69.7     | 0.816        | 68.4    | 37.1   | 98.2   |
| 6   | 136                      | 10                  | 0.1              | 0.05           | 59.5     | 0.892        | 59.2    | 40.5   | 99.5   |
| 7   | 91                       | 15                  | 0.05             | 0.075          | 41.2     | 0.894        | 40.3    | 64.5   | 97.8   |
| 8   | 91                       | 5                   | 0.05             | 0.075          | 42.2     | 0.902        | 41.9    | 54.4   | 99.3   |
| 9   | 182                      | 5                   | 0.05             | 0.075          | 68.4     | 0.855        | 62.9    | 35.3   | 92.0   |
| 10  | 136                      | 10                  | 0.1              | 0.05           | 58.4     | 0.882        | 57.7    | 41.2   | 98.8   |
| 11  | 182                      | 5                   | 0.15             | 0.025          | 64.8     | 0.830        | 58.1    | 33.9   | 89.7   |
| 12  | 91                       | 15                  | 0.15             | 0.075          | 41.3     | 0.897        | 40.4    | 66.1   | 97.8   |
| 13  | 91                       | 15                  | 0.15             | 0.025          | 40.8     | 0.879        | 40.2    | 59.7   | 98.6   |
| 14  | 182                      | 15                  | 0.05             | 0.025          | 69.0     | 0.824        | 66.4    | 36.4   | 96.2   |
| 15  | 136                      | 10                  | 0.1              | 0.05           | 58.3     | 0.875        | 57.9    | 39.6   | 99.4   |
| 16  | 136                      | 10                  | 0.1              | 0.05           | 58.4     | 0.878        | 57.9    | 41.3   | 99.2   |
| 17  | 136                      | 10                  | 0.1              | 0.05           | 58.1     | 0.871        | 57.8    | 41.3   | 99.5   |
| 18  | 182                      | 15                  | 0.15             | 0.075          | 71.0     | 0.831        | 69.7    | 36.4   | 98.2   |
| 19  | 136                      | 10                  | 0                 | 0.05           | 58.4     | 0.876        | 58.1    | 40.6   | 99.5   |
| 20  | 182                      | 15                  | 0.15             | 0.025          | 68.7     | 0.809        | 66.9    | 36.2   | 97.4   |
| 21  | 91                       | 15                  | 0.05             | 0.025          | 40.2     | 0.868        | 39.5    | 58.0   | 98.2   |
| 22  | 136                      | 0                   | 0.1              | 0.05           | 44.0     | 0.938        | 30.8    | 32.6   | 70.0   |
| 23  | 182                      | 5                   | 0.15             | 0.075          | 68.4     | 0.843        | 63.8    | 34.7   | 93.3   |
| 24  | 227                      | 10                  | 0.1              | 0.05           | 71.2     | 0.803        | 58.9    | 30.5   | 82.7   |
| 25  | 91                       | 5                   | 0.05             | 0.025          | 41.6     | 0.894        | 41.1    | 51.6   | 98.8   |
| 26  | 136                      | 10                  | 0.1              | 0               | 49.5     | 0.789        | 46.5    | 30.6   | 93.8   |
| 27  | 45                       | 10                  | 0.1              | 0.05           | 21.2     | 0.937        | 20.1    | 99.3   | 94.7   |
| 28  | 91                       | 5                   | 0.15             | 0.025          | 41.4     | 0.890        | 40.8    | 51.3   | 98.7   |
| 29  | 136                      | 10                  | 0.1              | 0.1            | 58.9     | 0.884        | 58.7    | 40.2   | 99.5   |
| 30  | 91                       | 5                   | 0.15             | 0.075          | 41.8     | 0.894        | 41.5    | 32.8   | 99.3   |
| 31  | 136                      | 10                  | 0.2              | 0.05           | 58.0     | 0.876        | 57.3    | 41.5   | 98.8   |
Table A.10. White must. Estimated regression coefficients of mannitol concentration, $C_M$ (g/L). *Note:* This model includes all the terms.

| Term                                      | Coefficient | Standard error coef. | T     | p     |
|-------------------------------------------|-------------|----------------------|-------|-------|
| Constant                                  | -9.83       | 9.692                | -1.014| 0.325 |
| Initial sugars (g/L)                      | 0.59        | 0.079                | 7.436 | 0.000 |
| Yeast extract (g/L)                       | 0.95        | 0.647                | 1.467 | 0.162 |
| MgSO$_4$·7H$_2$O (g/L)                    | -34.58      | 64.685               | -0.535| 0.600 |
| MnSO$_4$·H$_2$O (g/L)                     | 118.15      | 129.369              | 0.913 | 0.375 |
| Initial sugars (g/L) * Initial sugars (g/L) | 0.00        | 0.000                | -5.773| 0.000 |
| Yeast extract (g/L) * Yeast extract (g/L) | -0.06       | 0.019                | -3.214| 0.005 |
| MgSO$_4$·7H$_2$O (g/L) * MgSO$_4$·7H$_2$O (g/L) | 110.15     | 187.562               | 0.587 | 0.565 |
| MnSO$_4$·H$_2$O (g/L) * MnSO$_4$·H$_2$O (g/L) | -113.40     | 750.248               | -1.508| 0.151 |
| Initial sugars (g/L) * Yeast extract (g/L) | 0.00        | 0.003                | 1.459 | 0.164 |
| Initial sugars (g/L) * MgSO$_4$·7H$_2$O (g/L) | 0.00        | 0.276                | -0.017| 0.987 |
| Initial sugars (g/L) * MnSO$_4$·H$_2$O (g/L) | 0.38        | 0.552                | 0.687 | 0.502 |
| Yeast extract (g/L) * MgSO$_4$·7H$_2$O (g/L) | 0.80        | 2.507                | 0.318 | 0.755 |
| Yeast extract (g/L) * MnSO$_4$·H$_2$O (g/L) | -1.47       | 5.015                | -0.294| 0.772 |
| MgSO$_4$·7H$_2$O (g/L) * MnSO$_4$·H$_2$O (g/L) | 94.50       | 501.494              | 0.188 | 0.853 |

$S = 2.50747$  
$\text{PRESS} = 573.082$  
$R^2 = 97.82\%$  
$R^2 \text{(pred.)} = 87.59\%$  
$R^2 \text{(adjusted)} = 95.91\%$

Table A.11. White must. Analysis of variance of mannitol concentration, $C_M$ (g/L). *Note:* This model includes all the terms.

| Source                                      | df | Sum of squares | Mean squares | F     | p     |
|---------------------------------------------|----|----------------|--------------|-------|-------|
| Regression                                  | 14 | 4516.29        | 322.592      | 51.31 | 0.000 |
| Linear                                      | 4  | 4228.62        | 97.317       | 15.48 | 0.000 |
| Initial sugars (g/L)                        | 1  | 4135.69        | 347.567      | 55.30 | 0.000 |
| Yeast extract (g/L)                         | 1  | 53.31          | 13.538       | 2.15  | 0.162 |
| MgSO$_4$·7H$_2$O (g/L)                      | 1  | 0.01           | 1.797        | 0.29  | 0.600 |
| MnSO$_4$·H$_2$O (g/L)                       | 1  | 39.60          | 5.24         | 0.83  | 0.375 |
| Quadratic                                   | 4  | 269.92         | 67.481       | 10.73 | 0.000 |
| Initial sugars (g/L) * Initial sugars (g/L) | 1  | 189.36         | 209.565      | 33.33 | 0.000 |
| Yeast extract (g/L) * Yeast extract (g/L)   | 1  | 62.71          | 64.953       | 10.33 | 0.005 |
| MgSO$_4$·7H$_2$O (g/L) * MgSO$_4$·7H$_2$O (g/L) | 1  | 3.56           | 2.168        | 0.34  | 0.565 |
| MnSO$_4$·H$_2$O (g/L) * MnSO$_4$·H$_2$O (g/L) | 1  | 14.30          | 14.299       | 2.27  | 0.151 |
| Interaction                                 | 6  | 17.75          | 2.958        | 0.47  | 0.820 |
| Initial sugars (g/L) * Yeast extract (g/L)  | 1  | 13.38          | 13.377       | 2.13  | 0.164 |
| Initial sugars (g/L) * MgSO$_4$·7H$_2$O (g/L) | 1  | 0.00           | 0.002        | 0.00  | 0.987 |
| Initial sugars (g/L) * MnSO$_4$·H$_2$O (g/L) | 1  | 2.97           | 2.967        | 0.47  | 0.502 |
| Yeast extract (g/L) * MgSO$_4$·7H$_2$O (g/L) | 1  | 0.64           | 0.636        | 0.10  | 0.755 |
| Yeast extract (g/L) * MnSO$_4$·H$_2$O (g/L) | 1  | 0.54           | 0.544        | 0.09  | 0.772 |
| MgSO$_4$·7H$_2$O (g/L) * MnSO$_4$·H$_2$O (g/L) | 1  | 0.22           | 0.223        | 0.04  | 0.853 |
| Residual error                              | 16 | 100.60         | 6.287        |       |       |
| Lack of fit                                 | 10 | 99.15          | 9.915        | 41.12 | 0.000 |
| Pure error                                  | 6  | 1.45           | 0.241        |       |       |
| Total                                       | 30 | 4616.89        |             |       |       |
Table A.12. White must. Unusual observations of mannitol concentration, \( C_M \) (g/L). Note: This model includes all the terms.

| Obs | Mannitol (g/L) | Fit | SE Fit | Resid. | Std. Resid. |
|-----|----------------|-----|--------|--------|-------------|
| 22  | 43.990         | 49.622 | 1.915  | -5.632 | -3.48 | R |
| 26  | 49.540         | 53.234 | 1.915  | -3.694 | -2.28 | R |

R denotes an observation with a large standardized residue.

Table A.13. White must. Estimated regression coefficients of mannitol concentration, \( C_M \) (g/L), after removing terms with \( p > 0.50 \).

| Term                           | Coefficient | Standard error coef. | T      | p      |
|--------------------------------|-------------|----------------------|--------|--------|
| Constant                       | -14.25      | 5.491                | -2.595 | 0.016  |
| Initial sugars (g/L)           | 0.61        | 0.059                | 10.360 | 0.000  |
| Yeast extract (g/L)            | 0.98        | 0.464                | 2.107  | 0.046  |
| MnSO₄·H₂O (g/L)                | 169.21      | 66.589               | 2.541  | 0.018  |
| Initial sugars (g/L) * Initial sugars (g/L) | 0.00 | 0.000 | -6.818 | 0.000 |
| Yeast extract (g/L) * Yeast extract (g/L) | -0.06 | 0.016 | -3.828 | 0.001 |
| MnSO₄·H₂O (g/L) * MnSO₄·H₂O (g/L) | -1178.28 | 642.150 | -1.835 | 0.079 |
| Initial sugars (g/L) * Yeast extract (g/L) | 0.00 | 0.002 | 1.695 | 0.104 |

\[
S = 2.15843 \\
\text{PRESS} = 415.481 \\
\text{R-square = 97.68%} \\
\text{R-square(pred.)} = 91.00\% \\
\text{R-square(adjusted) =} 96.97\%
\]
Table A.14. White must. Analysis of variance of mannitol concentration, $C_M$ (g/L), after removing terms with $p > 0.50$.

| Source                          | df | Sum of squares Seq. | Sum of squares Adjust. | Mean squares Adjust. | F     | p       |
|---------------------------------|----|---------------------|------------------------|----------------------|-------|---------|
| Regression                      | 7  | 4509.73             | 4509.73                | 644.248              | 138.29| 0.000   |
| Lineal                          | 3  | 4228.60             | 524.88                 | 174.961              | 37.55 | 0.000   |
| Initial sugars (g/L)           | 1  | 4135.69             | 500.03                 | 500.033              | 107.33| 0.000   |
| Yeast extract (g/L)            | 1  | 53.31               | 20.69                  | 20.686               | 4.44  | 0.046   |
| MnSO$_4$·H$_2$O (g/L)          | 1  | 39.60               | 30.08                  | 30.083               | 6.46  | 0.018   |
| Quadratic                      | 3  | 267.75              | 267.75                 | 89.251               | 19.16 | 0.000   |
| Initial sugars (g/L) *         | 1  | 189.36              | 216.58                 | 216.576              | 46.49 | 0.000   |
| Initial sugars (g/L)            |    |                     |                        |                      |       |         |
| Yeast extract (g/L) *          | 1  | 62.71               | 68.28                  | 68.276               | 14.66 | 0.001   |
| Yeast extract (g/L)            |    |                     |                        |                      |       |         |
| MnSO$_4$·H$_2$O (g/L) *         | 1  | 15.69               | 15.69                  | 15.686               | 3.37  | 0.079   |
| MnSO$_4$·H$_2$O (g/L)           |    |                     |                        |                      |       |         |
| Interaction                    | 1  | 13.38               | 13.38                  | 13.377               | 2.87  | 0.104   |
| Initial sugars (g/L) * Yeast   | 1  | 13.38               | 13.38                  | 13.377               | 2.87  | 0.104   |
| extract (g/L)                  |    |                     |                        |                      |       |         |
| Residual error                 | 23 | 107.15              | 107.15                 | 4.659                |       |         |
| Lack of fit                    | 7  | 103.66              | 103.66                 | 4.809                | 67.89 | 0.000   |
| Pure error                     | 16 | 3.49                | 3.49                   | 0.218                |       |         |
| Total                          | 30 | 4616.89             |                        |                      |       |         |

Table A.15. White must. Unusual observations of mannitol concentration, $C_M$ (g/L), after removing terms with $p > 0.50$.

| Obs | Mannitol (g/L) | Fit  | SE Fit | Resid. | Std. Resid. | 
|-----|----------------|------|--------|--------|-------------|
| 22  | 43.990         | 49.786 | 1.631  | -5.796 | -4.10       |
| 24  | 71.230         | 74.221 | 1.631  | -2.991 | -2.12       |
| 26  | 49.540         | 53.398 | 1.631  | -3.858 | -2.73       |

R denotes an observation with a large standardized residue.
Table A.16. White grape must. Optimal values of nutrient addition calculated according to the RSM models. Estimated and experimental values are shown. Fermentation time 72 h.

| Model | Independent variables (RSM model) | Estimated responses (RSM model) | Experimental responses (n = 3) |
|-------|----------------------------------|---------------------------------|-----------------------------|
|       | Initial sugars (g/L) | Yeast extract (g/L) | MgSO₄·7H₂O (g/L) | MnSO₄·H₂O (g/L) | C_M (g/L) | Y_M (mol/mol) | π_M (mol/mol) | Lactic acid (g/L) | Acetic acid (g/L) | Ethanol (g/L) | ΔG (%) | ΔF (%) | ΔS (%) | Q_M [g/(L·h)] | Biomass (x10⁹ cells/mL) |
| W1    | Complete | 185.8 | 6.88 | 0.157 | 0.058 | 68.3 | 0.845 | 62.7 | 66.6 ± 0.87 | 0.812 ± 0.008 | 52.4 ± 0.92 | 16.5 ± 0.15 | 11.3 ± 0.10 | 0.41 ± 0.10 | 28.8 ± 0.64 | 78.6 ± 0.46 | 54.9 ± 0.26 | 0.925 ± 0.012 | 1.33 ± 0.15 |
| W2    | Simplified | 200.3 | 7.55 | 0 | 0.065 | 70.7 | 0.838 | 63.4 | 64.9 ± 0.88 | 0.799 ± 0.012 | 47.3 ± 0.58 | 16.2 ± 0.10 | 11.2 ± 0.06 | 0.43 ± 0.15 | 27.5 ± 0.18 | 72.8 ± 0.21 | 51.3 ± 0.20 | 0.901 ± 0.012 | 0.94 ± 0.05 |
| W3    | Simplified | 175.7 | 7.54 | 0 | 0.088 | 67.0 | 0.845 | 63.0 | 67.6 ± 0.67 | 0.817 ± 0.005 | 57.1 ± 0.85 | 16.9 ± 0.21 | 11.6 ± 0.14 | 0.34 ± 0.05 | 31.5 ± 0.44 | 84.4 ± 0.44 | 59.2 ± 0.37 | 0.940 ± 0.009 | 1.26 ± 0.08 |
Figure A.4. White must. Production of secondary metabolites as a function of mannitol concentration. These data correspond to the RSM optimisation experiments (Table A.9).

Figure A.5. White must. Contour plots for mannitol production based on the RSM model, including all independent variables.
Figure A.6. White must. Contour plots for mannitol production based on the RSM model, excluding terms with $p > 0.50$. 
Cost reduction: nature of inocula and use of wine lees

Table A.17. Effect of inoculum nature on grape must fermentation with *L. intermedius* NRRL B-3693 (t = 72 h). Asterisks (*) indicate the existence of significant differences (p < 0.05) between inocula for a certain fermentation broth.

| Inoculum | Fermentation broth | CM (g/L) | Lactic acid (g/L) | Acetic acid (g/L) | Ethanol (g/L) | YM (mol/mol) | ΔG (%) | ΔF (%) | ΔS (%) | Qm [g/(L·h)] | πM (g/L) | Biomass (x10^9 cells/mL) |
|----------|-------------------|----------|------------------|------------------|--------------|--------------|--------|--------|--------|--------------|--------|------------------------|
| Dilute   | Red must          | 72.7 ± 0.33* | 17.3 ± 0.06*     | 11.7 ± 0.05*     | 0 ± 0*       | 0.882 ± 0.005* | 39.7 ± 0.32* | 98.6 ± 0.13* | 69.2 ± 0.19* | 1.010 ± 0.005* | 71.7 ± 0.27* | 1.77 ± 0.21* |
|          | RWL + Mn          | 69.3 ± 0.82 | 16.7 ± 0.17      | 11.2 ± 0.10      | 0.31 ± 0.06  | 0.864 ± 0.01  | 36.5 ± 0.48  | 93.9 ± 0.76  | 66.3 ± 0.62  | 0.962 ± 0.011  | 66.5 ± 1.28 | 2.23 ± 0.21* |
|          | WWL + Mn          | 80.0 ± 0.81*| 18.7 ± 0.14*     | 12.6 ± 0.05*     | 0 ± 0*       | 0.901 ± 0.008*| 38.0 ± 0.81* | 93.6 ± 0.46* | 66.7 ± 0.46* | 1.112 ± 0.011* | 74.9 ± 0.85* | 1.70 ± 0.17* |
|          | No nutrients      | 69.9 ± 0.24 | 17.0 ± 0.03      | 11.5 ± 0.07      | 0.31 ± 0.04  | 0.846 ± 0.010 | 32.3 ± 0.25  | 87.0 ± 0.82  | 60.5 ± 0.30  | 0.971 ± 0.003  | 60.8 ± 0.51 | 1.60 ± 0.17* |
| MRS      | White must        | 72.7 ± 0.33*| 17.3 ± 0.06*     | 11.7 ± 0.05*     | 0 ± 0*       | 0.882 ± 0.005*| 39.7 ± 0.32* | 98.6 ± 0.13* | 69.2 ± 0.19* | 1.010 ± 0.005* | 71.7 ± 0.27* | 1.77 ± 0.21* |
|          | RWL + Mn          | 69.3 ± 0.82 | 16.7 ± 0.17      | 11.2 ± 0.10      | 0.31 ± 0.06  | 0.864 ± 0.01  | 36.5 ± 0.48  | 93.9 ± 0.76  | 66.3 ± 0.62  | 0.962 ± 0.011  | 66.5 ± 1.28 | 2.23 ± 0.21* |
|          | WWL + Mn          | 80.0 ± 0.81*| 18.7 ± 0.14*     | 12.6 ± 0.05*     | 0 ± 0*       | 0.901 ± 0.008*| 38.0 ± 0.81* | 93.6 ± 0.46* | 66.7 ± 0.46* | 1.112 ± 0.011* | 74.9 ± 0.85* | 1.70 ± 0.17* |
|          | No nutrients      | 69.9 ± 0.24 | 17.0 ± 0.03      | 11.5 ± 0.07      | 0.31 ± 0.04  | 0.846 ± 0.010 | 32.3 ± 0.25  | 87.0 ± 0.82  | 60.5 ± 0.30  | 0.971 ± 0.003  | 60.8 ± 0.51 | 1.60 ± 0.17* |

Table A.18. Kinetics of mannitol production from grape musts with *Lactobacillus intermedius* NRRL B-3693 using various nitrogen sources, based on data from Figure 1. All curves were fitted to Hill equation:

\[
C_M = \frac{at^b}{c^b + t^b}
\]

where \(C_M\) is mannitol concentration (g/L) and \(t\) is fermentation time (h).

| Grape must | Nitrogen source | a       | b       | c       | R-Square |
|------------|----------------|---------|---------|---------|----------|
| Red        | YE + Mn        | 69.8381 | 3.8975  | 16.0382 | 0.9999   |
|            | RWL + Mn       | 60.2118 | 2.5764  | 32.0529 | 0.9995   |
|            | WWL + Mn       | 52.1284 | 2.5119  | 32.8190 | 0.9995   |
|            | No nutrients   | 38.7833 | 1.7736  | 44.8140 | 0.9994   |
| White      | YE + Mn        | 81.0010 | 3.9959  | 16.3505 | 0.9995   |
|            | RWL + Mn       | 69.6691 | 1.8966  | 35.3073 | 0.9998   |
|            | WWL + Mn       | 61.8818 | 1.9502  | 38.9134 | 0.9984   |
|            | No nutrients   | 35.4832 | 1.3593  | 45.2474 | 0.9997   |

YE: yeast extract, RWL: red wine lees, WWL: white wine lees.