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Modelling of *S. cerevisiae* and *T. delbrueckii* pure culture fermentation in synthetic media using a compartmental nitrogen model

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ABSTRACT

Aim: The objective of the present work is to propose a model describing the evolution of the pure culture fermentation of two oenological yeasts: *S. cerevisiae* and *T. delbrueckii*.
Methods and results: For both yeasts, pure culture fermentation was performed in a synthetic medium with different initial concentrations of yeast available nitrogen. The datasets obtained from those experiments were used to identify the parameters of the proposed model.
Conclusions: The developed comprehensive model of wine-making fermentation is based on the partition of assimilated nitrogen between the constitutive and the storage compartments. It efficiently describes the evolution of *S. cerevisiae* and *T. delbrueckii* pure cultures. This mass-balance model provides a stoichiometric approach in biomass production, unlike nitrogen backboned models used in winemaking. Moreover, it gives an estimation of non-accessed data such as nitrogen partition between vacuole and cytosol during *T. delbrueckii* fermentation.
Significance and impact of the study: The developed model is robust enough to precisely describe the fermentation evolution of two pure culture yeasts and therefore has future potential for modelling mixed culture fermentations of *S. cerevisiae* and *T. delbrueckii*.

KEYWORDS

modelling, fermentation, *S. cerevisiae, T. delbrueckii*

NOMENCLATURE

a: correlation coefficient linking living cells concentration to mass concentration (10^9 cells/g)
E: ethanol concentration (g/L)
mortality coefficient (g/L)
G: glycerol concentration (g/L)
K_g:constant of glycerol production kinetics (1/h)
K_n: constant of nitrogen assimilation kinetics (L/mol/h)
K_s: constant of fermentation kinetics (1/h)
K_μ: constant of growth kinetics (1/h)
K_E: constant of growth inhibition by ethanol (g/L)
K_{EG}: constant of glycerol production inhibition (g/L)
K_{ES}: constant of ethanol production inhibition by ethanol (g/L)
K_{NS}: constant of ethanol production limitation by stored nitrogen (mg N/10^9 cells)
K_S: constant of ethanol production by sugar (g/L)
K_{SG}: constant of glycerol production limitation by sugar (g/L)
K_X: constant of growth limitation by stored nitrogen (mg N/10^9 cells)
M: yeast molar mass (g/mol)
M': yeast constitutive molar mass (g/mol)
N: nitrogen concentration in the medium (mg N/L)
N_s: stored nitrogen concentration (mg N/L)
S: sugar concentration (g/L)
X: yeast living cells concentration (10^9 cells/L)
X_{max}: maximum yeast living cells concentration (10^9 cells/L)
X_n: yeast constitutive molar concentration (mol/L)

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INTRODUCTION

Wine fermentation is a widely studied process in which hexoses are mainly converted into ethanol and carbon dioxide. *Saccharomyces* species are the yeasts most commonly used in winemaking, since they are tolerant to high ethanol levels (Pretorius, 2000). *T. delbrueckii* is of increasing interest, because it has been shown to have a positive impact on the organoleptic quality of wines and to produce low levels of undesirable compounds, such as acetic acid, ethyl acetate, acetaldehyde, acetoin, hydrogen sulphide and volatile phenols that lessen off-flavours (Ciani and Picciotti, 1995; Renault *et al.,* 2009; Ciani and Maccarelli, 1997; Loira *et al.,* 2015; Canonico *et al.,* 2016; Dutraive *et al.,* 2019; Benito *et al.,* 2019).

Yeasts are living organisms whose activity requires nutrients to provide both the basic elements and energy required for various biochemical syntheses. Nitrogen is one such nutrient, being essential for metabolism and growth during alcoholic fermentation (Jiranek *et al.*, 1995). A lot of studies have been undertaken, especially with *S. cerevisiae*, to help understand, quantify and overcome nitrogen limitation during fermentation (Anderson and Kirsop, 1974; Henschke and Jiranek, 1993; Albers *et al.,* 1996; Arias-Gil *et al.,* 2007; Taillandier *et al.,* 2007; Carrau *et al.,* 2008; Bergdahl *et al.,* 2012; Crepin *et al.,* 2012; Casalta *et al.,* 2013; Su *et al.,* 2020). A lack of nitrogen sources is the main cause of sluggish fermentation (Mendes-Ferreira *et al.*, 2004; Jolly *et al.*, 2014). Yeasts completely consume nitrogen during the first two days of fermentation (Monteiro and Bisson, 1991), and the assimilated nitrogen is distributed among cytosol, membranes and vacuoles. During fermentation there is an intracellular amino acids concentration gradient between the cytosol and the more concentrated vacuoles (Wiemken and Dürr, 1974). This compartmentalisation contributes to the regulation of the activity of many enzymes involved in the metabolism (Sumrada and Cooper, 1982).

Several mathematical models have been proposed in order to understand, simulate and control the fermentation process in winemaking (Sevely *et al.,* 1981; Williams *et al.,* 1986; Caro *et al.,* 1991; Marín, 1999; Colombié *et al.,* 2005; Coleman *et al.,* 2007; Goelzer *et al.,* 2009). They describe the macroscopic evolution of biomass, sugar, ethanol, carbon dioxide and sometimes nitrogen and glycerol. Among these, attention has focused on models in which growth is based on nitrogen sources (Cramer *et al.,* 2002; Malherbe *et al.,* 2004; David *et al.,* 2013; Mouret *et al.,* 2015; Henriques *et al.,* 2018). They describe the evolution of substrates and metabolites using biochemical reactions and mass balances. Even under limiting nitrogen conditions, nitrogen backbone models do not include mass balance and biochemical reaction to describe biomass evolution. The relationship between growth and nitrogen concentration is made by empirically linking the initial nitrogen concentration to the maximum living cell or to the maximum growth rate.

This paper describes a comprehensive kinetic model in which yeast growth is based on assimilated nitrogen partition. It aims to predict the evolution of major components and to contribute to understanding nitrogen limitation during winemaking fermentation. Experimental data has been acquired and a model has been developed for pure cultures of *S. cerevisiae* and *T. delbrueckii*.

MATERIALS AND METHODS

1. Yeast

Two commercial oenological yeasts were used in this study: *Torulaspora delbrueckii* Zymaflore alpha® supplied by Laffort S.A.S., France, and *Saccharomyces cerevisiae* QA23® supplied by Lallemand S.A.S., France. The yeasts were maintained on sterile YPD agar slants [yeast extract 1 % (w/v), peptone 1 % (w/v), glucose 2 % (w/v) and agar 2 % (w/v)] at 4 °C. When the inoculation cultures had been prepared, each yeast was transferred from the agar slant to a flask containing 50 mL of YPD [yeast extract 1 % (w/v), peptone 1 % (w/v), glucose 2 % (w/v)]. The yeasts were incubated at 25 °C with agitation (130 rpm) for 11 h. Subsequently, they were transferred to an Erlenmeyer flask containing 300 mL of YPD to give an initial concentration of 5.10^6 cells/mL, and then incubated with agitation (130 rpm) at 25 °C for 16 h.

2. Medium

Two synthetic media, MS170 and MS300, were used. They contained (per litre): 110 g glucose, 110 g fructose, 6 g L-malic acid, 6 g citric acid, mineral salts (750 mg KH_2PO_4, 500 mg K_2SO_4, 250 mg $MgSO_4 \cdot 7H_2O$, 155 mg $CaCl \cdot 2H_2O$,

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200 mg NaCl, 4 mg $MnSO_4 \cdot H_2O$, 4 mg $ZnSO_4 \cdot 7H_2O$, 1 mg $CuSO_4 \cdot 5H_2O$, 1 mg KI, 0.4 mg $CoCl_2 \cdot 6H_2O$, 1 mg H_3BO_3, and 1 mg $(NH_4)_6Mo_7O_{24}$), vitamins (20 mg myo-inositol, 2 mg nicotinic acid, 1.5 mg calcium pantothenate, 0.25 mg thiamin-HCl, 0.25 mg pyridoxine–HCl, and 0.003 mg biotin), anaerobic growth factors (15 mg ergosterol, 443 µg oleic acid, and 1 mL of a Tween80/ethanol solution (1:1, v/v)). The assimilable nitrogen was in the form of ammonium ions, NH_4Cl, and a concentrated solution of 19 α-amino acids. The composition of the concentrated amino acid solution was (per litre of solution): 1.4 g tyrosine, 13.7 g tryptophan, 2.5 g isoleucine, 3.4 g aspartic acid, 9.2 g glutamic acid, 28.6 g arginine, 3.7 g leucine, 5.8 g threonine, 1.4 g glycine, 38.6 g glutamine, 11.1 g alanine, 3.4 g valine, 2.4 g methionine, 2.9 g phenylalanine, 6.0 g serine, 2.5 g histidine, 1.3 g lysine, 1.0 g cysteine, and 46.8 g proline. The ammonium salts and α-amino acids (all amino acids except proline) in the medium were considered to be assimilable nitrogen (Salmon and Barre, 1998). Medium MS300 contained an equivalent of 324 mg N/L that was assimilable by yeasts (204 mg N/L from amino acid solution corresponding to 13 mL/L of amino acid solution and 120 mg N/L from NH_4Cl corresponding to 0.46 g/L). Medium MS170 contained only 176 mg N/L of assimilable nitrogen (108 mg N/L from amino acid solution corresponding to 7.4 mL/L and 68 mg N/L from NH_4Cl corresponding to 0.26 g/L). The pH of each medium was adjusted to 3.3 before autoclaving for 15 min at 121 °C. Vitamins were filtered and added after the thermal treatment.

3. Analytical methods

3.1. Biomass

Yeast growth was determined by cell counting, using a Thoma haemocytometer. Samples were withdrawn throughout the fermentations and diluted appropriately in 0.9 % (w/v) NaCl solution. Living and dead cells were differentiated by applying a methylene blue staining procedure.

3.2. Sugar, ethanol and glycerol

Samples were filtrated (0.45 µm), diluted ten times and kept at -20 °C until analysis. Metabolite concentrations were determined using high-performance liquid chromatography (Thermo Scientific, France) with a Rezex ROA-Organic acid H^+ (8 %), 250 × 4.6 mm phase-reverse column (Phenomenex, France). The column was eluted with a degassed mobile phase containing 10 mM sulfuric acid, at 30 °C and at a flow rate of 0.170 mL/min. The injection loop volume was 25 µL. Glucose, fructose, ethanol and glycerol peaks were detected using a refractive index detector (Finnigan Surveyor RI Plus detector, Thermo Scientific,France).

3.3. Nitrogen

Amino acid and ammonium chloride were the two sources of yeast assimilable nitrogen (YAN). Amino acid nitrogen was measured using an o-Phthaldialdehyde/N-Acetyl-L-Cysteine assay (K-PANOPA; Megazyme International Ireland Ltd). Free ammonium salts were measured using an enzymatic assay (K-AMIAR; Megazyme International Ireland Ltd). Taking into consideration ammonium chloride and nitrogen atoms only present in the α-amino position of amino acids, media MS300 and MS170 contained 324 and 176 mg N/L respectively (Taillandier *et al*., 2014). For stoichiometric purposes, all the nitrogen atoms assimilable by the yeast had to be taken in account, including those not in the α-amino position of amino acids; MS300 and MS170 thereby contained 429 and 243 mg N/L respectively. The measured YAN concentration with only nitrogen in the α-amino position is proportional to YAN concentration containing all nitrogen atoms in amino acids.

3.4 Elementary analysis

The sample was centrifuged for 5 minutes at 10000 rpm. The supernatant was withdrawn and ultrapure water was added to the precipitate. The mixture was then stirred and centrifuged. This washing step was repeated three times. The obtained precipitate was placedin a vacuum oven at 105 °C. Mass fractions of carbon, hydrogen and nitrogen were determined under dry combustion using a PERKIN ELMER 2400 micro analyser (Jimenez and Ladha, 1993).

4. Fermentation

Each fermentation was performed in a 2.5 L reactor filled with 1.5 L of medium. The reactor has a sampling probe and an opening at the top equipped with a filter (0.45 µm) to avoid overpressure. The inoculation volume was calculated to initially reach $5*10^8$ and 10^{10} cells/L, respectively, for *S. cerevisiae* and

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T. delbrueckii. Pure cultures of *S. cerevisiae* and *T. delbrueckii* were performed in MS170 and MS300. The duration of fermentation corresponded to the time needed to consume 97 % of the sugar initially present. All fermentations were carried out at 20 °C under magnetic agitation (250 rpm) and were performed in duplicate.

5. Statistical analysis

The data are given as mean values (± standard deviation). Statistical analyses were performed using the RStudio programme (Racine, 2012). Following verification of variance homogeneity (Fisher test, $p > 0.05$), one-way analysis of variance (ANOVA) ($p < 0.05$) was used to determine statistically significant differences between modalities. In the text, no significant differences are shown between the mean values compared (if p value is higher than 0.05) or significant differences (if p value is lower than 0.05).

MODEL FORMULATION

1. Minimal nitrogen and stored nitrogen

A structured model was developed based on nitrogen-based biomass growth. In this model, the nitrogen in the medium (N) is assimilated by a yeast cell and distributed between two compartments: the constitutive compartment and the storage compartment. The constitutive compartment contains the minimal quantity of nitrogen (N_{min}) required to ensure the vital functions of a cell. Apart from the vacuoles, the constitutive compartment corresponds to all the nitrogenous elements of the cytoplasm, the membranes and the contents of the organelles, including free amino acids. Like the constitutive compartment, the storage compartment contributes to cell activity; however, only the storage compartment contains the nitrogen reserves (N_s) available for growth. The storage compartment corresponds to the nitrogen content of the vacuoles. During fermentation, the size and composition of amino acids in the cytosolic pool is fairly constant, in contrast to those in the vacuolar pool (Huber-Wälchli and Wiemken, 1979; Kitamoto *et al.*, 1988). Therefore, it can be assumed that the amount of nitrogen in the constitutive compartment (N_{min}) is invariable and the amount of nitrogen in the storage compartment (N_s) varies during fermentation. As the cells multiply, the amount of nitrogen required for the formation of the additional constitutive compartment is drawn from the storage compartment of the mother cell. The remaining nitrogen stock is equitably distributed between the daughter cells (Figure 1.).

2. Additional assumptions

Some assumptions were made to describe the evolution of the fermentation. It was assumed that the reactors used were perfectly agitated and that the volumes sampled over time did not disturb the evolution of the fermentation. The model describes the evolution of total sugars and does not differentiate between glucose and fructose. Similarly, the model considers total assimilable nitrogen withé xwout discerning between ammonia and amino acids. Ethanol and glycerol production are two reactions which are considered independent and instantaneous and which use sugar as a substrate. There is therefore

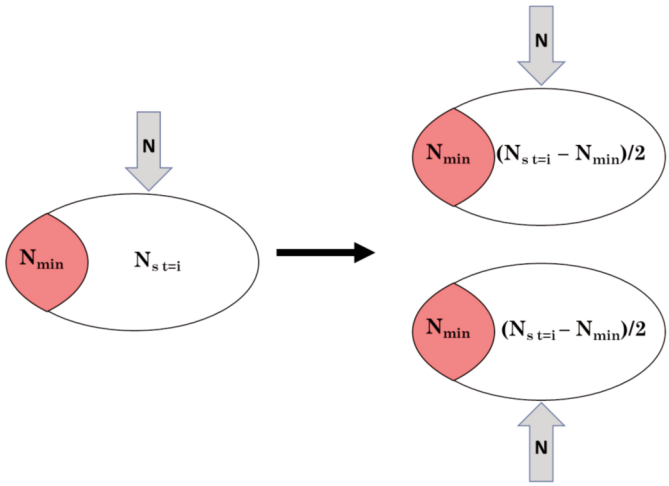


FIGURE 1. Nitrogen partition during cell multiplication.
N_S t=i represents the amount of nitrogen in the storage compartment before a cell multiplication occurs at time t=i.

no accumulation of sugars in the cells. For each yeast, molar mass (*M*) and chemical formula ($CH_xO_yN_z$) vary during fermentation. It is assumed that molar mass (*M'*) and chemical formula ($CH_xO_yN_{z'}$) of a cell containing an empty storage compartment do not vary. The minimum amount of nitrogen contained in the constituent compartment is the only nitrogen contribution to this molar mass. This molar mass is called the constitutive molar mass.

3. Stoichiometry

The model is based on five reactions: growth (*r1*), ethanol production (*r2*), YAN absorption (*r3*), yeast death (*r4*), and glycerol production (*r5*).

$$(r1)\ 1\ C_6H_{12}O_6 + g1\ N_S \rightarrow g2\ CH_xO_yN_{z'} + g3\ CO_2 + g4\ H_2O$$
$$(r2)\ 1\ C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$$
$$(r3)\ 1\ N \rightarrow 1\ N_s$$
$$(r4)\ 1\ CH_xO_yN_{z'}\ (alive) \rightarrow 1\ CH_xO_yN_{z'}\ (dead)$$
$$(r5)\ 1\ C_6H_{12}O_6 + 4H^+ + 4\acute{e} \rightarrow 2\ C_3H_8O_3$$

Nitrogen mass balance in growth reaction (*r1*) allows us to establish a relationship between stoichiometric coefficients *g1* and *g2* (Eq. (6)):

$$z' = \frac{g1}{g2}$$

Therefore, the growth reaction rate is written as follows (Eq. (7)):

$$(r1)\ 1\ C_6H_{12}O_6 + g1\ N_S \rightarrow g2\ CH_xO_yN_{(g1/g2)} + g3\ CO_2 + g4\ H_2O$$

The molar mass containing the lowest fraction of nitrogen experimentally observed was $CH_{1.741}O_{0.698}N_{0.097}$ and $CH_{1.710}O_{0.632}N_{0.113}$ for *S. cerevisiae* and *T. delbrueckii* respectively. These values were used to determine the growth reaction stoichiometric coefficients for each yeast (8-9).

S. cerevisiae:

$$(r1)\ 1\ C_6H_{12}O_6 + g1\ N_S \rightarrow 5.525\ CH_{1.741}O_{0.698}N_{(g1/5.525)} + 0.475\ CO_2 + 1.191\ H_2O$$

T. delbrueckii:

$$(r1)\ 1\ C_6H_{12}O_6 + g1\ N_S \rightarrow 5.398\ CH_{1.710}O_{0.632}N_{(g1/5.398)} + 0.602\ CO_2 + 1.386\ H_2O$$

The stoichiometric coefficient *g1* is identified alongside parameters described in the following section.

4. Kinetics

Reaction rates are expressed in mol/L/h. and *a* represent the concentration of living yeast cells and the correlation coefficient linking the concentration of living cells to mass concentration respectively. The value of *a* is $37.6*10^9$ and $71.6*10^9$ cells/g for *S. cerevisiae* and *T. delbrueckii* respectively. , the constitutive yeast molar concentration, is defined by (Eq. (10)):

$$X_n = \frac{X}{a*M'}$$

Microbial growth rate (*r1*) is assumed to be inhibited by ethanol (*E*) and limited by the nitrogen stock (N_s). It is expressed using the following logistic-like equation (Eq. (11)):

$$r1 = X_n.k_\mu.\left(1-\frac{X}{X_{max}}\right).\left(\frac{K_E}{K_E+E}\right).\left(\frac{\frac{N_S}{X}}{\frac{N_S}{X}+K_X}\right)$$

where, K_μ is the growth kinetics constant, X_{max} is the maximum living cells concentration, K_E is the constant of growth inhibition by ethanol and K_x is the constant of growth limitation by stored nitrogen. Ethanol production rate (*r2*) is assumed to be inhibited by ethanol (*E*) and limited by sugar (*S*) and nitrogen stock (N_s) (Eq. (12)).

$$r2 = k_s.X_n.\left(\frac{S}{S+K_S}\right).\left(\frac{K_{ES}}{K_{ES}+E}\right).\left(\frac{\frac{N_S}{X}}{\frac{N_S}{X}+K_{NS}}\right)$$

The terms $K_{s,}\ K_{S,}\ K_{ES}\ and\ K_{NS}$ represent the constants of ethanol production kinetics, ethanol production limitation by sugar, ethanol production inhibition by ethanol and ethanol production limitation by stored nitrogen respectively.

Nitrogen uptake rate is related to the concentration of assimilable nitrogen and the number of cells present in the medium and is described as follows (Eq. (13)) :

$$r3 = k_N.X_n.N$$

Where K_N is the constant of nitrogen assimilation kinetics. In the death reaction (*r4*), E_{lim} is the mortality coefficient (Eq. (14)).

$$r4 = X_n.e^{\left(\frac{E-E_{lim}}{10}\right)}$$

Glycerol production (*r5*) is limited by low sugar concentration. The production of glycerol (*G*) is coupled to the oxidation of NADH to NAD^+. Regarding the surplus of NADH produced

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during the growth of biomass, the production of glycerol contributes to maintaining the redox equilibrium (Nordstöm, 1968). In addition, the production of ethanol is coupled with the oxidation of NADH to NAD^+; the more ethanol produced the less glycerol is needed to maintain the redox equilibrium. The rate of glycerol production is described as follows (Eq. (15)):

$$r5 = k_G.X_n.\left(\frac{S}{S+K_{SG}}\right).\left(\frac{K_{EG}}{K_{EG}+E}\right)$$

where K_G is the constant of glycerol production kinetics, is the constant of glycerol production limitation by sugar, and is the constant of glycerol production inhibition while ethanol is being produced. The dynamic evolution of each of the six variables of the model is given by the system (A):

$$(A)\quad \begin{cases} \frac{dX}{dt} = (g2 * r1 - r4) \\ \frac{dS}{dt} = -(r1 + r2 + r5) \\ \frac{dN}{dt} = -r3 \\ \frac{dN_s}{dt} = (r3 - g1 * r1) \\ \frac{dE}{dt} = 2 * r2 \\ \frac{dG}{dt} = r5 \end{cases}$$

5. Calculation methods

All calculations were performed using Matlab® 2014a. Integration of the ordinary differential equations system (*A*) uses the function ode15 of Shampine and Reichelt (1997). Parameter estimation is based on a constrained non-linear multivariable function minimisation. The function to be minimised is the least-square sum expressing the difference between experimental values and calculated values of the five measured variables: concentration of living yeast cells (*X*), sugar (*S*), YAN (*N*), ethanol (*E*) and glycerol (*G*). A Sequential Quadratic Programming (SQP) method was used to minimise the least-square sum. The confidence intervals given for the estimated parameters correspond to a 10 % quasi-invariance region of the least-square sum. The initial value of the specific nitrogen in storage compartment was 0.1 mg/10^9 cells for both yeasts.

COMPARISON OF THE MODEL WITH EXPERIMENTAL DATA

Pure cultures of *S. cerevisiae* and *T. delbrueckii* were created in two media with different initial YAN concentrations. The evolution of 5 variables (concentration of living yeast cells (*X*), sugar (*S*), YAN (*N*), ethanol (*E*) and glycerol

TABLE 1. Parameters of the model.

Parameters	*S. cerevisiae*	*T. delbrueckii*	Unit
k_μ	0.032 ± 0.003	0.029 ± 0.005	1/h
X_{max}	305 ± 40	604 ± 73	10^9 cell/L
K_X	(1±1) *10^{-3}	(5 ±4) *10^{-4}	mg N/10^9 cells
$g1$	0.41 ± 0.06	0.42 ± 0.03	(-)
K_E	20 ± 7	12 ± 5	g/L
k_s	0.18 ± 0.03	0.17 ± 0.02	1/h
K_S	71 ± 66	79 ± 17	g/L
K_{ES}	102 ± 74	33 ± 7	g/L
K_{NS}	(5 ± 3) *10^{-6}	0	mg N/10^9 cells
k_N	6.1 ± 4.0	1.0 ± 0.4	L/mol/h
E_{lim}	155 ± 14	145 ± 9	g/L
k_G	1.58 ± 1.26	1.20 ± 0.98	1/h
K_{SG}	140 ± 124	126 ± 117	g/L
K_{EG}	1.6 ± 1.4	1.2 ± 0.9	g/L

The confidence intervals given for the estimated parameters corresponding to a 10 % quasi-invariance region of the least-square sum.

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(*G*)) were assessed for each performed fermentation. The datasets obtained from those experiments were used to identify the kinetic constants and the stoichiometric coefficient *g1*. The results of this identification process are given in Table 1. Figures 2 and 3 show both experimental data and simulated curves.

1. Yeast growth

Passing from MS170 medium to MS300 medium induced a 77 % increase in initial YAN concentration, engendering a slight increase in concentration of *S. cerevisiae* maximum living cells (from 240 (±10) $*10^9$ to 281 (±13) $*10^9$ cells/L). The increase in initial YAN concentration also induced an increase in *T. delbrueckii* maximum living cells (from 420 (±20) $*10^9$ to 542 (±53) $*10^9$ cells/L), which corresponds to an 29 % increase, whereas that of *S. cerevisiae* was 17 %.

In terms of population evolution, the model faithfully describes the latency, growth, stationary and decline phases of each culture of *S. cerevisiae* and *T. delbrueckii* (Figures 2 and 3). In each fermentation, the profile of the nitrogen in the storage compartment showed an accumulation phase followed by a depletion phase (Figures 2 and 3). In medium MS170, yeast growth stopped when there was no longer enough stored nitrogen for the production of new cells (Figures 2a and 3a). The model suggests that not enough nitrogen was assimilated from this medium to satisfy requirements for reaching the maximal concentration of living cells (). This is in accordance with the hypothesis of a limiting nitrogen concentration in MS170 for this

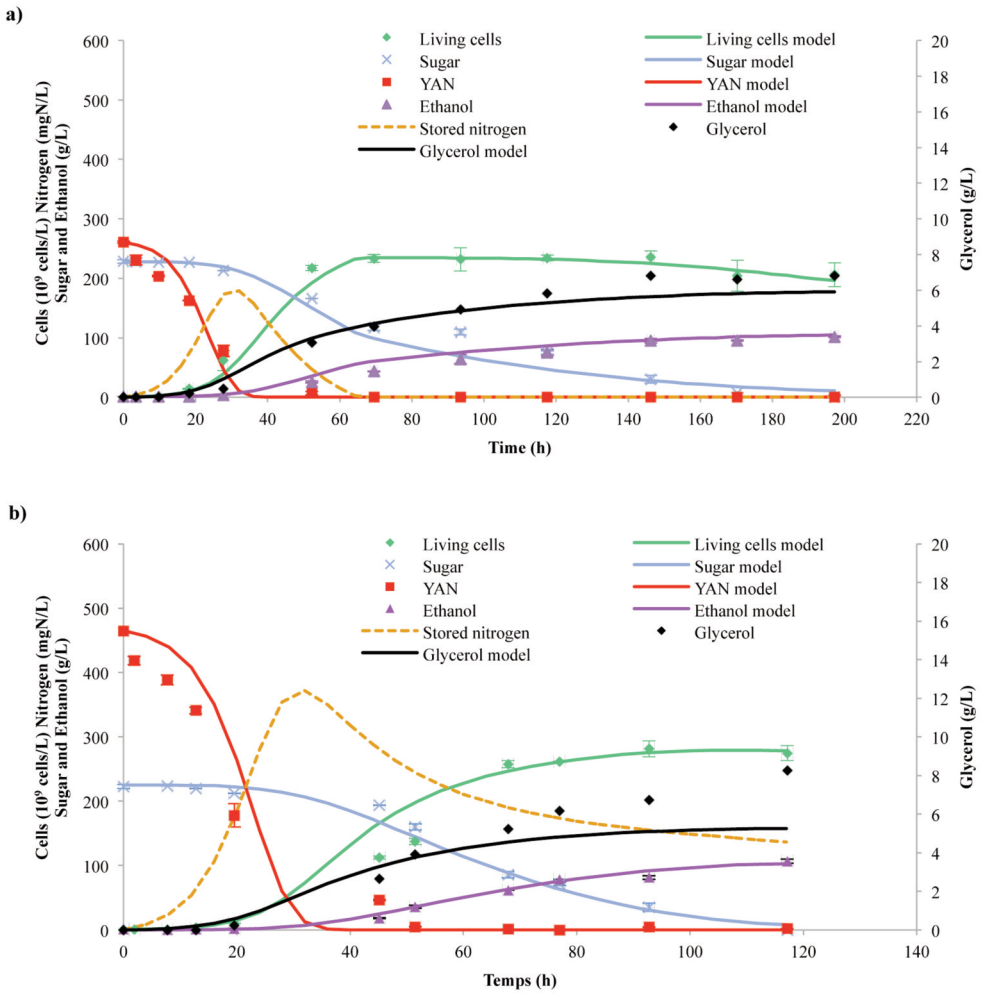


FIGURE 2. *S. cerevisiae* pure culture fermentations in synthetic media: MS170 (a) and in MS300 (b). Data points are experimental measurements, while solid lines are dynamic model predictions. Vertical bars represent the standard deviations.

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specific strain of *S. cerevisiae* and another strain of *T. delbrueckii* (Taillandier *et al.*, 2014). In medium MS300, nitrogen is not limiting and yeast growth stops when is reached.

2. Nitrogen partition

Early on in the performed fermentations, the yeasts assimilated nitrogen and the content of the storage compartment increased and rapidly became higher than that of the constitutive compartment (Figure 4). In the latency and growth phases, the storage compartment of *S. cerevisiae* contained a nitrogen pool which reached a concentration 5 to 20 times higher than that of the constitutive compartment (Figure 4a). Amino acids were accumulated in vacuoles at levels 5 to 40-fold higher than the corresponding cytosolic concentration of *S. cerevisiae* (Ohsumi and Anraku, 1981; Sato *et al.,* 1984; Klionsky, 1990). The hypothesis regarding the association of the storage compartment with vacuoles can therefore be confirmed.

The model showed that the ratio N_s/N_{min} was always under 2.5 in *T. delbrueckii* fermentations (Figure 4b). This is much lower than ratios calculated in *S. cerevisiae* fermentations. As far as *T. delbrueckii* is concerned, we have not found data about nitrogen partitioning during fermentation in the literature. The modelling therefore gives an estimation of non-accessed data and additional experiments are necessary to determine the reliability of such an estimation.

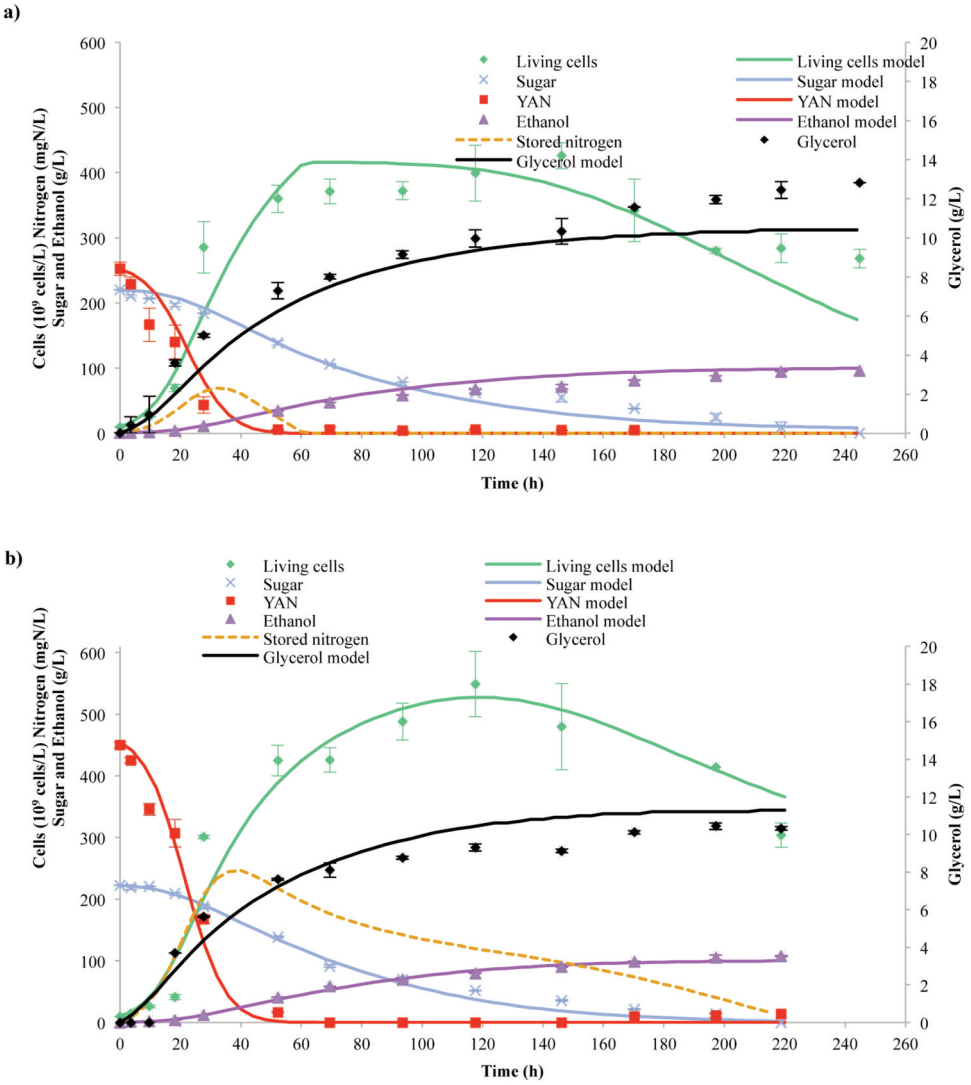


FIGURE 3. *T. delbrueckii* pure culture fermentations in synthetic media: MS170 (a) and MS300 (b).
Data points are experimental measurements, while solid lines are dynamic model predictions. Vertical bars represent the standard deviations.

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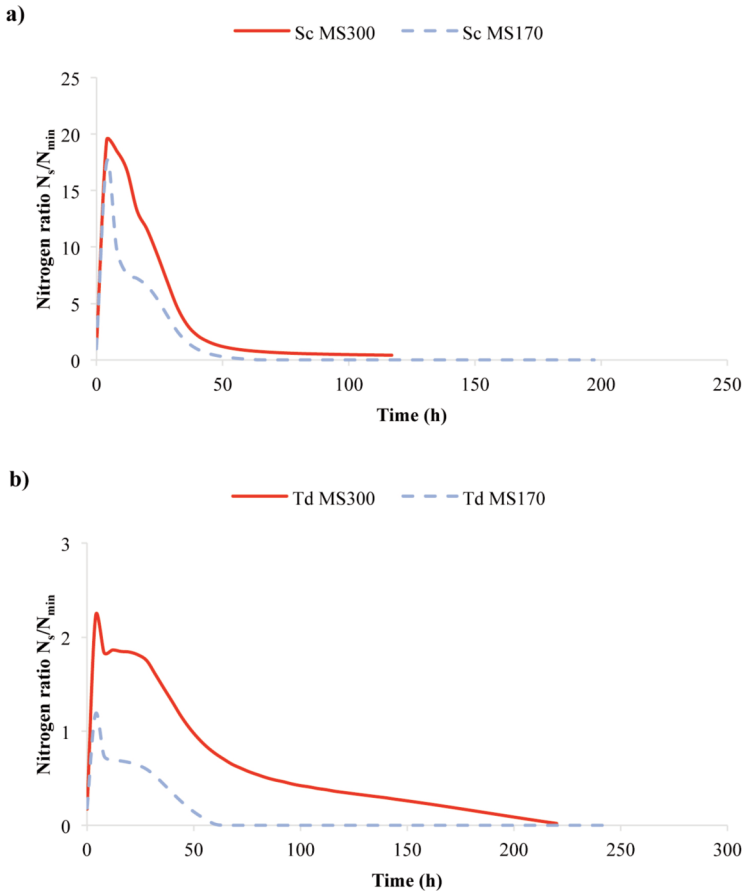


FIGURE 4. Nitrogen partition in *S. cerevisiae* (a) and *T. delbrueckii* (b) pure cultures.

The transport of amino acids from cytosol to vacuole is performed through an energy-requiring process (Okorokov *et al.,* 1985). The higher the N_s/N_{min} ratio, the higher the energy required to maintain the transport of amino acids against the concentration gradient. The N_s/N_{min} ratios for *S. cerevisiae* and *T. delbrueckii* were revealed to be different. Our model therefore allows us to conclude that the energy requirements for amino acid transport from cytosol to vacuole in *T. delbrueckii* may be different to those of *S. cerevisiae.*

3. Nitrogen requirements

In both media, *S. cerevisiae* (Figure 2) and *T. delbrueckii* (Figure 3) completely consumed the YAN initially present in ≈55 h. In each fermentation, the stored nitrogen profile showed an accumulation phase followed by a depletion phase (Figures 2 and 3). In medium MS170, yeast growth stopped when there was no longer enough stored nitrogen for the production of new cells (Figures 2a and 3a). The model suggests that there is not enough nitrogen assimilated in this medium to satisfy requirements for reaching the maximal concentration of living cells (). This is in accordance with the hypothesis of a limiting nitrogen concentration in MS170 for this specific strain of *S. cerevisiae* and another strain of *T. delbrueckii* (Taillandier *et al.*, 2014). In medium MS300, nitrogen is not limiting and yeast growth stops when is reached.

The stoichiometric coefficient *g1* links the consumption of nitrogen in the storage compartment to cell production. Its value is 0.41 ± 0.06 and 0.42 ± 0.03 respectively for *S. cerevisiae* and *T. delbrueckii*. However, the yeast stoichiometric coefficients *g1* are not significantly different and the maximal concentration of *T. delbrueckii* living cells () is twice as high as that of *S. cerevisiae*. This means that twice as much nitrogen is required to produce one *S. cerevisiae* cell than to produce one *T. delbrueckii* cell. However, the maximum

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biomass concentration was close to 8 g/L for all fermentations of *S. cerevisiae* and *T. delbrueckii*. Despite both yeasts having a similar maximal dry weight, *T. delbrueckii* performs fermentations with a higher maximal concentration of living cells. The latter observation, alongside the nitrogen requirements suggested by the model, are coherent with the fact that *S. cerevisiae* cells size is higher than that of *T. delbrueckii* (Hernández-López *et al*., 2007).

The determined yeast constitutive molar mass was $CH_{1.741}O_{0.698}N_{0.074}$ and $CH_{1.710}O_{0.632}N_{0.078}$ for *S. cerevisiae* and *T. delbrueckii* respectively. For both yeasts, nitrogen fractions experimentally measured during the fermentations were higher than the estimated nitrogen fraction of yeast with an empty storage compartment (data not shown). The minimum amount of nitrogen required to ensure the vital functions of cells was 38 and 40 mg N per gram of yeast for *S. cerevisiae* and *T. delbrueckii* respectively.

4. Ethanol production

Sugar had been completely consumed by the end of all performed fermentations. For both yeasts, final ethanol concentration was ≈105 g/L and it was not significantly affected by initial YAN concentration. By increasing the initial concentration of YAN, the fermentation time decreased from 197 ±11 to 117 ±7 h and from 245 ±11 to 218 ±7 h for *S. cerevisiae* and *T. delbrueckii* respectively. The ethanol production reaction (*r2*) is limited by sugar and stored nitrogen and is inhibited by ethanol. The evolution of sugar limitation , stored nitrogen limitation and ethanol inhibition are represented as a function of the consumed sugar in figure 5. For both yeasts, sugar limitation and ethanol inhibition continuously occurred while sugar was being consumed. The identified value of was 0 mg N/10^9 cells for *T. delbrueckii*. In both media the production of ethanol by *T. delbrueckii* was not limited by stored nitrogen. Therefore, the lower ethanol production rate observed in

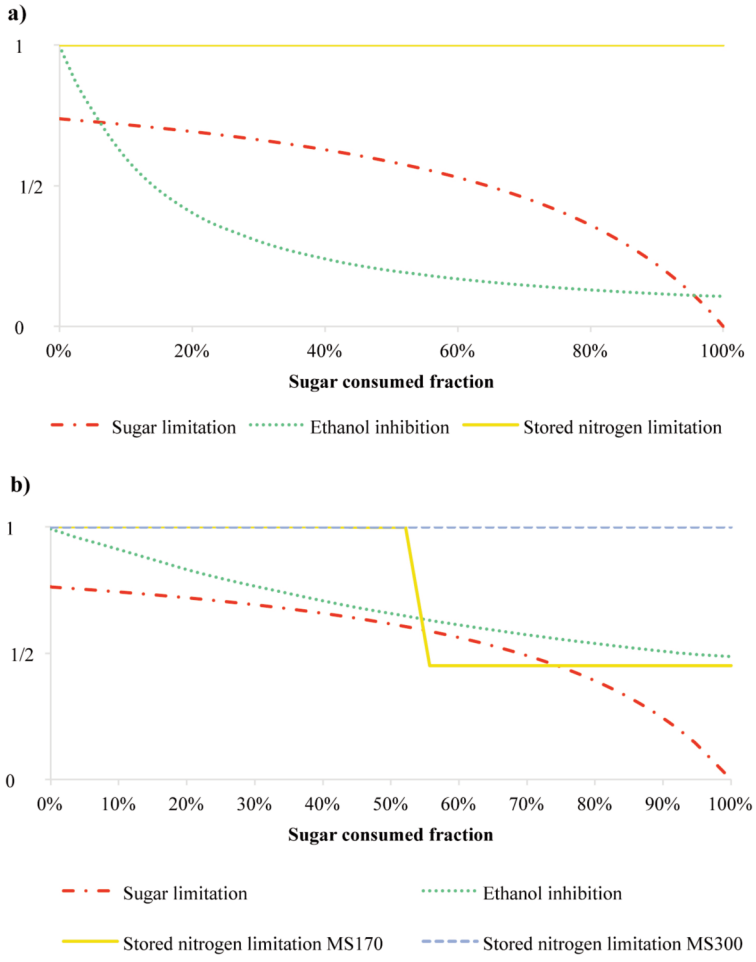


FIGURE 5. Fermentation limitations and inhibition in *T. delbrueckii* (a) and *S. cerevisiae* (b) pure cultures. *T. delbrueckii* stored nitrogen limitation has the same profile in MS170 and MS300 media.

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T delbrueckii fermentation in MS170 was caused by the lower concentration of living cells rather than a limitation of stored nitrogen. The model enabled us to rank the effect of limitations and inhibition on ethanol productivity, and therefore to rank these phenomena.

The identified value of was (5 ± 3) $*10^{-6}$ mg $N/10^9$ cells for *S. cerevisiae.* In MS300, ethanol production of *S. cerevisiae* was not limited by stored nitrogen. In MS170, *S. cerevisiae* consumed the stored nitrogen until there was not enough for the production of new cells. Subsequently, the stored nitrogen suddenly became limiting for ethanol production and the ethanol production rate sharply decreased by 55 % (Figure 5b). Unlike that of *T. delbrueckii,* the ethanol production rate of *S. cerevisiae* was impacted by the limited stored nitrogen in MS170.

CONCLUSION AND FUTURE WORK

1. Conclusion

The present comprehensive model of wine-making fermentation is based on the partition of assimilated nitrogen between the constitutive and the storage compartments and includes the following variables: yeast living cells, sugar, YAN, ethanol, glycerol and stored nitrogen. It contains a set of five ordinary differential equations in order to efficiently predict the evolution of *S. cerevisiae* and *T. delbrueckii* pure culture fermentations. This mass-balance model provides a stoichiometric approach to biomass production, unlike nitrogen backboned models used in winemaking. The developed model gives an estimation of non-accessed data, such as nitrogen partition between vacuole and cytosol during fermentation. Although this estimation is coherent with the partition of amino acids observed in *S. cerevisiae* fermentation, additional experiments are necessary to determine its reliability.

2. Future work

The developed model is robust enough to give a precise description of the fermentation evolution of the pure cultures of two yeasts. The next step will be to validate the model with more initial concentrations of yeast assimilable nitrogen. The model will be further developed so that it can be used for modelling the mixed culture fermentation of *S. cerevisiae* and *T. delbrueckii*.

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REFERENCES

Albers E., Larsson C., Lidén G., Niklasson C. and Gustafsson L., 1996. Influence of the nitrogen source on *Saccharomyces cerevisiae* anaerobic growth and product formation. *Applied and Environmental Microbiology*, *62*(9), 3187–3195. doi:10.1128/AEM.62.9.3187-3195.1996

Anderson R.G. and Kirsop B.H., 1974. The control of volatile ester synthesis during the fermentation of wort of high specific gravity. *Journal of the Institute of Brewing*, *80*(1), 48–55. doi:10.1002/j.2050-0416.1974.tb03582.x

Arias-Gil M., Garde-Cerdán T. and Ancín-Azpilicueta C., 2007. Influence of addition of ammonium and different amino acid concentrations on nitrogen metabolism in spontaneous must fermentation. *Food Chemistry*, *103*(4), 1312–1318. doi:10.1016/j.foodchem.2006.10.037

Benito A., Calderón F. and Benito S., 2019. The influence of non-*Saccharomyces* species on wine fermentation quality parameters. *Fermentation*, *5*(3), 54. doi:10.3390/fermentation5030054

Bergdahl B., Heer D., Sauer U., Hahn-Hägerdal B. and van Niel, EW., 2012. Dynamic metabolomics differentiates between carbon and energy starvation in recombinant *Saccharomyces cerevisiae* fermenting xylose. *Biotechnology for Biofuels*, *5*(1), 34. doi:10.1186/1754-6834-5-34

Canonico L., Agarbati A., Comitini F. and Ciani M., 2016. *Torulaspora delbrueckii* in the brewing process: A new approach to enhance bioflavour and to reduce ethanol content. *Food Microbiology*, *56*, 45–51. doi:10.1016/j.fm.2015.12.005

Caro I., Pérez L. and Cantero D., 1991. Development of a kinetic model for the alcoholic fermentation of must. *Biotechnology and Bioengineering*, *38*(7), 742–748. doi:10.1002/bit.260380708

Carrau F.M., Medina K., Farina L., Boido E., Henschke P.A. and Dellacassa E., 2008. Production of fermentation aroma compounds by *Saccharomyces cerevisiae* wine yeasts: Effects of yeast assimilable nitrogen on two model strains. *FEMS Yeast Research*, *8*(7), 1196–1207. doi:10.1111/j.1567-1364.2008.00412.x

Ciani M. and Maccarelli F., 1997. Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. *World Journal of Microbiology and Biotechnology*, *14*(2), 199–203. doi:10.1023/A:1008825928354

Ciani M. and Picciotti G., 1995. The growth kinetics and fermentation behaviour of some non-*Saccharomyces* yeasts associated with wine-making.

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Biotechnology Letters, *17*(11), 1247–1250. doi:10.1007/BF00128395

Coleman M.C., Fish R. and Block D.E., 2007. Temperature-dependent kinetic model for nitrogen-limited wine fermentations. *Applied and Environmental Microbiology*, *73*(18), 5875–5884. doi:10.1128/AEM.00670-07

Colombié S., Malherbe S. and Sablayrolles J.M., 2005. Modeling alcoholic fermentation in enological conditions: feasibility and interest. *American Journal of Enology and Viticulture*, *56*(3), 238–245. https://www.ajevonline.org/content/56/3/238

Cramer A.C., Vlassides S. and Block D.E., 2002. Kinetic model for nitrogen-limited wine fermentations. *Biotechnology and Bioengineering*, *77*(1), 49–60. doi:10.1002/bit.10133

Crepin L., Nidelet T., Sanchez I., Dequin S. and Camarasa C., 2012. sequential use of nitrogen compounds by *Saccharomyces cerevisiae* during wine fermentation: a model based on kinetic and regulation characteristics of nitrogen permeases. *Applied and Environmental Microbiology*, *78*(22), 8102–8111. doi:10.1128/AEM.02294-12

David R., Dochain D., Mouret, JR., Vande Wouwer A. and Sablayrolles, JM., 2013. Nitrogen-backboned modeling of wine-making in standard and nitrogen-added fermentations. *Bioprocess and Biosystems Engineering*, *37*(1), 5–16. doi:10.1007/s00449-013-0914-6

Dutraive O., Benito S., Fritsch S., Beisert B., Patz CD. and Rauhut D., 2019. Effect of sequential inoculation with non-*Saccharomyces* and *Saccharomyces* yeasts on Riesling wine chemical composition. *Fermentation*, *5*(3), 79. doi:10.3390/fermentation5030079

Goelzer A., Charnomordic B., Colombié S., Fromion V. and Sablayrolle J.M., 2009. Simulation and optimization software for alcoholic fermentation in winemaking conditions. *Food Control*, *20*(7), 635–642. doi:10.1016/j.foodcont.2008.09.016

Henriques D., Alonso-del-Real J., Querol A. and Balsa-Canto E., 2018. *Saccharomyces cerevisiae* and *S. kudriavzevii* synthetic wine fermentation performance dissected by predictive modeling. *Frontiers in Microbiology*, *9*. doi:10.3389/fmicb.2018.00088

Henschke P.A. and Jiranek V., 1993. *Yeasts – Metabolism of nitrogen compounds. Wine Microbiol Biotechnol 77–164.* https://www.researchgate.net/publication/294697283_Yeasts-metabolism_of_nitrogen_compounds_in_Wine_Microbiology_and_Biotechnology

Hernández-López M.J., Pallotti C., Andreu P., Aguilera J., Prieto, JA. and Randez-Gil F., 2007. Characterization of a *Torulaspora delbrueckii* diploid strain with optimized performance in sweet and frozen sweet dough. *International Journal of Food Microbiology*, *116*(1), 103–110. doi:10.1016/j.ijfoodmicro.2006.12.006

Huber-Wälchli V. and Wiemken A., 1979. Differential extraction of soluble pools from the cytosol and the vacuoles of yeast (*Candida utilis*) using DEAE-dextran. Arch. Microbiol. 120, 141–149 doi:10.1007/BF00409100

Jimenez R.R. and Ladha J.K., 1993. Automated elemental analysis: A rapid and reliable but expensive measurement of total carbon and nitrogen in plant and soil samples. *Communications in Soil Science and Plant Analysis*, *24*(15–16), 1897–1924. doi:10.1080/00103629309368926

Jiranek V., Langridge P. and Henschke P.A., 1995. Amino acid and ammonium utilization by *Saccharomyces cerevisiae* wine yeasts from a chemically defined medium. *American Journal of Enology and Viticulture*, *46*(1), 75–83. https://www.ajevonline.org/content/46/1/75.short

Jolly N.P., Varela C. and Pretorius I.S., 2014. Not your ordinary yeast: Non- *Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Research*, *14*(2), 215–237. doi:10.1111/1567-1364.12111

Kitamoto K., Yoshizawa K., Ohsumi Y. and Anraku Y., 1988. Dynamic aspects of vacuolar and cytosolic amino acid pools of *Saccharomyces cerevisiae*. Journal bacteriol., 170 (6), 2683-2686. doi:10.1128/JB.170.6.2683-2686.1988

Klionsky D.J., 1990. The Fungal Vacuole: Composition, Function, and Biogenesis. *Microbiol. Rev.*, *54*, 27. doi:10.1128/MMBR.54.3.266-292.1990

Loira I., Morata A., Comuzzo P., Callejo M.J., González C., Calderón F. and Suárez-Lepe J.A., 2015. Use of *Schizosaccharomyces pombe* and *Torulaspora delbrueckii* strains in mixed and sequential fermentations to improve red wine sensory quality. *Food Research International*, *76*, 325–333. doi:10.1016/j.foodres.2015.06.030

Malherbe S., Fromion V., Hilgert N. and Sablayrolles J.M., 2004. Modeling the effects of assimilable nitrogen and temperature on fermentation kinetics in enological conditions. *Biotechnology and Bioengineering*, *86*(3), 261–272. doi:10.1002/bit.20075

Marín M.R., 1999. Alcoholic Fermentation Modelling: Current State and Perspectives. *American Journal of Enology and Viticulture*, *50*(2), 166–178. https://www.ajevonline.org/content/50/2/166.short

Mendes-Ferreira A., Mendes-Faia A. and Leao C., 2004. Growth and fermentation patterns of *Saccharomyces cerevisiae* under different ammonium concentrations and its implications in winemaking industry. *Journal of Applied Microbiology*, *97*(3), 540–545. doi:10.1111/j.1365-2672.2004.02331.x

Monteiro F.F. and Bisson L.F., 1991. Amino acid utilization and urea formation during vinification

fermentations. *American Journal of Enology and Viticulture*, *42*(3), 199. https://www.ajevonline.org/content/42/3/199.short

Mouret J.R., Farines V., Sablayrolles J.M. and Trelea I.C., 2015. Prediction of the production kinetics of the main fermentative aromas in winemaking fermentations. *Biochemical Engineering Journal*, *103*, 211–218. doi:10.1016/j.bej.2015.07.017

Nordstöm K., 1968. Yeast growth and glycerol formation II. Carbon and redox balances. *J Inst Brew*, *74*, 429–432. doi:10.1002/j.2050-0416.1968.tb03154. x

Ohsumi Y. and Anraku Y., 1981. Active transport of basic amino acids driven by a proton motive force in vacuolar membrane vesicles of *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, *256*(5), 2079–2082. http://www.jbc.org/content/256/5/2079.short

Okorokov L.A., Kulakovskaya T.V., Lichko LP. and Polorotova E.V., 1985. H^+ ion antiport as the principal mechanism of transport systems in the vacuolar membrane of the yeast *Saccharomyces carlsbergensis*. *FEBS Letters*, *192*(2), 303–306. doi:10.1016/0014-5793(85)80130-7

Pretorius I.S., 2000. Tailoring wine yeast for the new millennium: Novel approaches to the ancient art of winemaking. *Yeast*, *16*(8), 675–729. doi:10.1002/1097-0061(20000615)16:8<675::AID-YEA585>3.0.CO;2-B

Racine JS., 2012. RStudio: A Platform-Independent IDE for R and Sweave. *Journal of Applied Econometrics*, *27*(1), 167–172. doi:10.1002/jae.1278

Renault P., Miot-Sertier C., Marullo P., Hernández-Orte P., Lagarrigue L., Lonvaud-Funel A. and Bely M., 2009. Genetic characterization and phenotypic variability in *Torulaspora delbrueckii* species: Potential applications in the wine industry. *International Journal of Food Microbiology*, *134*(3), 201–210. doi:10.1016/j.ijfoodmicro.2009.06.008

Salmon J.M. and Barre P., 1998. Improvement of nitrogen assimilation and fermentation kinetics under enological conditions by derepression of alternative nitrogen-assimilatory pathways in an industrial *Saccharomyces cerevisiae* strain. *Applied and Environmental Microbiology*, *64*(10), 3831–3837. doi:10.1128/AEM.64.10.3831-3837.1998

Sato T., Ohsumi Y. and Anraku Y., 1984. Substrate specificities of active transport systems for amino acids in vacuolar-membrane vesicles of *Saccharomyces cerevisiae*. Evidence of seven independent proton/amino acid antiport systems. *Journal of Biological Chemistry*, *259*(18), 11505–11508. http://www.jbc.org/content/ 259/18/11505.short

Sevely Y., Pourciel J.B., Rauzy G. and Bovee J.P., 1981. Modelling, Identification and Control of the Alcohol Fermentation in a Cascade Reactor. *IFAC Proceedings Volumes*, *14*(2), 2821–2828. doi:10.1016/S1474-6670(17)63891-6

Shampine L.F. and Reichelt M.W., 1997. The MATLAB ODE Suite. *SIAM Journal on Scientific Computing*, *18*(1), 1–22. doi:10.1137/S1064827594276424

Su Y., Seguinot P., Sanchez I., Ortiz-Julien A., Heras J.M., Querol A., Camarasa C. and Guillamón J.M., 2020. Nitrogen sources preferences of non-*Saccharomyces* yeasts to sustain growth and fermentation under winemaking conditions. *Food Microbiology, 85*, 103287. doi:10.1016/j.fm.2019.103287

Sumrada R.A. and Cooper T.G., 1982. Isolation of the CAR1 Gene from *Saccharomyces cerevisiae* and analysis of its expression. *Molecular and Cellular Biology*, *2*(12), 1514–1523. doi:10.1128/MCB.2.12.1514

Taillandier P., Lai, QP., Julien-Ortiz A. and Brandam C., 2014. Interactions between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in wine fermentation: Influence of inoculation and nitrogen content. *World Journal of Microbiology and Biotechnology*, *30*(7), 1959–1967. doi:10.1007/s11274-014-1618-z

Taillandier P., Ramon Portugal F., Fuster A. and Strehaiano P., 2007.Effect of ammonium concentration on alcoholic fermentation kinetics by wine yeasts for high sugar content. *Food Microbiology*, *24*(1), 95–100. doi:10.1016/j.fm.2006.04.002

Wiemken A. and Dürr M., 1974.Characterization of amino acid pools in the vacuolar compartment of *Saccharomyces cerevisiae*. *Archives of Microbiology*, *101*(1), 45–57. doi:10.1016/j.fm.2006.04.002

Williams D., Yousefpour P. and Wellington E.M.H., 1986. On-line adaptive control of a fed-batch fermentation of *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering*, *28*(5), 631–645. doi:10.1002/bit.260280502

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